Interleukin-1 has Opposing Effects on Connective Tissue Growth Factor and Tenascin-C Expression in Human Cardiac Fibroblasts

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Abstract

Cardiac fibroblasts (CF) play a central role in the repair and remodeling of the heart following injury and are important regulators of inflammation and extracellular matrix (ECM) turnover. ECM-regulatory matricellular proteins are synthesized by several myocardial cell types including CF. We investigated the effects of pro-inflammatory cytokines on matricellular protein expression in cultured human CF. cDNA array analysis of matricellular proteins revealed that interleukin-1α (IL-1α, 10 ng/ml, 6 h) down-regulated connective tissue growth factor (CTGF/CCN2) mRNA by 80% and up-regulated tenascin-C (TNC) mRNA levels by 10-fold in human CF, without affecting expression of thrombospondins 1-3, osteonectin or osteopontin. Western blotting confirmed these changes at the protein level. In contrast, tumor necrosis factor α (TNFα) did not modulate CCN2 expression and had only a modest stimulatory effect on TNC levels. Signaling pathway inhibitor studies suggested an important role for the p38 MAPK pathway in suppressing CCN2 expression in response to IL-1α. In contrast, multiple signaling pathways (p38, JNK, PI3K/Akt and NFκB) contributed to IL-1α-induced TNC expression. In conclusion, IL-1α reduced CCN2 expression and increased TNC expression in human CF. These observations are of potential value for understanding how inflammation and ECM regulation are linked at the level of the CF.

Key words: cardiac fibroblasts; interleukin-1; connective tissue growth factor; tenascin-C; signal transduction

1. Introduction

The acute inflammatory response observed following myocardial infarction (MI)[1] plays an integral role in tissue repair and the heart's adaptation to injury (Nian et al., 2004; Frangogiannis et al., 2002). However, inflammatory mediators can also be detrimental and promote adverse cardiac remodeling and heart failure progression (Nian et al., 2004). The pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) are two key players in the myocardial inflammatory response to injury. The inflammatory cells that infiltrate the infarct zone are the predominant source of these cytokines in the early stages following MI, however in the longer-term pro-inflammatory cytokines may be produced by myocardial cells at sites remote from the initial injury (Irwin et al., 1999; Deten et al., 2002). In addition to hematopoietic cells, cardiomyocytes and fibroblasts (the two major resident cell types of the heart) have the capacity to secrete TNFα, and fibroblasts appear to be the main source of IL-1 (Jugdutt, 2003; Long, 2001). In turn these cytokines can have profound effects on the pathophysiology of cardiac myocytes and fibroblasts. Cardiomyocytes undergo apoptosis and/or hypertrophy in response to many pro-inflammatory cytokines, whilst fibroblasts adopt a myofibroblast phenotype and undergo increased proliferation and collagen turnover and thereby contribute to adverse post-MI remodeling (Porter and Turner, 2009; Jugdutt, 2003). IL-1 and TNFα have each been shown to induce distinct patterns of extracellular matrix (ECM) protein and/or protease expression in the heart that contribute to adverse remodeling in heart failure (Bradham et al., 2002; Turner et al., 2010; Turner and Porter, 2012).

The matricellular proteins are a group of ECM proteins that are synthesized by a number of different cell types including fibroblasts, macrophages and endothelial cells

[1] Abbreviations: CF, cardiac fibroblast; CCN2/CTGF, connective tissue growth factor; ECM, extracellular matrix; HK, housekeeping gene; IL-1, interleukin-1; MI, myocardial infarction; TGFβ, transforming growth factor beta; TNC, tenascin-C; TNF, tumor necrosis factor.

(Frangogiannis, 2012). However, unlike structural matrix proteins, they do not play a principal role in tissue architecture, but serve as biological mediators of cell function by interacting with cells directly or by modulating the activity of cytokines, growth factors, proteases and other ECM proteins (Frangogiannis, 2012). Matricellular proteins can regulate cell movement, proliferation and differentiation during morphogenesis and the inflammatory and reparative response following tissue injury. The expanding family of matricellular proteins includes the thrombospondins (THBS 1-5), the CCN family (most notably CCN2; also known as connective tissue growth factor, CTGF), osteonectin (SPARC), osteopontin (SPP1), tenascin-C (TNC), tenascin-X, periostin and galectin (Frangogiannis, 2012).

Most matricellular proteins show increased expression in the heart following MI or stress where they act as transducers of key molecular signals in cardiac repair and as modulators of cell migration, proliferation and adhesion (Frangogiannis, 2012). Hence they play a vital function in tissue repair and post-MI remodeling. There is however, increasing evidence to suggest that the matricellular proteins may also modulate the inflammatory and fibrotic process in myocardial remodeling, which leads to cardiac dysfunction and heart failure (Dobaczewski et al., 2010; Okamoto and Imanaka-Yoshida, 2012; Schellings et al., 2004).

The aims of the present study were to determine whether IL-1 and TNFα could modulate expression of matricellular proteins in human cardiac fibroblasts (CF) and to identify the intracellular signaling pathways responsible for these effects.

2. Results

2.1. Myofibroblast phenotype

Human CF spontaneously adopted a myofibroblast phenotype when cultured *in vitro,* as defined by co-expression of α-smooth muscle actin and vimentin (data not shown), in agreement with our previous reports (Porter et al., 2004b; Turner et al., 2003; Mughal et al.,

2009). Such a phenotypic transition bears similarities to that observed when CF differentiate to myofibroblasts *in vivo* (Santiago et al., 2010) and so our findings with cultured human CF are particularly relevant to myofibroblast function in the remodeling heart.

2.2. Effect of IL-1α on mRNA expression of matricellular proteins in human CF

CF were stimulated with 10 ng/ml IL-1α for 6 h before extracting RNA and measuring mRNA levels of seven matricellular proteins as part of a focused RT-PCR array (Table 1). Although the array did not include all the known members of the matricellular protein family, it did facilitate evaluation of the most well-studied members. Unstimulated cells expressed particularly high basal mRNA levels of THBS1, SPARC (osteonectin) and CTGF/CCN2, and appreciable levels of THBS2. Basal TNC and THBS3 mRNA levels were relatively low, and SPP1 (osteopontin) levels were below the threshold of detection. IL-1α treatment had opposing effects on CCN2 and TNC expression; it decreased CCN2 expression by 80% and increased TNC expression by almost 10-fold (Table 1). mRNA expression levels of the other matricellular proteins were not markedly altered in response to IL-1α treatment (Table 1).

2.3. Opposing effects of IL-1α on CCN2 and TNC expression

Additional experiments were performed to confirm the array results for CCN2 and TNC. CF from 3 different patients were stimulated with IL-1α, RNA extracted over a 2-24 h time course, and CCN2 and TNC mRNA levels determined by real-time RT-PCR with Taqman primer/probes sets. In agreement with the 6 h array data, IL-1α potently reduced CCN2 mRNA levels, and this reduction of up to 80% was maintained over the full 2-24 h time course (Fig. 1A). TNC mRNA levels were increased over the same time period, with peak expression (10-fold increase) observed 4 h after IL-1α treatment, and levels remaining elevated by 7-fold even 24 h after IL-1α stimulation (Fig. 1B).

