

Short communication

Small molecule-mediated inhibition of CD40-TRAF6 reduces adverse cardiac remodelling in pressure overload induced heart failure☆

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ABSTRACT

Background: CD40 signalling is involved in chronic inflammation, a condition that plays an important role in non-ischemic heart failure (HF). Small molecule inhibitors of CD40-TRAF6 have shown to be effective in multiple animal-models of chronic inflammatory disease, such as obesity and atherosclerosis.

Methods & results: Mice were subjected to transverse aortic constriction (TAC) and randomized to small molecule inhibition of CD40-TRAF6 or placebo. CD40-TRAF6 inhibition resulted in less cardiac remodelling 10 weeks after TAC with a reduced end systolic volume (TAC-placebo group: 71.9 ± 8.8 vs TAC-CD40-TRAF6 inhibitor: $53.7 \pm 6.1 \mu\text{l}$, $p = 0.03$) and improved ejection fraction (EF) compared to placebo (TAC-placebo group: 25.6 ± 2.8 vs TAC-CD40-TRAF6 inhibitor: $35.5 \pm 3.3\%$, $p = 0.02$). Within the myocardium, CD40-TRAF6 inhibition resulted in decreased macrophage and T-cell infiltration 10 weeks after TAC compared to placebo. In addition, a decrease in fibrosis and cardiomyocyte hypertrophy was observed in the CD40-TRAF6 inhibitor group compared to placebo.

Conclusion: CD40-TRAF6 inhibition improves cardiac function in non-ischemic HF in mice. This effect is mediated by a reduction in macrophage and T-cell influx in the myocardium, accompanied by a reduction in cardiac fibrosis and hypertrophy.

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1. Introduction

The development of heart failure (HF) is characterized by adverse remodelling of the myocardium and involves processes such as hypertrophy and fibrosis. It is appreciated that immune activation and chronic inflammation play an important role in HF initiation and progression [1]. Co-stimulatory molecules are critical regulators of inflammation and have been implicated in chronic inflammatory diseases such as atherosclerosis, hypertension and obesity. The CD40-CD40L receptor/ligand is a well-known co-stimulatory pathway and a known driver of chronic inflammatory diseases [2]. The immune checkpoint protein CD40 is expressed on the surface of a variety of immune cells such as monocytes, dendritic cells and also on non-immune cells including endothelial cells [2]. The ligand of CD40, CD40L, is mainly expressed

on activated T-cells and also exists as soluble protein (sCD40L). Circulating levels of sCD40L are elevated in patients with HF and correlate with left ventricular (LV) dysfunction and clinical severity [3]. Also hypertensive patients show increased sCD40L plasma levels. In animal models of angiotensin II induced hypertension CD40L/CD40 interactions are instrumental in aortic inflammation, immune cell recruitment and disease progression [4]. Activation of CD40 leads to intracellular recruitment of TNF receptor-associated factors (TRAFs). The intracellular C-terminus of CD40 has a distal binding site able to bind to TRAF 2, 3, and 5 and a proximal binding site for TRAF6 [5]. CD40-TRAF6 signalling, and not CD40-TRAF 2,3,5 signalling, is involved in the development of insulin resistance [6], obesity [7] and atherosclerosis [8] and small molecule inhibitors of CD40-TRAF6 interaction were effective in animal models of these chronic inflammatory diseases. Inhibition leads to less macrophage recruitment and reductions in CD4⁺ and CD8⁺ T-cells [6,7,9]. In the current study the CD40-TRAF6 inhibitor (6877002) was tested in a mouse model of transverse aortic constriction (TAC) leading to non-ischemic HF. It was previously shown that TRAF-STOPs (including 6877002) abolish CD40-TRAF6, and not CD40-TRAF2/3/5 interactions [9].

☆ All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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2. Material and methods

A comprehensive material & methods section can be found in the supplemental material. In brief, 10–12 week old male C57BL/6 mice (Charles River) were subjected to TAC or sham surgery as described previously [10]. All experiments were conducted with consent of the Central Commission for Animal Research, The Hague, The Netherlands. Mice were randomized to either placebo (vehicle) or CD40-TRAF6 inhibition every other day i.p. for the duration of 10 weeks, starting after TAC surgery. All analysis, including echocardiography and histology, were performed by investigators blinded for group assignments. Data are expressed as mean \pm SEM. A two-sided p -value of 0.05 was regarded statistically significant.

