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Complement component C3 and the TLR co-receptor CD14 are not involved in angiotensin II induced cardiac remodelling

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Abstract

Inflammation is centrally involved in the development of cardiac hypertrophy and the processes of remodelling. The complement system and Toll-like receptor (TLR) family, two upstream arms of the innate immune system, have previously been reported to be involved in cardiac remodelling. However, the role of complement component 3 (C3), TLR co-receptor CD14 and the synergy between them have not been addressed during pressure overload-induced cardiac remodelling. Here, we examined angiotensin II-induced cardiac hypertrophy and remodelling for 7 days in male C57Bl/6J mice deficient in C3, CD14, or both (C3CD14), and WT controls. Angiotensin II infusion induced a mild concentric hypertrophic phenotype in WT mice with increased left ventricle weight, wall thicknesses and reduced ventricular internal diameter, associated with increased cardiac fibrosis. However, there were no differences between WT mice and mice deficient for C3, CD14 or C3CD14, as systolic blood pressure, cardiac function and structure and levels of fibrosis were comparable between WT mice and the three other genotypes. C5a did not change in angiotensin II treated mice, whereas Mac2 levels were increased in angiotensin II treated mice, but did not differ between genotypes. The inflammatory IL-6 response was comparable between WT and C3 deficient mice, however, it was decreased in CD14 and C3CD14 deficient mice. We conclude that deficiency in C3, CD14 or C3CD14 had no effect on cardiac remodelling following angiotensin II-induced pressure overload. This suggests that C3 and CD14 are not involved in angiotensin II-induced adverse cardiac remodelling.

Keywords

Complement, Toll-like receptor, C3, CD14, cardiac remodelling, angiotensin

Introduction

Cardiac remodelling is a compensatory response to increased cardiac workload that over time can turn maladaptive and cause cardiac dysfunction and heart failure (HF) [1]. The remodelling process is characterized by increased cardiomyocyte size, *i.e.* cardiomyocyte hypertrophy, changes in the geometry of the left ventricle (LV), and development of cardiac fibrosis [1, 2]. An increasing number of studies suggest that inflammation plays an important role in the development of cardiac hypertrophy and in turning the remodelling processes from adaptive to maladaptive [3-5]. Extensive fibrosis formation is a hallmark of pressure overload-induced cardiac remodelling which is regulated through several distinct inflammatory pathways [6]. Angiotensin II (AngII), an effector molecule of the renin-angiotensin-aldosterone system (RAAS), exacerbates the hypertensive-induced cardiac remodelling at least partly by enhancing inflammatory responses and oxidative stress [7-9]. These inflammatory responses during cardiac hypertrophy and remodelling involve inflammatory cytokines like tumor necrosis factor (TNF), interleukin (IL)-1β and IL-6 [10, 11]. However, the initiating mechanisms and the exact role of inflammation in cardiac hypertrophy including hypertensive-induced cardiac remodelling are still not clear.

The complement system and the Toll-like receptor (TLR) family are two main upstream activators of innate immunity and initiate downstream inflammatory signals. Although the complement and TLR systems represent separate signalling pathways, there is considerable cross-talk between them and both seem to be involved in pressure overload-induced cardiac remodelling [12, 13].

The complement system can be activated through the classical, lectin, and/or alternative pathway leading to cleavage of the central complement component 3 (C3). Cleavage of C3 leads to production of the opsonin C3b and the anaphylatoxin C3a, which may act either pro- or anti-inflammatory by binding to the C3a receptor [14]. Subsequent cleavage of C5 leads to

release of the anaphylatoxin C5a, which acts mainly pro-inflammatory through its binding to C5aR1 and partly anti-inflammatory when binding to C5aR2. C5b binds to C6, C7, C8 and C9, ultimately leading to the assembly of the terminal C5b-9 complement complex (TCC) that as effector molecule is able, when inserted into the membrane, to lyse cells or activate cells in sublytic concentrations. Previously, increased levels of C3a and C5a have been shown in AngII-induced cardiac hypertrophy [15, 16]. Also, inhibition of the C5a receptor 1 (C5aR1) has been shown to prevent AngII-induced hypertrophy [15, 17, 18], but data on C3 inhibition in models of cardiac hypertrophy are lacking. The TLR system contains a number of co-receptors, of which CD14 is of particular interest since it interacts with several TLRs, including TLR2 and TLR4 [19]. The interactions between CD14 and TLRs leads to cellular activation and expression of several pro- and anti-inflammatory cytokines [20]. Experimental mouse models have shown the crucial role of TLR2- and TLR4-mediated inflammatory signals in cardiac hypertrophy, [13, 21], but data on any modulation of the co-receptor CD14 has not been reported.