Western blotting studies confirmed robust basal expression of CCN2 protein by human CF that was rapidly suppressed by IL-1α (Fig. 1C). CCN2 protein levels were decreased by 80% within 4 h of IL-1α treatment, and remained low for at least 24 h (Fig. 1C). CCN2 resolved as a doublet of 35-40 kDa, in agreement with previous studies (Abraham et al., 2000). Western blotting also revealed that IL-1α stimulated protein expression of TNC, which was elevated 3.2-fold within 6 h and continued to rise to a 10-fold induction by 24 h (Fig. 1D). TNC resolved as a doublet of >250 kDa, in agreement with previous studies (Nakoshi et al., 2008).

2.4. Comparison of effects of IL-1α and TNFα

We have previously shown that TNFα is another important proinflammatory cytokine that can induce human CF migration, proliferation and altered gene expression (Porter et al., 2004a; Turner et al., 2007; Turner et al., 2011). We therefore compared the effects of TNFα (10 ng/ml, 6 h) with those of IL-1α (10 ng/ml, 6 h) on CCN2 and TNC mRNA and protein expression in a side-by-side manner. In stark contrast to the 70-80% inhibition in CCN2 mRNA and protein observed with IL-1α, TNFα treatment had no significant effect on CCN2 mRNA (Fig. 2A) or protein (Fig. 2C) levels. In contrast, TNFα increased TNC mRNA levels by 2.6-fold (Fig. 2B), and appeared to induce TNC protein expression slightly (Fig. 2D), although this was not statistically significant and was much less than the response to IL-1α.

2.5. Signaling pathways mediating the effects of IL-1α on CCN2 and TNC expression

IL-1α rapidly stimulates several intracellular signaling pathways in human CF, including ERK, p38 MAPK, JNK, PI3K/Akt and NFκB (Turner et al., 2009). To determine which of these pathways were responsible for directly mediating the effects of IL-1α on CCN2 and

TNC expression, we pretreated cells with selective pharmacological inhibitors (PD98059, SB203580, SP600125, LY294002 and IMD-0354 respectively) before stimulating cells with IL-1α and measuring mRNA levels after 2 h or protein levels after 6 h (Fig. 3 and 4). These relatively early time points were selected to evaluate direct roles of the signaling pathways on gene expression, rather than any contribution via autocrine/paracrine signaling mechanisms. Inhibitors were used at concentrations that we have previously shown to effectively and selectively inhibit relevant pathways in this cell type (Turner et al., 2007).

The IL-1α-mediated decrease in CCN2 expression at both mRNA (Fig. 3A) and protein (Fig. 3B) levels was attenuated by the p38 MAPK inhibitor SB203580, suggesting an important role for p38 MAPK in mediating the inhibitory effects of IL-1α. None of the other inhibitors specifically affected the ability of IL-1α to reduce CCN2 mRNA expression, although SP600125, LY294002 and IMD-0354 did increase basal CCN2 mRNA levels (Fig. 3A). The NFκB pathway inhibitor IMD-0354 overcame the suppressive effect of IL-1α on CCN2 expression at the protein level, resulting in elevated CCN2 levels relative to control cells (Fig. 3B). However, this may have been due to an effect on basal expression, as IMD-0354 alone induced a doubling of CCN2 mRNA levels in the absence of IL-1α (Fig. 3A).

The IL-1α-induced increase in TNC mRNA expression was partially attenuated by inhibitors of p38 MAPK, JNK, PI3K/Akt and NFκB pathways, but not the ERK pathway inhibitor PD98059 (Fig. 4A). PD98059 was similarly ineffective in modulating IL-1α-induced TNC protein levels, whereas the other inhibitors partially reduced TNC protein levels, with the NFκB pathway inhibitor IMD-0354 being most effective (60% inhibition) (Fig. 4B).

3. Discussion

The key finding of our study was that IL-1α simultaneously down-regulated CCN2 and up-

regulated TNC expression in human CF, without affecting expression of several other matricellular proteins. The TNC response to IL-1α was reproducibly higher than that induced by TNFα, whereas TNFα had no significant effect on CCN2 expression in these cells. Signaling inhibitor studies suggested an important role for the p38 MAPK pathway in IL-1α-mediated suppression of CCN2 levels, whereas a combination of several pathways (p38, JNK, PI3K/Akt and NFκB) contributed to IL-1α-induced TNC expression.

It is clear that the matricellular proteins play important roles in cardiac pathophysiology (Schellings et al., 2004; Frangogiannis, 2012). These molecules are minimally expressed in the healthy adult heart but are markedly upregulated in the injured and remodeling myocardium, where they regulate inflammatory, reparative, fibrotic and angiogenic processes. THBS1 is expressed in the early stages following MI and serves to limit the expansion of the inflammatory phase of infarct healing and so attenuates the spread of damage to the non-infarcted myocardium (Frangogiannis et al., 2005). In contrast, CCN2, TNC, SPARC and SPP1 are primarily expressed during the granulation and scar formation stages and are important for myofibroblast recruitment, collagen deposition and fiber assembly (Komatsubara et al., 2003; Tamaoki et al., 2005; Dobaczewski et al., 2006; Trueblood et al., 2001; Daniels et al., 2009; McCurdy et al., 2011).

In the present study, we were able to detect mRNA expression of THBS1-3, SPARC, CCN2 and TNC mRNA in human CMF, but SPP1 mRNA was not detectable. The high basal levels of expression of these matricellular proteins was likely due to the activated myofibroblast phenotype observed when human CF are grown in culture. Marked changes in mRNA expression were observed for CCN2 (decrease) and TNC (increase) following stimulation with IL-1α, and the importance of these findings is discussed below.

3.1. CCN2

CCN2 levels are elevated in various fibrotic disorders and it is reported to be an excellent surrogate marker for activated fibroblasts in wound healing and fibrosis (Daniels et al., 2009; Leask et al., 2009). CCN2 is strongly induced by transforming growth factor β (TGFβ) in human dermal fibroblasts (Leask et al., 2003) and there is evidence to suggest that CCN2 is a cofactor required to enhance the fibrotic response to TGFβ (Kennedy et al., 2007; Shi-wen et al., 2006). More recently a direct profibrotic effect of CCN2 (through the transcriptional activation of the collagen I α2 gene) was demonstrated in murine lung fibroblasts (Ponticos et al., 2009), and the loss of CCN2 was shown to provide resistance to bleomycin-induced skin fibrosis (Liu et al., 2011). On the basis of these profibrotic effects, it could be predicted that a down regulation of CCN2 by IL-1α may act to suppress profibrotic mechanisms during the inflammatory phase of post-MI remodeling (van Nieuwenhoven and Turner, 2013).

A recent transgenic study using cardiac-restricted overexpression of CCN2 demonstrated that rather than restricting cardiac function, as might be expected, CCN2 was found to elicit a number of cardioprotective effects, notably the induction of myocardial genes known to be involved in the regulation of cardiac growth and cardioprotection (Ahmed et al., 2011; Gravning et al., 2012). Moreover, mice with this transgene displayed remarkable resistance to dilated cardiomyopathy and heart failure and were protected against ischemia/reperfusion injury (Ahmed et al., 2011; Gravning et al., 2012).