3. Results

3.1. Adverse cardiac remodelling is attenuated by CD40-TRAF6 inhibition

CD40-TRAF6 inhibitor treated animals showed reduced cardiac hypertrophy, indicated by decreased heart weight/tibia length (Fig. 1A&B: TAC-placebo: 1.71 ± 0.12 vs TAC-CD40-TRAF6 inhibitor: 1.40 ± 0.07 g/mm, $p = 0.03$). LV-mass did not differ between groups up to 4 weeks of TAC. After 6 weeks of TAC the inhibitor treated animals

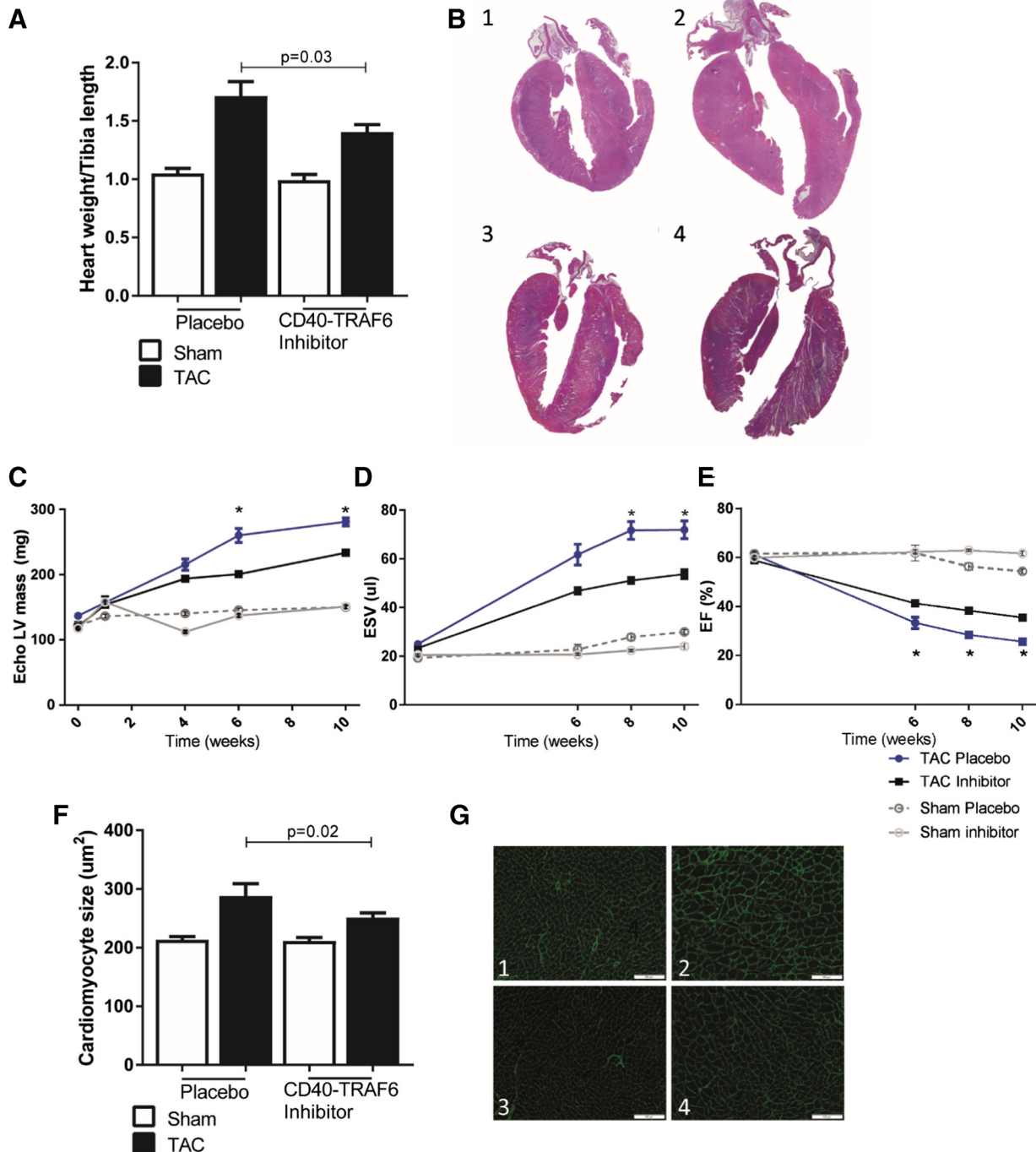


Fig. 1. A. Reduced heart weight/tibia length in CD40-TRAF6 inhibitor treated animals after TAC compared to placebo. B. Representative images of HE staining of 1) sham-placebo, 2) TAC-placebo, 3) sham-CD40-TRAF6 inhibitor, 4) TAC-CD40-TRAF6 inhibitor. C. Echo LV-mass, D. end systolic volume (ESV) and E. ejection fraction (EF) timelines. F&G. WGA-staining showing reduced cardiomyocyte size after CD40-TRAF6 inhibitor treatment compared to placebo. $n = 6$ for sham placebo, sham CD40-TRAF6 inhibitor, TAC placebo and $n = 11$ for TAC CD40-TRAF6 inhibitor. * $p < 0.05$ TAC CD40-TRAF6 inhibitor vs TAC placebo.

showed a significantly lower LV-mass compared to placebo ($p = 0.05$) which was maintained 10 weeks after TAC ($p = 0.02$; Fig. 1C).

On echocardiography the CD40-TRAF6 inhibitor treated animals showed less cardiac remodelling ten weeks after TAC. End systolic volume (ESV) was significantly lower (Fig. 1D: TAC placebo: 71.9 ± 8.8 vs TAC CD40-TRAF6 inhibitor: $53.7 \pm 6.1 \mu\text{l}$, $p = 