Combined inhibition of C3 and CD14 during experimental models of sepsis has been shown to attenuate inflammatory responses and promote increased survival [22]. However, this combined inhibition of C3 and CD14, potentially representing a bottle neck in the initiation of the innate immune response, has not been evaluated in sterile inflammatory processes such as those occurring during cardiac pressure overload. Thus, we hypothesized that deficiency of these central components of the complement (C3) and TLR (CD14) systems could attenuate the inflammatory response and have beneficial effects on cardiac remodelling during pressure overload. To test this hypothesis, we evaluated the effect of C3 and CD14 deficiency and the combination thereof on AngII-induced cardiac remodelling in an experimental mouse model.

Materials and Methods

The following animals, reagents, measurements and procedures were used and are described in detail in the online Material file: Mice were B6.129S4-C3tm1Crr/J ($C3^{-/-}$) and B6.129S4-Cd14tm1Frm/J ($CD14^{-/-}$) [23], and double $C3^{-/-}/CD14^{-/-}$ mice were supervised according to the Norwegian Animal Welfare Act, which conforms to the National Institute of Health guideline (NIH publication number 85-23, revised 1996). The experimental animal protocol was approved by the Norwegian National Animal Research Committee (FOTS id 7783).

Angiotensin delivery, blood pressure and echocardiography were performed as detailed in the online material. **Blood and tissue samples** were obtained after strict criteria and **histology and immunohistochemistry** were performed as detailed in the online file. **Hydroxyproline** was measured by HPLC as previously described [24]. **Inflammatory markers** were measured by ELISA and RT-qPCR as detailed online.

Statistical analysis

GraphPad Prism 7.0 software was used for data analysis (GraphPad Software, CA). Significance of difference between the groups was evaluated using univariate analysis of variance (ANOVA). Multiple comparisons were tested by two-way ANOVA with Fisher's LSD post-tests. When performing repeated measures ANOVA (blood pressure), Bonferroni's *post hoc* test was applied. Values are presented as mean ± standard error of mean (SEM). P-values <0.05 were considered statistically significant.

Results

Systolic blood pressure during AngII infusion is not attenuated in mice deficient for C3, CD14 or C3CD14

To evaluate the role of C3 and CD14 in blood pressure regulation, WT, $C3^{-/-}$, $CD14^{-/-}$ and $C3CD14^{-/-}$ mice were continuously infused with AngII or PBS for 7 days. At baseline, all groups had comparable SBP levels (Suppl. Figure 1). Infusion of AngII in WT mice resulted in a steady and significant increase in SBP (116±5% at day 3, 119±5% increase at day 6, p=0.01 and p=0.001, respectively) compared to baseline, and notably, this pattern was not influenced by C3, CD14 or C3CD14 deficiency.

Increased heart weights in $C3^{-/-}$, $CD14^{-/-}$, $C3CD14^{-/-}$ and WT mice after AngII infusion

Whereas C3, CD14 or C3CD14 deficiency did not influence the AngII induced increase in SBP, the deficiency of these molecules could potentially affect cardiac remodelling by influencing the direct effects of AngII on the myocardium. However, after 7 days of subcutaneous AngII continuous infusion, LV weight (Figure 1A) and total heart weight (Figure 1B) were increased in all AngII treated mice compared to their respective PBS controls with the same pattern in all genotypes. Lung weight was modestly increased in WT and in $C3CD14^{-/-}$ mice ($p<0.05$) with a similar tendency in $C3^{-/-}$ and $CD14^{-/-}$ mice (p=0.05 and p=0.1, respectively; Figure 1C). Moreover, when cardiomyocyte hypertrophy was examined by measuring the cardiomyocyte area in WGA-stained sections, no significant differences were observed in cardiomyocyte area neither between mice treated with PBS or AngII, nor between the different genotypes (Figure 1D-E).

C3, CD14 and C3CD14 deficiency do not influence cardiac structure and function in AngII-induced cardiac pressure overload

Cardiac structure was measured by echocardiography at day 6. AngII infusion induced a significant increase in end-diastolic intraventricular septum (IVSd) thickness in all genotypes (Suppl. Figure 2A) and LV end-diastolic posterior wall (LVPWd) thickness in WT ($p<0.01$), $C3^{-/-}$ ($p<0.001$) and $CD14^{-/-}$ ($p=0.001$) mice with a similar trend for $C3CD14^{-/-}$ mice ($p=0.10$; Suppl. Figure 2B). In contrast, LV end-diastolic internal diameter (LVIDd) decreased significantly after AngII infusion, and again, with the same pattern in all genotypes (Suppl. Figure 2C). To assess geometrical changes, we calculated relative wall thickness (RWT) [25], showing a marked concentric remodelling pattern in AngII infused mice, *i.e.* RWT >0.42 with no differences between the genotypes (Suppl. Figure 2D).