Previous studies in fibroblasts of non-cardiac origin have determined that CCN2 expression can be stimulated by profibrotic molecules such as TGFβ or angiotensin II, and inhibited by proinflammatory molecules such as IL-1 or TNFα. For example, TGFβ-induced CCN2 expression was inhibited by TNFα (Abraham et al., 2000; Beddy et al., 2006; Lin et al., 1998) or IL-1α (Nowinski et al., 2002; Nowinski et al., 2010) in human dermal fibroblasts or fibroblasts from bowel biopsies. In human dermal fibroblasts, IL-1α reduced TGFβ-induced CCN2 transcription via inhibitory effects on Smad3 (Nowinski et al., 2010),

whereas in mouse embryonic fibroblasts stimulated with TNFα a role for Smad4 was proposed (Yu et al., 2009). Although we observed a consistent reduction in CCN2 expression following IL-1α treatment in human CF, no reduction was observed with TNFα. We have previously reported that TNFα responses in human CF are variable between cells from different patients (Porter et al., 2004a). However this observation is unlikely to explain the lack of effect on CCN2 expression, as TNC mRNA levels were elevated by TNFα treatment in identical samples. Other groups have also reported that TNFα does not inhibit basal CCN2 expression in fibroblasts from non-cardiac sources, but can inhibit the TGFβ-induced increment in CCN2 expression (Abraham et al., 2000; Beddy et al., 2006; Yu et al., 2009).

CCN2 gene transcription is known to be positively or negatively influenced by a range of stimuli in different cell types (Daniels et al., 2009; Leask et al., 2009). Positive regulatory signals including TGFβ, angiotensin II, endothelin-1 and mechanical stretch can induce CCN2 gene expression through activation of Smads, protein kinase C, ERK and RhoA signaling pathways (Daniels et al., 2009; Leask et al., 2009). In contrast, inhibitors of CCN2 gene transcription appear to act via the NFκB, cyclic AMP, cyclic GMP and PPARγ signaling pathways (Daniels et al., 2009; Leask et al., 2009). Our data revealed that IL-1α strongly attenuated the high basal CCN2 expression in human CF at both mRNA and protein levels, and that this could be overcome by selective inhibition of the p38 MAPK pathway. The p38 MAPK pathway is an important signaling axis regulating CF function and represents an attractive therapeutic target for ameliorating cardiac dysfunction post-MI (Turner, 2011). Given that SB203580 inhibits the α and β subtypes of p38, but not p38-γ or -δ (Clark et al., 2007), and our previously observation that human CF express the α, γ and δ subtypes of p38, but not p38-β (Sinfield et al., 2013), our data most likely indicate a role for p38-α in the IL-1α-mediated inhibition of CCN2 expression in this cell type.

3.2. TNC

TNC plays an active role in the early stages of tissue repair (Chiquet-Ehrismann and Chiquet, 2003; Imanaka-Yoshida et al., 2001; Tamaoki et al., 2005). Following MI, TNC can promote myocardial repair and prevent ventricular dilation by recruiting myofibroblasts to the site of injury and enhancing collagen fiber contraction (Tamaoki et al., 2005; Toma et al., 2005). On the other hand, TNC may also promote adverse myocardial remodeling. TNC has been shown to up-regulate MMP-2 and MMP-9 expression in a number of cell types (Kalembeyi et al., 2003; Nishiura et al., 2005), enhance inflammatory responses through activation of NFκB and cytokine upregulation (El-Karef et al., 2007; Midwood et al., 2009; Nakahara et al., 2006), and inhibit the strong linkages that occur between cardiomyocytes and connective tissues (Imanaka-Yoshida et al., 2001; Imanaka-Yoshida et al., 2004). Although these functions are useful for clearing damaged tissue and releasing residual cardiomyocytes from connective tissue for rearrangement, they may also contribute to progressive degradation of ECM and slippage of myocytes within the myocardial wall, ultimately resulting in left ventricle wall thinning and dilation. Consistent with this, high serum levels of TNC in patients following MI have been associated with a greater incidence of adverse cardiac remodeling and poor prognosis, supporting the view that excessive and sustained increments of TNC could lead to detrimental myocardial remodeling (Sato et al., 2006).

Our data revealed that IL-1α strongly induced TNC expression in human CF, and that this could be partially reduced by inhibitors of the p38, JNK, PI3K/Akt and NFκB pathways, with the NFκB inhibitor being most potent. A recent study investigating the effects of IL-1β on TNC expression in synovial fibroblasts reported an upregulation of TNC expression, but only under hypoxic conditions (Tojyo et al., 2008). Although little is known regarding the mechanisms underlying transcription of the human TNC gene, there is accumulating evidence that the NFκB pathway is pivotal (Nakoshi et al., 2008; Goh et al., 2010). In a study on

cultured human chondrocytes, NFκB inhibition was found to prevent TNFα-induced TNC mRNA and protein expression (Nakoshi et al., 2008). Moreover, in human monocyte-derived dendritic cells, lipopolysaccharide was shown to induce TNC expression via activation of the NFκB and PI3K/Akt pathways, but not the p38, JNK or ERK pathways (Goh et al., 2010). The authors identified numerous putative NFκB-binding sites within the promoter and first intron of the human TNC gene promoter that potentially mediate the stimulatory effect of lipopolysaccharide and other proinflammatory molecules (Goh et al., 2010). The induction of TNC in neonatal rat cardiac myocytes subjected to mechanical deformation also involved the activation of NFκB, but occurred independently of PKC and MAPK activation (Yamamoto et al., 1999). Our data provide the first insight into regulation of TNC expression in human CF, and support a key role for NFκB and PI3K/Akt pathways as well as the stress-activated kinases p38 and JNK.

3.3. Conclusion

We have demonstrated that the proinflammatory cytokine IL-1α has opposing effects on expression of CCN2 and TNC in human CF, whilst TNFα has little or no effect. IL-1α-mediated inhibition of CCN2 expression occurred via a p38-dependent pathway and may act to suppress profibrotic mechanisms during the inflammatory phase of post-MI remodeling. Moreover, IL-1-induced upregulation of TNC may aid myofibroblast recruitment and drive early myocardial repair mechanisms following MI.

4. Experimental Procedures

4.1. Reagents

Recombinant human IL-1α and TNFα were purchased from Invitrogen. IMD-0354, PD98059, SB203580 and SP600125 were purchased from Calbiochem (Nottingham, UK)

and LY294002 was from Alexis Biochemicals (Nottingham, UK).

4.2. Cell culture

Right atrial appendage biopsies from multiple patients undergoing elective coronary artery bypass surgery at the Leeds General Infirmary were obtained following local ethical committee approval and informed patient consent. Primary cultures of CF were harvested, cultured and characterized as >99% pure population of α-smooth muscle actin and vimentin co-expressing cells (i.e. myofibroblasts), as we have described previously (Porter et al., 2004b; Turner et al., 2003; Mughal et al., 2009). Experiments were performed on early passage cells (P3-P5) from several different patients (indicated by *n* number). Cells were cultured under serum-free conditions for 48 h before performing experiments in medium supplemented with 0.4% fetal calf serum.

4.3. RT-PCR Array

Cells from 3 different patients were treated with or without 10 ng/ml IL-1α for 6 h before extracting RNA using the Aurum Total RNA kit (BioRad). Equivalent RNA samples from each of the 3 patients were pooled before preparing cDNA and measuring expression levels of matricellular proteins as part of a SYBR Green-based real-time PCR array (RT^2 Profiler Human Extracellular Matrix and Adhesion Molecules, SABiosciences, Qiagen), as we described previously (Turner et al., 2010; Turner et al., 2011). ΔC_T values for the target genes were calculated by subtracting the mean C_T value (threshold cycle number) of the 5 housekeeping (HK) genes on the array (β2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13A, β-actin and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) from the C_T value of the target genes. Data are expressed as a percentage of the mean of HK genes using the formula $2^{-\Delta C_T}$ x100.