0.03$) resulting in improved ejection fraction (EF) (Fig. 1E: TAC placebo: 25.6 ± 2.8 vs TAC CD40-TRAF6 inhibitor: $35.5 \pm 3.3\%$, $p = 0.02$). No differences were observed in global longitudinal strain or reverse longitudinal strain rate. Supplemental Table I summarizes echocardiographic data. The level of cardiomyocyte hypertrophy was assessed using WGA staining (Fig. 1F&G). As expected TAC induced hypertrophy and a significant decrease in cell size was witnessed after CD40-TRAF6 inhibition ($252 \pm 7.5 \mu\text{m}^2$) compared to placebo ($288 \pm 20.8 \mu\text{m}^2$, $p = 0.02$).

3.2. CD40-TRAF6 inhibition reduced myocardial macrophage and T-cell influx and was accompanied by a reduction in cardiac fibrosis

Ten weeks after TAC decreased macrophage influx in the myocardium was seen in CD40-TRAF6 inhibitor treated animals (Fig. 2A&B: TAC-placebo: 12.9 ± 2.3 vs TAC-CD40-TRAF6 inhibitor: 6.1 ± 1.7 cells/ mm^2 , $p = 0.01$). No difference in the number of circulating monocytes or monocytes in draining lymph nodes was present (data not shown). A trend toward decreased expression of pro-inflammatory M1-macrophage related genes (CD68, MHCII & TNF α , supplemental Fig. 1A–C) was observed in the myocardium, while within the draining lymph nodes a non-significant increase in CD206 $^{+}$ -macrophages (M2-phenotype) was seen in inhibitor treated animals (supplemental Fig. 1D). CD3 $^{+}$ T-cell influx in the myocardium increased after TAC in