We subsequently investigated LV volumes, which were diminished by AngII infusion as reflected by a significant reduction in end diastolic volume (EDV) and end systolic volume (ESV) with no differences between the four different genotypes (i.e., WT, $C3^{-/-}$, $CD14^{-/-}$, $C3CD14^{-/-}$**)** (Figure 2A-B). In addition, stroke volume, computed as subtraction of ESV from EDV, was significantly diminished in $C3^{-/-}$ and $CD14^{-/-}$ while there were no AngII-induced changes in stroke volume in WT and $C3CD14^{-/-}$ mice (Figure 2C). Additional echocardiographic measurements supporting the deteriorated cardiac function in AngII infused mice are shown in Supplemental Table 1.

Finally, atrial natriuretic peptide (ANP) mRNA, as a marker of cardiac wall stress, increased significantly in AngII infused $C3^{-/-}$ mice, with a similar trend in WT, $CD14^{-/-}$ and $C3CD14^{-/-}$ mice infused with AngII compared to PBS controls ($p=0.06$, 0.09 and 0.07, respectively) (Figure 2D).

C3, CD14 and C3CD14 deficiency do not affect AngII-induced myocardial fibrosis

Cardiac fibrosis is a hallmark of pressure overload-induced cardiac remodelling and was significantly increased in all the groups infused with AngII compared to PBS controls as

assessed by Sirius Red staining with no difference between the different genotypes (Figure 3A-B). Additionally, there were no differences between WT and mice deficient in C3, CD14, or C3CD14 for the expression of collagen type I and collagen type III (Figure 3C-D). Finally, also total collagen levels evaluated by hydroxyproline assay showed no differences between the genotypes (Figure 3E).

CD14 deficiency, but not C3 deficiency, attenuates cardiac IL-6 mRNA expression in AngII-induced cardiac hypertrophy

Lastly we measured inflammatory parameters in heart tissue as well as in the circulation. Assessment of the amount of macrophages with immunohistochemistry showed an increase in Mac2 positive staining in AngII treated groups compared to PBS controls for WT, C3 and C3CD14 deficient mice ($p= 0.04$, 0.04 and 0.003, respectively) with a similar tendency in CD14 deficient mice with no significant differences between the genotypes (Figure 4A). Plasma C5a levels measured by ELISA did not differ in PBS compared to AngII treated mice, neither was there any difference between the different genotypes (Figure 4B). Finally, cardiac gene expression levels showed a tendency towards an increased expression of IL-6 in WT and C3 deficient mice infused with AngII compared to PBS controls ($p=0.05$ and 0.07, respectively, Figure 4C), and notably, this increase was markedly attenuated in CD14 and C3CD14 deficient mice ($p=0.02$ versus WT for both; Figure 4C).

Discussion

Innate immune activation is suggested to be involved in the cardiac remodelling response to pressure overload [26]. The complement system and TLRs represent two interacting arms of innate immunity [27]. We investigated if a single or combined inhibition of these systems could improve the adverse cardiac response to chronic infusion of AngII As expected we found that AngII infusion led to increased SBP and a mild concentric hypertrophic response, as well as cardiac fibrosis. However, deficiency in C3, CD14 or a combination thereof did not influence SBP nor structure, function or fibrosis within the myocardium, suggesting that C3 and/or CD14 inhibition does not affect the maladaptive remodelling.

Evidence from recent studies indicates that complement inhibition at the C5a-C5aR1 axis could be beneficial in cardiac pressure overload [15, 18]. In contrast, herein we show that elimination of C3, a component that is proximal to C5 in the complement system, did not affect the cardiac response up to 7 days of AngII infusion. Our results are, however, in line with those of Coles *et al.* and also with findings in C3aR deficient mice in a similar model of cardiac hypertrophy [15, 28].