4.4. Real-time RT-PCR

RNA was extracted from cells (Aurum Total RNA kit) following appropriate treatments. Real-time RT-PCR was performed using intron-spanning human CCN2/CTGF (Hs00170014_m1) and TNC (Hs01115665_m1) primers and Taqman probes (Applied Biosystems). Data are expressed as percentage of GAPDH endogenous control mRNA expression (Hs99999905_m1 primers) using the formula $2^{-\Delta C_T}$ x 100.

4.5. Western Blotting

Protein homogenates were prepared by directly scraping cell layers into sample buffer, and equal amounts of protein (typically 30 µg/lane) were resolved by SDS-PAGE before immunoblotting as described previously (Turner et al., 2001). Western blotting was performed using 1:1000 diluted goat polyclonal anti-CCN2 antibody (sc-14939, Santa Cruz Biotechnology) or 1:1000 diluted rabbit polyclonal anti-human TNC antibody (sc-20932, Santa Cruz Biotechnology), and immunolabelled proteins detected and densitometry quantified as described previously (Turner et al., 2001). Equal protein loading was confirmed with 1:7500 diluted mouse anti-β-actin monoclonal antibody (ab8226, Abcam).

4.6. Statistical analysis

Results are mean ± SEM with *n* representing the number of experiments on cells from different patients. Data were analyzed as ratios using repeated measures one-way ANOVA and Newman-Keuls post hoc test (GraphPad Prism software, www.graphpad.com), with $P<0.05$ considered statistically significant.

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References

Abraham, D.J., Shiwen, X., Black, C.M., Sa, S., Xu, Y., Leask, A., 2000. Tumor necrosis factor α suppresses the induction of connective tissue growth factor by transforming growth factor-beta in normal and scleroderma fibroblasts. J. Biol. Chem. 275, 15220-15225.

Ahmed, M.S., Gravning, J., Martinov, V.N., von Lueder, T.G., Edvardsen, T., Czibik, G., Moe, I.T., Vinge, L.E., Oie, E., Valen, G., Attramadal, H., 2011. Mechanisms of novel cardioprotective functions of CCN2/CTGF in myocardial ischemia-reperfusion injury. Am. J. Physiol Heart Circ. Physiol 300, H1291-H1302.

Beddy, D., Mulsow, J., Watson, R.W., Fitzpatrick, J.M., O'Connell, P.R., 2006. Expression and regulation of connective tissue growth factor by transforming growth factor β and tumour necrosis factor α in fibroblasts isolated from strictures in patients with Crohn's disease. Br. J. Surg. 93, 1290-1296.

Bradham, W.S., Moe, G., Wendt, K.A., Scott, A.A., Konig, A., Romanova, M., Naik, G., Spinale, F.G., 2002. TNF-alpha and myocardial matrix metalloproteinases in heart failure: relationship to LV remodeling. Am. J. Physiol Heart Circ. Physiol 282, H1288-H1295.

Chiquet-Ehrismann, R., Chiquet, M., 2003. Tenascins: regulation and putative functions during pathological stress. J. Pathol. 200, 488-499.

Clark, J.E., Sarafraz, N., Marber, M.S., 2007. Potential of p38-MAPK inhibitors in the treatment of ischaemic heart disease. Pharmacol. Ther. 116, 192-206.

Daniels, A., Van Bilsen, M., Goldschmeding, R., van Der Vusse, G.J., van Nieuwenhoven, F.A., 2009. Connective tissue growth factor and cardiac fibrosis. Acta Physiol (Oxf) 195, 321-338.

Deten, A., Volz, H.C., Briest, W., Zimmer, H.G., 2002. Cardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats. Cardiovasc. Res. 55, 329-340.

Dobaczewski, M., Bujak, M., Zymek, P., Ren, G., Entman, M.L., Frangogiannis, N.G., 2006. Extracellular matrix remodeling in canine and mouse myocardial infarcts. Cell Tissue Res. 324, 475-488.

Dobaczewski, M., Gonzalez-Quesada, C., Frangogiannis, N.G., 2010. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. J. Mol. Cell Cardiol. 48, 504-511.

El-Karef, A., Yoshida, T., Gabazza, E.C., Nishioka, T., Inada, H., Sakakura, T., Imanaka-Yoshida, K., 2007. Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice. J. Pathol. 211, 86-94.

Frangogiannis, N.G., 2012. Matricellular proteins in cardiac adaptation and disease. Physiol Rev. 92, 635-688.

Frangogiannis, N.G., Ren, G., Dewald, O., Zymek, P., Haudek, S., Koerting, A., Winkelmann, K., Michael, L.H., Lawler, J., Entman, M.L., 2005. Critical role of endogenous thrombospondin-1 in preventing expansion of healing myocardial infarcts. Circulation 111, 2935-2942.

Frangogiannis, N.G., Smith, C.W., Entman, M.L., 2002. The inflammatory response in myocardial infarction. Cardiovasc. Res. 53, 31-47.

Goh, F.G., Piccinini, A.M., Krausgruber, T., Udalova, I.A., Midwood, K.S., 2010. Transcriptional regulation of the endogenous danger signal tenascin-C: a novel autocrine loop in inflammation. J. Immunol. 184, 2655-2662.

Gravning, J., Orn, S., Kaasboll, O.J., Martinov, V.N., Manhenke, C., Dickstein, K., Edvardsen, T., Attramadal, H., Ahmed, M.S., 2012. Myocardial connective tissue

growth factor (CCN2/CTGF) attenuates left ventricular remodeling after myocardial infarction. PLoS. One. 7, e52120.

Imanaka-Yoshida, K., Hiroe, M., Nishikawa, T., Ishiyama, S., Shimojo, T., Ohta, Y., Sakakura, T., Yoshida, T., 2001. Tenascin-C modulates adhesion of cardiomyocytes to extracellular matrix during tissue remodeling after myocardial infarction. Lab Invest 81, 1015-1024.

Imanaka-Yoshida, K., Hiroe, M., Yoshida, T., 2004. Interaction between cell and extracellular matrix in heart disease: multiple roles of tenascin-C in tissue remodeling. Histol. Histopathol. 19, 517-525.

Irwin, M.W., Mak, S., Mann, D.L., Qu, R., Penninger, J.M., Yan, A., Dawood, F., Wen, W.H., Shou, Z., Liu, P., 1999. Tissue expression and immunolocalization of tumor necrosis factor-α in postinfarction dysfunctional myocardium. Circulation 99, 1492-1498.

Jugdutt, B.I., 2003. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? Circulation. 108, 1395-1403.

Kalembeyi, I., Inada, H., Nishiura, R., Imanaka-Yoshida, K., Sakakura, T., Yoshida, T., 2003. Tenascin-C upregulates matrix metalloproteinase-9 in breast cancer cells: direct and synergistic effects with transforming growth factor beta1. Int. J. Cancer 105, 53-60.

Kennedy, L., Liu, S., Shi-wen, X., Chen, Y., Eastwood, M., Sabetkar, M., Carter, D.E., Lyons, K.M., Black, C.M., Abraham, D.J., Leask, A., 2007. CCN2 is necessary for the function of mouse embryonic fibroblasts. Exp. Cell Res. 313, 952-964.

Komatsubara, I., Murakami, T., Kusachi, S., Nakamura, K., Hirohata, S., Hayashi, J., Takemoto, S., Suezawa, C., Ninomiya, Y., Shiratori, Y., 2003. Spatially and temporally different expression of osteonectin and osteopontin in the infarct zone of experimentally induced myocardial infarction in rats. Cardiovasc. Pathol. 12, 186-194.