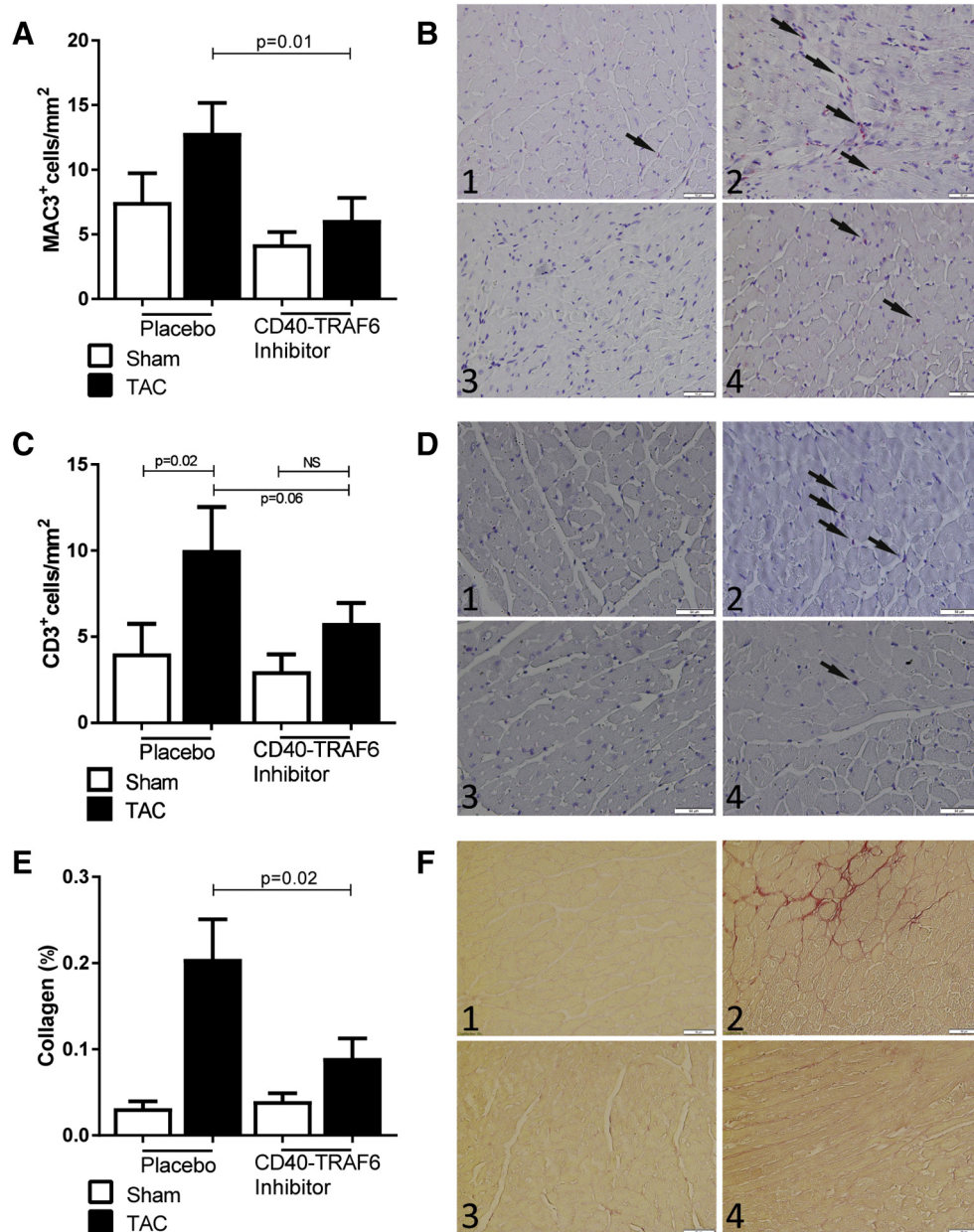


Fig. 2. A&B. Decreased macrophage influx in the myocardium in CD40-TRAF6 treated animals compared to placebo 10 weeks after TAC. C&D. Increased CD3 $^{+}$ T-cell infiltration after TAC in the placebo group, this increase was not seen in the CD40-TRAF6 inhibitor treated animals. E&F. Reduced collagen percentage in the myocardium 10 weeks after TAC compared to placebo. Order representative images 1) sham-placebo, 2) TAC-placebo, 3) sham-CD40-TRAF6 inhibitor, 4) TAC-CD40-TRAF6 inhibitor. $n = 6$ for sham placebo, sham CD40-TRAF6 inhibitor, TAC placebo and $n = 11$ for TAC CD40-TRAF6 inhibitor.