The apparent discrepancies in results from studies that target C5a/C5aR1 *versus* our approach of targeting C3 could have several explanations. First, not all studies have been able to establish a role for C5aR1 in cardiac remodelling. In a model of cardiac pressure overload induced by transverse aortic constriction, Haan *et al*. did not find any effect of C5aR1 deficiency on cardiac hypertrophy and cardiac function [29]. Additionally, a very recent study by Chen *et al.* revealed that C5aR1 and C3aR double deficiency prevented AngII-induced blood pressure elevation and kidney injury, whereas single deficiency of C3aR and C5aR1 did not [17]. In contrast, Weiss *et al.* used an aggravated model of hypertension with unilateral nephrectomized mice receiving AngII and salt, found that whereas C5aR1 deficiency attenuated renal injury, cardiac injury was accelerated with significantly increased cardiac fibrosis and

heart weight in C5aR1-deficient mice [30]. Finally, although the literature frequently refers to C3a, as an inflammatory anaphylatoxin, accumulating evidence indicates that C3a also may have important anti-inflammatory effects [14]. Thus, elimination of C3 may not only reduce the inflammatory consequences of C5a generation, but also potentially the beneficial effects of C3a, and these could neutralize each other.

CD14 acts as a co-receptor for TLR4 *e.g.* by enhancing LPS responsiveness. However, CD14 has also been suggested as an accessory molecule for other TLRs (*i.e.* TLR2, TLR3, TLR7, and TLR9) [19]. Several studies support a role for CD14 in infectious diseases [22], and importantly, CD14-deficiency protects against LPS-induced cardiac inflammation and dysfunction [21, 31]. To the best of our knowledge this is the first study that addresses the role of CD14 in sterile cardiac remodelling. Notably, deficiency in CD14 and C3CD14, but not C3 defiency, markedly decreased cardiac expression levels of IL-6, supporting an anti-inflammatory effect within the myocardium in the absence of CD14. CD14 deficiency was, however, not protective in measures of other parameters involved in cardiac remodelling (*e.g.* fibrosis levels, echocardiographic parameters). The reason for this apparently discrepancy is at present not clear, but could suggest that other inflammatory mediators are of more importance than IL-6 in AngII induced cardiac remodelling. Thus, neither C3 nor CD14 deficiency attenuated cardiac macrophage infiltration in AngII- treated mice and these cells have been implicated in adverse cardiac remodelling following AngII infusion [32].

The AngII model used in the current study induced an acute, but mild version of cardiac hypertrophy, accompanied by marked cardiac fibrosis and inflammation. The reported AngII-induced increase in SBP and coherent cardiac remodelling varies among different mouse strains [33, 34]. The C57B1/6J mice used in this study, displayed a rather low increase in SBP, which potentially could explain the modest cardiac remodelling and coherent inflammatory response. As such, in the study by Zhang *et al*., although they used the same mouse background at about

the same age, the same AngII dose and the same study period as in the present study they showed higher elevation of SBP and a higher collagen deposition in response to AngII infusion [18]. Thus, it could be argued that complement-dependent mechanisms potentially come into play at a higher level of cardiac stress, but at present, there is no evidence to support this hypothesis.

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Disclosures

None

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Figure Legends

Figure 1. Heart weight and lack of cardiomyocyte hypertrophy in WT, C3, CD14 and C3CD14 deficient mice after AngII infusion. Left ventricle (LV) (**A**), total heart (**B**), and lung (**C**) weights measured 7 days after AngII infusion and normalized by tibia length (TL) in mice deficient for C3 (PBS, n=6; AngII, n=14), CD14 (PBS, n=5; AngII, n=10) and C3CD14 (PBS, n=7; AngII, n=12) as well as in wild type (WT) mice (PBS, n=8; AngII, n=10). Cardiomyocyte size was determined by measuring cardiomyocyte cross-sectional area in Wheat germ agglutinin-stained cardiac sections (**D-E,** left panel for PBS and right panel for AngII treated. Scale bars are 100 μM). Values are mean ± standard error of mean (SEM), *p<0.05; **p<0.01; ***p<0.001 vs PBS with 2-way ANOVA and Fisher's LSD *post hoc* test.

Figure 2. Cardiac function and increased cardiac wall stress after AngII infusion in both WT and mice lacking C3, CD14 and C3CD14. Echocardiographic parameters of (**A**) end diastolic volume (EDV), (**B**) end systolic volume (ESV), and (**C**) stroke volume were measured at day 6 in both PBS and their corresponding AngII infused mice. Morphometric data are normalized by tibia length (TL). Cardiac mRNA expression of atrial natriuretic peptide (ANP) was assessed after 7 days of AngII infusion (**D**) and was normalized by GAPDH as a housekeeping gene. WT: PBS, n=4; AngII, n=8, $C3^{-/-}$: PBS, n=6; AngII, n=14, $CD14^{-/-}$: PBS, n=6; AngII, n=10, $C3CD14^{-/-}$: PBS, n=6; AngII, n=10. Values are mean± standard error of the mean (SEM), *p<0.05; **p<0.01; ***p<0.001 vs PBS with 2-way ANOVA and Fisher's LSD *post hoc* test.