Leask, A., Holmes, A., Black, C.M., Abraham, D.J., 2003. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. J. Biol. Chem. 278, 13008-13015.

Leask, A., Parapuram, S.K., Shi-wen, X., Abraham, D.J., 2009. Connective tissue growth factor (CTGF, CCN2) gene regulation: a potent clinical bio-marker of fibroproliferative disease? J. Cell Commun. Signal. 3, 89-94.

Lin, J., Liliensiek, B., Kanitz, M., Schimanski, U., Bohrer, H., Waldherr, R., Martin, E., Kauffmann, G., Ziegler, R., Nawroth, P.P., 1998. Molecular cloning of genes differentially regulated by TNF-alpha in bovine aortic endothelial cells, fibroblasts and smooth muscle cells. Cardiovasc. Res. 38, 802-813.

Liu, S., Shi-wen, X., Abraham, D.J., Leask, A., 2011. CCN2 is required for bleomycin-induced skin fibrosis in mice. Arthritis Rheum. 63, 239-246.

Long, C.S., 2001. The role of interleukin-1 in the failing heart. Heart Fail. Rev. 6, 81-94.

McCurdy, S.M., Dai, Q., Zhang, J., Zamilpa, R., Ramirez, T.A., Dayah, T., Nguyen, N., Jin, Y.F., Bradshaw, A.D., Lindsey, M.L., 2011. SPARC mediates early extracellular matrix remodeling following myocardial infarction. Am. J. Physiol Heart Circ. Physiol 301, H497-H505.

Midwood, K., Sacre, S., Piccinini, A.M., Inglis, J., Trebaul, A., Chan, E., Drexler, S., Sofat, N., Kashiwagi, M., Orend, G., Brennan, F., Foxwell, B., 2009. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nat. Med. 15, 774-780.

Mughal, R.S., Warburton, P., O'Regan, D.J., Ball, S.G., Turner, N.A., Porter, K.E., 2009. Peroxisome proliferator-activated receptor γ-independent effects of thiazolidinediones on human cardiac myofibroblast function. Clin. Exp. Pharmacol. Physiol. 36, 478-486.

Nakahara, H., Gabazza, E.C., Fujimoto, H., Nishii, Y., D'Alessandro-Gabazza, C.N., Bruno, N.E., Takagi, T., Hayashi, T., Maruyama, J., Maruyama, K., Imanaka-Yoshida, K., Suzuki, K., Yoshida, T., Adachi, Y., Taguchi, O., 2006. Deficiency of tenascin C attenuates allergen-induced bronchial asthma in the mouse. Eur. J. Immunol. 36, 3334-3345.

Nakoshi, Y., Hasegawa, M., Sudo, A., Yoshida, T., Uchida, A., 2008. Regulation of tenascin-C expression by tumor necrosis factor-α in cultured human osteoarthritis chondrocytes. J. Rheumatol. 35, 147-152.

Nian, M., Lee, P., Khaper, N., Liu, P., 2004. Inflammatory cytokines and postmyocardial infarction remodeling. Circ. Res. 94, 1543-1553.

Nishiura, R., Noda, N., Minoura, H., Toyoda, N., Imanaka-Yoshida, K., Sakakura, T., Yoshida, T., 2005. Expression of matrix metalloproteinase-3 in mouse endometrial stromal cells during early pregnancy: regulation by interleukin-1alpha and tenascin-C. Gynecol. Endocrinol. 21, 111-118.

Nowinski, D., Hoijer, P., Engstrand, T., Rubin, K., Gerdin, B., Ivarsson, M., 2002. Keratinocytes inhibit expression of connective tissue growth factor in fibroblasts in vitro by an interleukin-1α-dependent mechanism. J. Invest Dermatol. 119, 449-455.

Nowinski, D., Koskela, A., Kiwanuka, E., Bostrom, M., Gerdin, B., Ivarsson, M., 2010. Inhibition of connective tissue growth factor/CCN2 expression in human dermal fibroblasts by interleukin-1alpha and beta. J. Cell Biochem. 110, 1226-1233.

Okamoto, H., Imanaka-Yoshida, K., 2012. Matricellular proteins: new molecular targets to prevent heart failure. Cardiovasc. Ther. 30, e198-e209.

Ponticos, M., Holmes, A.M., Shi-wen, X., Leoni, P., Khan, K., Rajkumar, V.S., Hoyles, R.K., Bou-Gharios, G., Black, C.M., Denton, C.P., Abraham, D.J., Leask, A., Lindahl, G.E.,

2009. Pivotal role of connective tissue growth factor in lung fibrosis: MAPK-dependent transcriptional activation of type I collagen. Arthritis Rheum. 60, 2142-2155.

Porter, K.E., Turner, N.A., 2009. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacol. Ther. 123, 255-278.

Porter, K.E., Turner, N.A., O'Regan, D.J., Ball, S.G., 2004a. Tumor necrosis factor α induces human atrial myofibroblast proliferation, invasion and MMP-9 secretion: inhibition by simvastatin. Cardiovasc. Res. 64, 507-515.

Porter, K.E., Turner, N.A., O'Regan, D.J., Balmforth, A.J., Ball, S.G., 2004b. Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. Cardiovasc. Res. 61, 745-755.

Santiago, J.J., Dangerfield, A.L., Rattan, S.G., Bathe, K.L., Cunnington, R.H., Raizman, J.E., Bedosky, K.M., Freed, D.H., Kardami, E., Dixon, I.M., 2010. Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. Dev. Dyn. 239, 1573-1584.

Sato, A., Aonuma, K., Imanaka-Yoshida, K., Yoshida, T., Isobe, M., Kawase, D., Kinoshita, N., Yazaki, Y., Hiroe, M., 2006. Serum tenascin-C might be a novel predictor of left ventricular remodeling and prognosis after acute myocardial infarction. J. Am. Coll. Cardiol. 47, 2319-2325.

Schellings, M.W., Pinto, Y.M., Heymans, S., 2004. Matricellular proteins in the heart: possible role during stress and remodeling. Cardiovasc. Res. 64, 24-31.

Shi-wen, X., Stanton, L.A., Kennedy, L., Pala, D., Chen, Y., Howat, S.L., Renzoni, E.A., Carter, D.E., Bou-Gharios, G., Stratton, R.J., Pearson, J.D., Beier, F., Lyons, K.M., Black, C.M., Abraham, D.J., Leask, A., 2006. CCN2 is necessary for adhesive

responses to transforming growth factor-beta1 in embryonic fibroblasts. J. Biol. Chem. 281, 10715-10726.

Sinfield, J.K., Das, A., O'Regan, D.J., Ball, S.G., Porter, K.E., Turner, N.A., 2013. p38 MAPK alpha mediates cytokine-induced IL-6 and MMP-3 expression in human cardiac fibroblasts. Biochem. Biophys. Res. Commun. 430, 419-424.

Tamaoki, M., Imanaka-Yoshida, K., Yokoyama, K., Nishioka, T., Inada, H., Hiroe, M., Sakakura, T., Yoshida, T., 2005. Tenascin-C regulates recruitment of myofibroblasts during tissue repair after myocardial injury. Am. J. Pathol. 167, 71-80.

Tojyo, I., Yamaguchi, A., Nitta, T., Yoshida, H., Fujita, S., Yoshida, T., 2008. Effect of hypoxia and interleukin-1beta on expression of tenascin-C in temporomandibular joint. Oral Dis. 14, 45-50.