the placebo group, while this influx was not observed in the treated animals (Fig. 2C&D: TAC-placebo: 10.0 ± 2.5 vs sham-placebo: 4.0 ± 1.7 , $p = 0.02$ and TAC-CD40-TRAF6 inhibitor: 5.8 ± 1.2 vs sham-CD40-TRAF6 inhibitor: 3.0 ± 1.0 cells/mm², $p = 0.2$, TAC-placebo vs TAC-CD40-TRAF6, $p = 0.06$). No difference was seen in CD4⁺ or CD8⁺ T-cells in draining lymph nodes or the circulation (supplemental Fig. 1E–H). The reduction of infiltrating macrophages and T-cells was accompanied by a 2.3-fold decrease in ventricular fibrosis in CD40-TRAF6 inhibitor treated hearts after TAC (Fig. 2E&F, $p = 0.02$). No differences were seen in cell death by TUNEL staining (supplemental Fig. 1J). IL-6, IL-1 β , TNF α , IFN γ and MCP1 levels were below detection limit in the plasma 10 weeks after TAC. In the myocardium 10 weeks after TAC only TNF α was detectable and no differences were seen between the groups (TAC placebo: 1.28 ± 0.12 , TAC inhibitor 1.36 ± 0.13 , sham placebo: 1.7 ± 0.15 , sham inhibitor: 1.75 ± 0.34 pg/mg).

4. Discussion

In the present study we were able to successfully reduce adverse cardiac remodelling in a mouse model of pressure overload induced HF by targeting the CD40-TRAF6 interaction using a small molecule inhibitor. Processes involved in adverse cardiac remodelling such as fibrosis and hypertrophy are, at least partly, mediated by immune mechanisms [11]. Progression of non-ischemic HF, induced by pressure overload, involves a significant inflammatory component, negatively impacting disease outcome [11,12]. Especially in the field of HF there is room for new anti-inflammatory treatment strategies, and modulating immune checkpoints, including co-stimulatory molecules, were effective in models of other chronic inflammatory diseases [6,7,9]. In a recent study abatacept, an FDA approved drug inhibiting CD80/86-induced co-stimulatory activation of T-cells resulted in a ~25% increase in EF after TAC, in similar degree to our results with CD40-TRAF6 inhibition [12].

In the current study, a reduction in macrophage influx was observed in CD40-TRAF6 inhibitor treated mice after TAC. This is in line with previous studies using CD40-TRAF6 inhibitors, showing for instance a reduction in macrophage influx in the atherosclerotic plaque [9]. Atherosclerotic CD40-TRAF6 deficient mice display a polarization of macrophages toward an anti-inflammatory regulatory M2-signature [8]. We were unable to show statistical significant differences in pro-inflammatory M1 or anti-inflammatory M2-macrophages. However, in concordance with previous findings on the influence of CD40-TRAF6 inhibition on polarization of macrophages toward an anti-inflammatory M2-signature [8] we hypothesise that a shift toward M2-macrophages might be present in the treated animals after TAC. In addition, T-cell influx in the myocardium significantly increased after TAC in the placebo group, while this increase was not witnessed after CD40-TRAF6 inhibition. T-cells have been implicated in fibrotic disease, and suggested to be involved in the development of cardiac fibrosis. T-helper-1 (Th1) cells in particular can selectively activate cardiac fibroblast, resulting in the production of TGF- β and subsequently fibrosis [13]. Also, inhibition of T-cell co-stimulation with abatacept, completely inhibited fibrosis after TAC, resulting in reduced hypertrophy [12]. In agreement, we show that CD40-TRAF6 inhibition results in a reduction of cardiac fibrosis and hypertrophy, possibly due to the reduction of T-cell influx.

Limitations: This proof of concept study showed that CD40-TRAF6 inhibition has potential therapeutic implications in non-ischemic HF. However, treatment was started directly after TAC placement, before initiation of cardiac remodelling. In clinical practice, patients usually present

with when adverse remodelling already started. Future translational studies will have to establish if and when anti-inflammatory strategies, including CD40-TRAF6 inhibition, are effective in already established HF. This temporal approach is required to translate findings from animal studies to the large number of chronic HF patients worldwide [1].

In conclusion, small molecule inhibition of CD40-TRAF6 reduces adverse remodelling in pressure overload induced HF in mice. A reduction of macrophage and T-cell influx was seen in the myocardium, accompanied by a reduction in cardiac fibrosis and hypertrophy.

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Disclosures

Nothing to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijcard.2018.12.076>.

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