Figure 3. Cardiac fibrosis in WT and mice lacking C3, CD14 and C3CD14 after AngII infusion. Heart sections were stained with Sirius Red to visualise fibrosis area (**A**) after 7 days of AngII infusion in both PBS and AngII treated mice (**B**, left and right panel, respectively. Scale bars are 100 μM). Cardiac mRNA expression of collagen I (**C**), collagen III (**D**) were

assessed after 7 days of AngII infusion in both AngII and PBS treated mice. Values are normalized by GAPDH as a housekeeping gene (C-D). Total myocardial collagen content, quantitative analysis of tissue contents of hydroxyproline (**E**) was measured after 7 days of AngII infusion in both AngII and PBS treated mice. WT: PBS, n=4; AngII, n=8, $C3^{-/-}$: PBS, n=6; AngII, n=14, $CD14^{-/-}$: PBS, n=6; AngII, n=10, $C3CD14^{-/-}$: PBS, n=6; AngII, n=10. Values are mean± standard error of the mean (SEM), *p<0.05; **p<0.01; ***p<0.001 vs PBS with 2-way ANOVA and Fisher's LSD *post hoc* test.

Figure 4. Circulating and Cardiac inflammatory markers in WT and mice deficient for C3, CD14 and C3CD14 after AngII infusion. Seven days after AngII infusion (**A**) ELISA was used to measure plasma C5a levels, (**B-C**) Mac2 staining was used to visualise macrophages in heart sections (scale bars are 200 μM), (**D)** qPCR gene expression was performed to examine cardiac expression levels of IL-6. Values are normalized by GAPDH as a housekeeping gene. WT: PBS, n=4; AngII, n=8, C3-/-: PBS, n=6; AngII, n=14, CD14-/-: PBS, n=6; AngII, n=10, C3CD14-/-: PBS, n=6; AngII, n=10. Values are mean± standard error of the mean (SEM), $^{\#}$p<0.05 vs WT with 2-way ANOVA and Fisher's LSD *post hoc* test.