Toma, N., Imanaka-Yoshida, K., Takeuchi, T., Matsushima, S., Iwata, H., Yoshida, T., Taki, W., 2005. Tenascin-C-coated platinum coils for acceleration of organization of cavities and reduction of lumen size in a rat aneurysm model. J. Neurosurg. 103, 681-686.

Trueblood, N.A., Xie, Z., Communal, C., Sam, F., Ngoy, S., Liaw, L., Jenkins, A.W., Wang, J., Sawyer, D.B., Bing, O.H., Apstein, C.S., Colucci, W.S., Singh, K., 2001. Exaggerated left ventricular dilation and reduced collagen deposition after myocardial infarction in mice lacking osteopontin. Circ. Res. 88, 1080-1087.

Turner, N.A., 2011. Therapeutic regulation of cardiac fibroblast function: targeting stress-activated protein kinase pathways. Fut. Cardiol. 7, 673-691.

Turner, N.A., Ball, S.G., Balmforth, A.J., 2001. The mechanism of angiotensin II-induced extracellular signal-regulated kinase-1/2 activation is independent of angiotensin AT_{1A} receptor internalisation. Cell. Signal. 13, 269-277.

Turner, N.A., Das, A., O'Regan, D.J., Ball, S.G., Porter, K.E., 2011. Human cardiac fibroblasts express ICAM-1, E-selectin and CXC chemokines in response to proinflammatory cytokine stimulation. Int. J. Biochem. Cell Biol. 43, 1450-1458.

Turner, N.A., Das, A., Warburton, P., O'Regan, D.J., Ball, S.G., Porter, K.E., 2009. Interleukin-1α stimulates pro-inflammatory cytokine expression in human cardiac myofibroblasts. Am. J. Physiol Heart Circ. Physiol 297, H1117-H1127.

Turner, N.A., Mughal, R.S., Warburton, P., O'Regan, D.J., Ball, S.G., Porter, K.E., 2007. Mechanism of TNFα-induced IL-1α, IL-1β and IL-6 expression in human cardiac fibroblasts: Effects of statins and thiazolidinediones. Cardiovasc. Res. 76, 81-90.

Turner, N.A., Porter, K.E., 2012. Regulation of myocardial matrix metalloproteinase expression and activity by cardiac fibroblasts. IUBMB. Life 64, 143-150.

Turner, N.A., Porter, K.E., Smith, W.H., White, H.L., Ball, S.G., Balmforth, A.J., 2003. Chronic β_2-adrenergic receptor stimulation increases proliferation of human cardiac fibroblasts via an autocrine mechanism. Cardiovasc. Res. 57, 784-792.

Turner, N.A., Warburton, P., O'Regan, D.J., Ball, S.G., Porter, K.E., 2010. Modulatory effect of interleukin-1α on expression of structural matrix proteins, MMPs and TIMPs in human cardiac myofibroblasts: role of p38 MAP kinase. Matrix Biol. 29, 613-620.

van Nieuwenhoven, F.A., Turner, N.A., 2013. The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction. Vascul. Pharmacol. In press doi: 10.1016/j.vph.2012.07.003.

Yamamoto, K., Dang, Q.N., Kennedy, S.P., Osathanondh, R., Kelly, R.A., Lee, R.T., 1999. Induction of tenascin-C in cardiac myocytes by mechanical deformation. Role of reactive oxygen species. J. Biol. Chem. 274, 21840-21846.

Yu, F., Chou, C.W., Chen, C.C., 2009. TNF-α suppressed TGF-β-induced CTGF expression by switching the binding preference of p300 from Smad4 to p65. Cell Signal. 21, 867-872.

Figure Legends

Fig. 1. Time course of effect of IL-1α on CCN2 and TNC expression. CF from 3 different patients were exposed to 10 ng/ml IL-1α for 2-24 h before measuring mRNA levels by RT-PCR with primers for CCN2 **(A)** or TNC **(B)**. Data are expressed as percentage GAPDH mRNA levels. ***P<0.001, **P<0.01 for effect of IL-1α (n=3). CF from a further 3 patients were exposed to 10 ng/ml IL-1α for 2-24 h before measuring protein levels of CCN2 **(C)** or TNC **(D)** by Western blotting. Approximate positions of molecular weight markers (kDa) are shown. Pooled densitometry data (mean ± SEM) are expressed relative to control. ***P<0.001, **P<0.01, *P<0.05 vs. control for pooled densitometry data (n=3).

Fig. 2. Comparison of effects of IL-1α and TNFα on CCN2 and TNC expression. CF from 4 different patients were exposed to 10 ng/ml IL-1α or 10 ng/ml TNFα for 6 h before measuring mRNA levels by RT-PCR with primers for CCN2 **(A)** or TNC **(B)**. Data are expressed as percentage GAPDH mRNA levels. ***P<0.001, **P<0.01, NS = not significant vs. control (n=4). CF from a further 3 patients were exposed to 10 ng/ml IL-1α or 10 ng/ml TNFα for 6 h before measuring protein levels of CCN2 **(C)** or TNC **(D)** by Western blotting. Approximate positions of molecular weight markers (kDa) are shown. Pooled densitometry data (mean ± SEM) are expressed relative to control. **P<0.01, *P<0.05, NS = not significant vs. control for pooled densitometry data (n=3).

Fig. 3. Effect of signaling pathway inhibitors on IL-1α-mediated suppression of CCN2 mRNA and protein expression. CF from multiple patients were exposed to 30 μM PD98059 (PD; ERK pathway inhibitor), 10 μM SB203580 (SB; p38 MAPK pathway inhibitor), 10 μM SP600125 (SP; JNK pathway inhibitor), 10 μM LY294002 (LY; PI3K/Akt pathway

inhibitor) or 10 μM IMD-0354 (IMD; NFκB pathway inhibitor) for 1 h before stimulation with or without 10 ng/ml IL-1α for a further 2 h (for mRNA studies) or 6 h (for protein studies). **(A)** CCN2 mRNA levels were measured by RT-PCR. Data are normalized to GAPDH mRNA levels. $^{**}P<0.01$, $^{*}P<0.05$, NS = not significant for effect of IL-1 (n=5). **(B)** CCN2 protein levels were measured by Western blotting. Approximate position of molecular weight marker (kDa) is shown. Pooled densitometry data for CCN2 (mean ± SEM) are expressed relative to control (n=4).

Fig. 4. Effect of signaling pathway inhibitors on IL-1α-induced TNC mRNA and protein expression. CF from multiple patients were exposed to 30 μM PD98059 (PD; ERK pathway inhibitor), 10 μM SB203580 (SB; p38 MAPK pathway inhibitor), 10 μM SP600125 (SP; JNK pathway inhibitor), 10 μM LY294002 (LY; PI3K/Akt pathway inhibitor) or 10 μM IMD-0354 (IMD; NFκB pathway inhibitor) for 1 h before stimulation with or without 10 ng/ml IL-1α for a further 2 h (for mRNA studies) or 6 h (for protein studies). **(A)** TNC mRNA levels were measured by RT-PCR. Data are normalized to GAPDH mRNA levels. $^{**}P<0.01$, $^{*}P<0.05$, NS = not significant for effect of IL-1 (n=5). **(B)** TNC protein levels were measured by Western blotting. Approximate position of molecular weight marker (kDa) is shown. Pooled densitometry data for TNC (mean ± SEM) are expressed relative to control (n=4).

Figure 1

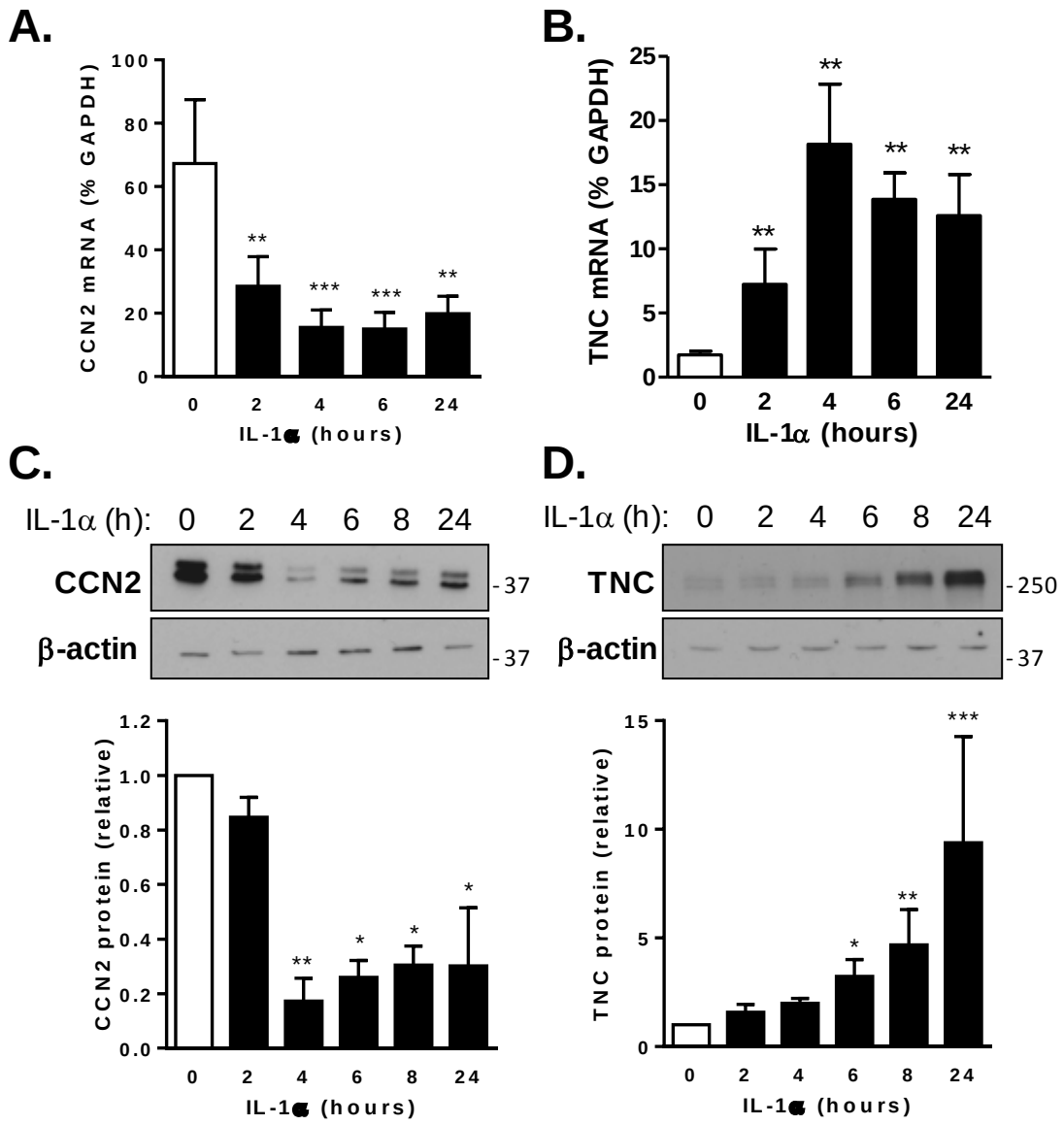

A.
CCN2 mRNA (% GAPDH)
100
80
60
40
20
0
**
***
***
**
0
2
4
6
24
IL-1α (hours)
B.
TNC mRNA (% GAPDH)
25
20
15
10
5
0
**
**
**
**
0
2
4
6
24
IL-1α (hours)
C.
IL-1α (h): 0 2 4 6 8 24
CCN2
β-actin
-37
-37
CCN2 protein (relative)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
*
*
0
2
4
6
8
24
IL-1α (hours)
D.
IL-1α (h): 0 2 4 6 8 24
TNC
β-actin
-250
-37
TNC protein (relative)
15
10
5
0
***
**
*
0
2
4
6
8
24
IL-1α (hours)