Figures

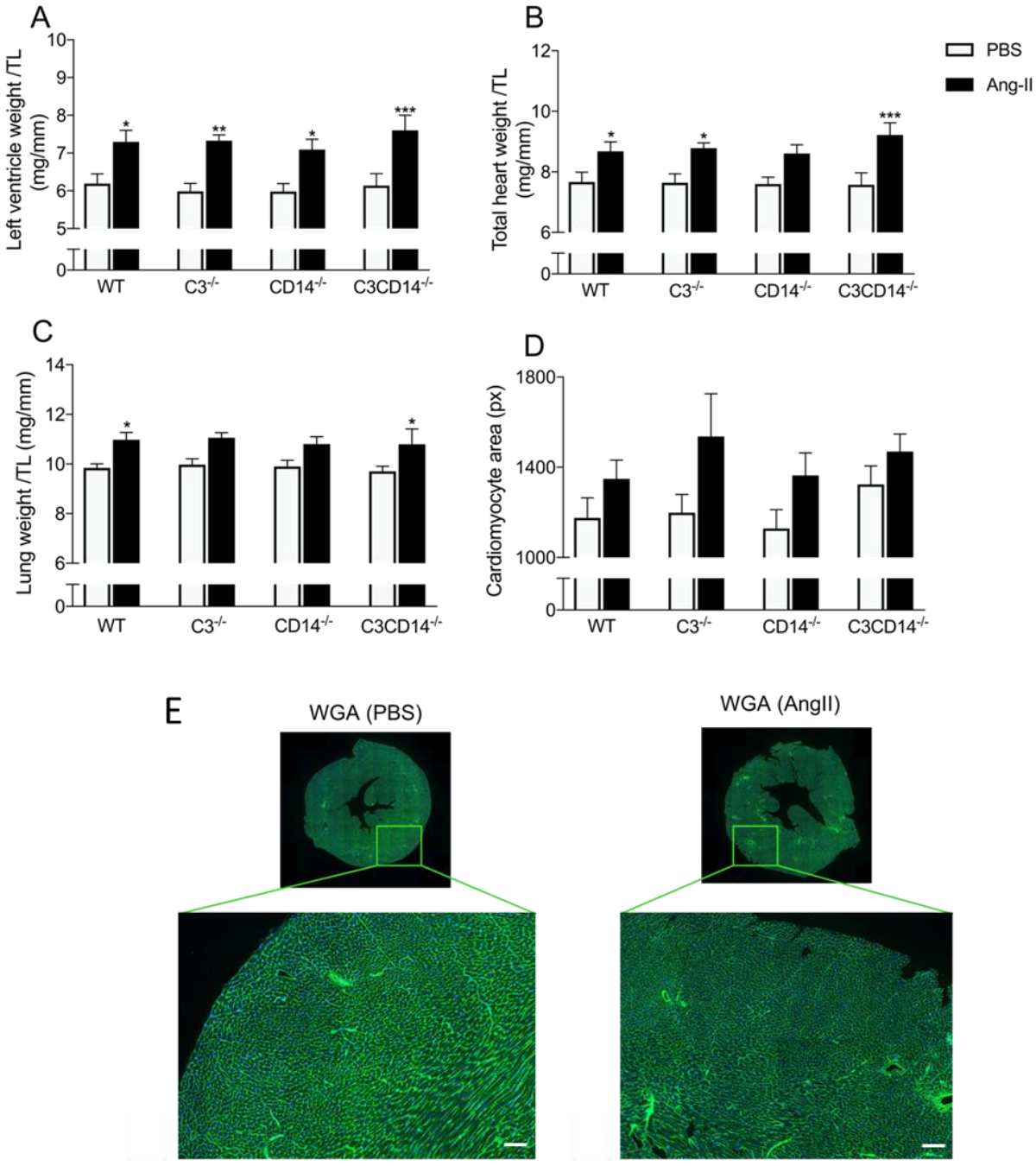

Figure 1.

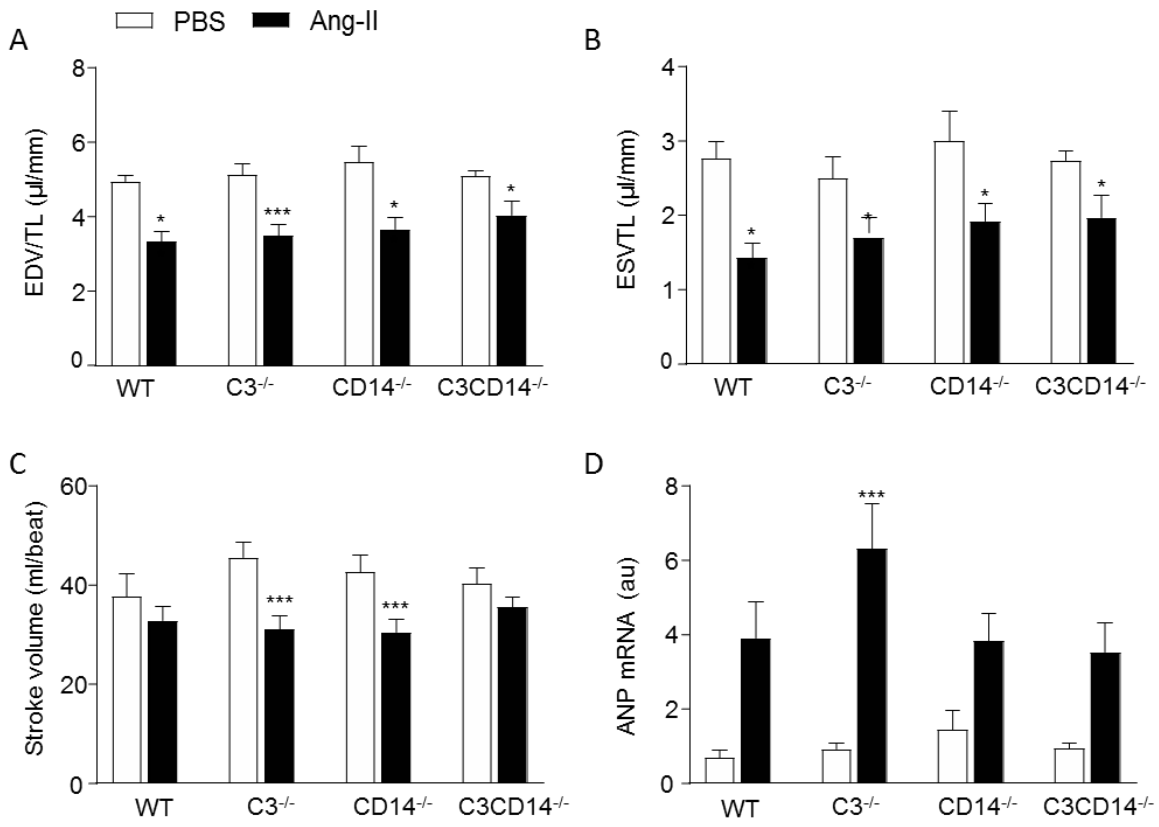
A
PBS
Ang-II
EDV/TL (µl/mm)
B
ESVTL (µl/mm)
C
Stroke volume (ml/beat)
D
ANP mRNA (au)
WT
C3-/-
CD14-/-
C3CD14-/-

Figure 2.