Figure 2

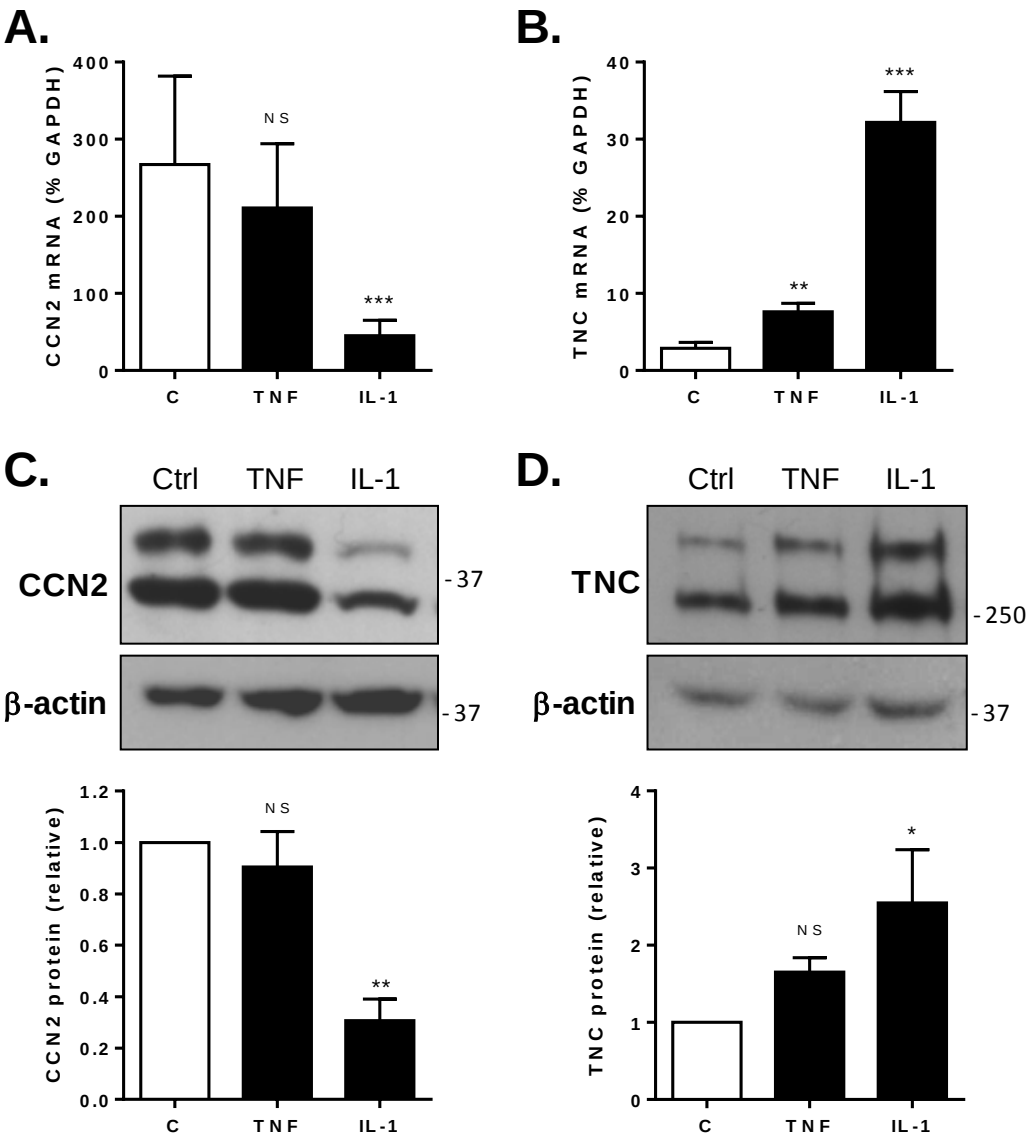

A.
CCN2 mRNA (% GAPDH)
400
300
200
100
0
NS
***
C
TNF
IL-1
B.
TNC mRNA (% GAPDH)
40
30
20
10
0
**
***
C
TNF
IL-1
C.
Ctrl
TNF
IL-1
CCN2
β-actin
-37
-37
CCN2 protein (relative)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NS
**
C
TNF
IL-1
D.
Ctrl
TNF
IL-1
TNC
β-actin
-250
-37
TNC protein (relative)
4
3
2
1
0
NS
*
C
TNF
IL-1

Figure 3

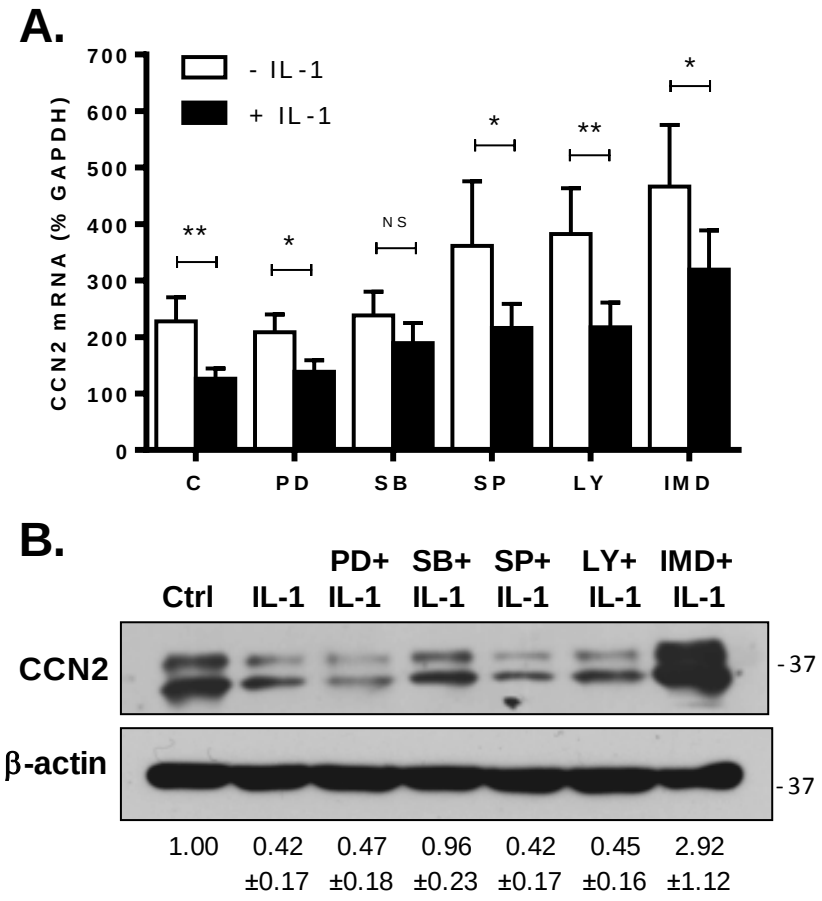
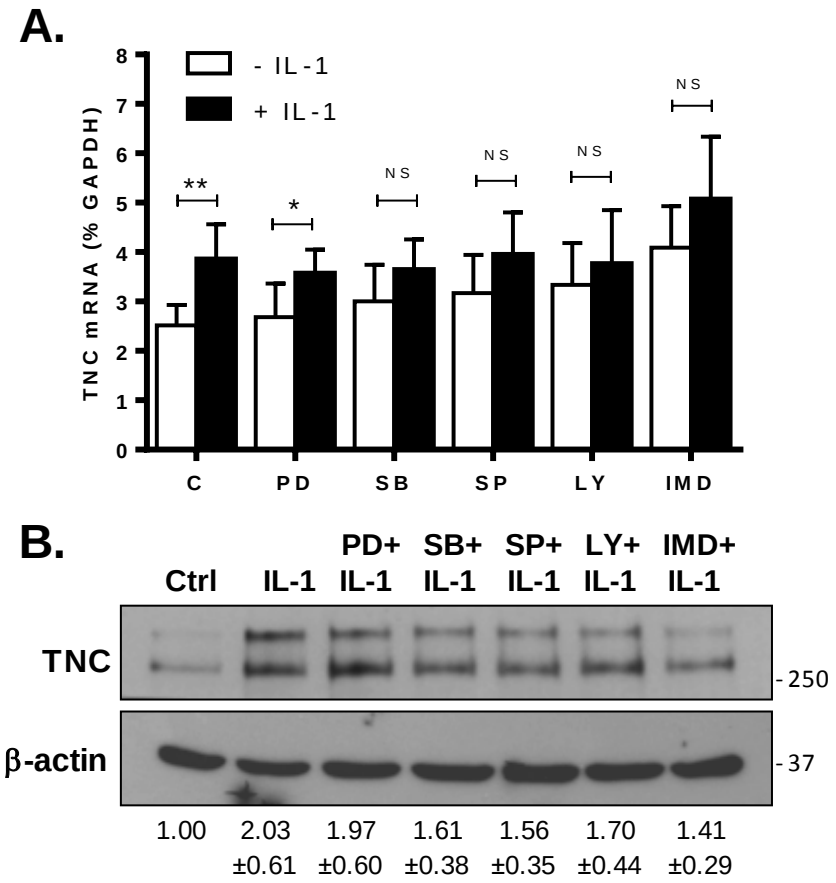

A.
- IL-1
+ IL-1
CCN2 mRNA (% GAPDH)
700
600
500
400
300
200
100
0
**
*
NS
*
**
*
C
PD
SB
SP
LY
IMD
B.
Ctrl
IL-1
PD+ IL-1
SB+ IL-1
SP+ IL-1
LY+ IL-1
IMD+ IL-1
CCN2
β-actin
-37
-37
1.00
0.42 ±0.17
0.47 ±0.18
0.96 ±0.23
0.42 ±0.17
0.45 ±0.16
2.92 ±1.12

Figure 4


A.
- IL-1
+ IL-1
TNC mRNA (% GAPDH)
0
1
2
3
4
5
6
7
8
**
*
NS
NS
NS
NS
C
PD
SB
SP
LY
IMD
B.
Ctrl
IL-1
PD+ IL-1
SB+ IL-1
SP+ IL-1
LY+ IL-1
IMD+ IL-1
TNC
β-actin
250
37
1.00
2.03 ±0.61
1.97 ±0.60
1.61 ±0.38
1.56 ±0.35
1.70 ±0.44
1.41 ±0.29