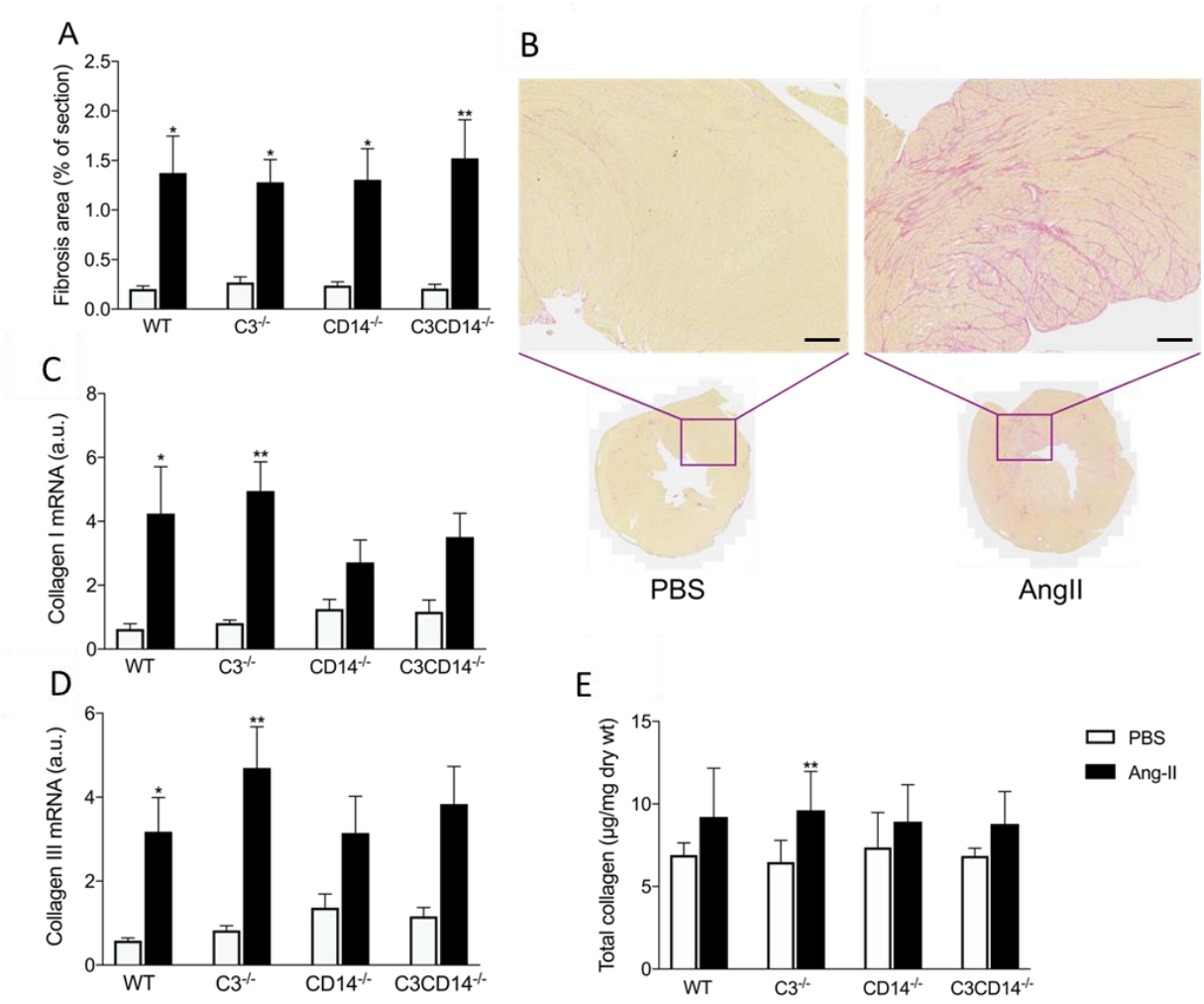
A
Fibrosis area (% of section)
2.5
2.0
1.5
1.0
0.5
0.0
WT
C3-/-
CD14-/-
C3CD14-/-
B
PBS
AngII
C
Collagen I mRNA (a.u.)
8
6
4
2
0
WT
C3-/-
CD14-/-
C3CD14-/-
D
Collagen III mRNA (a.u.)
6
4
2
0
WT
C3-/-
CD14-/-
C3CD14-/-
E
Total collagen (µg/mg dry wt)
15
10
5
0
WT
C3-/-
CD14-/-
C3CD14-/-
PBS
Ang-II

Figure 3.

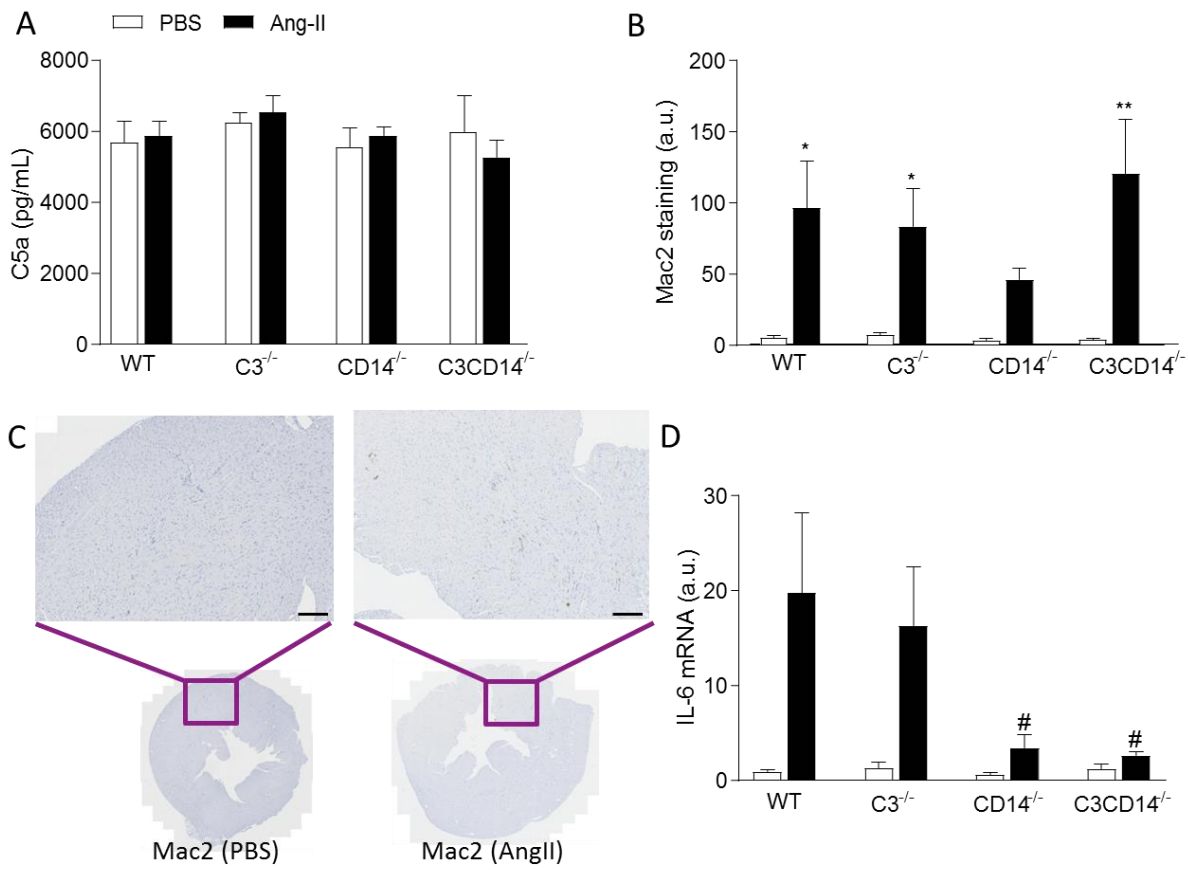
A
PBS
Ang-II
C5a (pg/mL)
8000
6000
4000
2000
0
WT
C3-/-
CD14-/-
C3CD14-/-
B
Mac2 staining (a.u.)
200
150
100
50
0
*
*
**
WT
C3-/-
CD14-/-
C3CD14-/-
C
Mac2 (PBS)
Mac2 (AngII)
D
IL-6 mRNA (a.u.)
30
20
10
0
#
#
WT
C3-/-
CD14-/-
C3CD14-/-

Figure 4.