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Expression and function of toll-like receptor 4 and inflammasomes in cardiac fibroblasts and myofibroblasts: IL-1β synthesis, secretion, and degradation

Pia Boza[a,1], Pedro Ayala[a,1], Raúl Vivar[a], Claudio Humeres[a], Felipe Tapia Cáceres[a], Claudia Muñoz[a], Lorena García[b,d], Marcela A. Hermoso[c], Guillermo Díaz-Araya[a,d,*]

[a] Departamento de Química Farmacológica y Toxicológica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Chile
[b] Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Chile
[c] Instituto de Ciencias Biomédicas, Programa de Inmunología, Facultad de Medicina, Universidad de Chile, Chile
[d] Centro Avanzado de Enfermedades Crónicas (ACCDis), Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Chile

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ABSTRACT

Cardiac inflammation can be produced by pathogen-associated molecular patterns (PAMPs), from parasitic, bacterial or viral origin; or by danger-associated molecular patterns (DAMPs), released from dead cells after cardiac tissue damage, for example by cardiac infarction. Both, PAMPS and DAMPS activate TLR4 on resident immune cells and heart tissue cells, triggering an inflammatory process necessary to begin the wound healing process. Cardiac fibroblasts (CF) are the most abundant cells in the heart and are critical to wound healing, along with cardiac myofibroblasts (CMF), which are differentiated from CF through a TGF-β1-mediated process. While TLR4 and the inflammasome complex are known to play important roles in CF function, the effects of TGF-β1 on TLR4 and inflammasome expression and activity remain unknown. To elucidate this important point, we evaluated the effect of TGF-β1 on TLR4, and the inflammasome protein expression and activity through activation by LPS, mimicking a myocarditis condition by bacterial origin.

We found that TGF-β1 increased TLR4 expression in CF and that the process was mediated by the TGFβRI and p38 signaling pathways. In both CF and CMF, LPS triggered ERK1/2, PI3K-Akt, and p65-NF-κB phosphorylation. All of these effects were blocked by TAK-242, a TLR4 signaling pathway inhibitor. LPS increased pro-IL-1β levels, which were dependent on the ERK1/2, PI3K-Akt, and NF-κB signaling pathways, and levels were higher in CF than CMF. NLRP3 and ASC levels were similar in CF and CMF, while pro-caspase-1 levels and caspase-1 activity were higher in CMF. LPS + ATP treatment induced inflammasome complex assembly and activation, triggering the release of IL-1β in both CMF and CF. Finally, the unsecreted pro-IL-1β in the CF was degraded by autophagy.

Conclusion: TGF-β1 increases TLR4 expression in CF. Despite different pro-IL-1β and caspase-1 activity levels in CF versus CMF, the two cell types secreted similar levels of IL-1β after LPS + ATP treatment. These findings suggest that both cell types are active participants in inflammation.

1. Introduction

Cardiac inflammation or myocarditis can be triggered by a wide variety of insults to the myocardium, including parasitic, bacterial or viral infections (Caforio et al., 2013), through pathogen associated molecular patterns (PAMPs), and by intracellular mediators known as damage danger-associated molecular patterns (DAMPs), which are released from dead cells after cardiac tissue damage by autoimmunity or cardiac infarction (Arslan et al., 2011). Both, PAMPs and DAMPs activate TLR4 on resident immune cells and heart tissue cells, triggering a strong inflammatory process necessary to start wound healing process.

Cardiac fibroblasts (CF), the most abundant cells in the heart, are largely responsible for maintaining extracellular matrix (ECM) homeostasis. As sentinel cells (Díaz-Araya et al., 2015), CF release cytokines and growth factors and express cognate receptors (Chen

* Corresponding author at: Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Olivos 1007, Santiago 8380492, Chile.
E-mail address: gadiaz@ciq.uchile.cl (G. Díaz-Araya).
[1] The first two authors contributed equally to the manuscript.

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and Frangogiannis, 2013). CF also differentiates into cardiac myofibroblasts (CMF) after tissue damage. This process occurs through mechanical stress and the actions of substances like TGF-β1. Wound healing relies heavily on these differentiated CMF, which synthesize abundant ECM proteins, especially collagen type I, collagen type III, and fibronectin (Porter and Turner, 2009).

Toll-like receptors (TLR) belong to the type-I IL1-R receptor superfamily that recognizes PAMPs such as bacterial lipopolysaccharides (LPS) (Wong et al., 2011) and also DAMPs, such as HMGB1 (Tsung et al., 2005), fibronectin ED-A, and heparan sulfate (Okamura et al., 2001). TLR are the first line of immune defense against pathogen infection, triggering a strong inflammatory response when they are activated (Akira and Takeda, 2004; Frangogiannis, 2012). Recently there has been extensive research into TLR activity in non-immune cells like fibroblasts. TLR2 activation in gingival fibroblasts, for example, has been found to promote cytokine release (Into et al., 2010), and TLR3 has been characterized as a pivotal factor in lung fibroblast-to-myofibroblast differentiation (Sugiura et al., 2009). TLR4 promotes lung fibroblast proliferation (He et al., 2012), cytokine release (Zeuke et al., 2002), fibrosis, and maladaptive left ventricular remodeling (Timmers et al., 2008).

Once activated, TLR4 dimerizes to in turn activate ERK1/2, PI3K-Akt kinases, and transcription factor NF-κB (Verstrepen et al., 2008; Laird et al., 2009), triggering the synthesis and release of pro-inflammatory cytokines such as IL-1β.

IL-1β is a potent pro-inflammatory cytokine that is crucial for host defenses against infection and injury (Dinarello, 2009). IL-1β signals through IL-1R1 to produce a strong proinflammatory effect and recruit myeloid cells such as neutrophils to inflammation sites (Rider et al., 2011). IL-1β production is tightly regulated; it is produced as an inactive pro-form (pro-IL-1β) that is cleaved by caspase-1 to create the mature and bioactive cytokine. Caspase-1 is then activated by the inflammasome, a large multimeric structure that includes the intracellular NOD-like receptor (NLR) NLRP3, the adaptor protein ASC, and pro-caspase-1 (Davis et al., 2011). IL-1β secretion mechanisms do not follow the conventional protein secretion route but rather operate via one or more non-conventional pathways (Lopez-Castejon and Brough, 2011). The above authors suggest that all of these mechanisms are part of a single secretion continuum or spectrum, in which the secretion route is dictated by the strength of the inflammatory stimulus. Excess, unsecreted intracellular pro-IL-1β may be degraded by autophagy or proteasomes (Harris et al., 2011).

LPS is the major component of the bacterial outer membrane and it is the principal cause of cardiac dysfunction by sepsis (Fallach et al., 2010). TLR4 activation by LPS triggers cardiomyocyte hypertrophy (Magi et al., 2015) and decrease cardiac contractility (Avlas et al., 2011). In addition, TLR4 plays an important role after viral myocarditis (Roberts et al., 2013), whereas TLR2 contributes to inflammation after *trypanosome cruzi* infection (Campos and Gazzinelli, 2004; Petersen et al., 2005). Although the role of TLR4 in CF is well-documented, the effects of TGF-β1 on TLR4, NLRP3, and ASC protein expression have not been reported. The effects of inflammasome activity on CMF are also unknown. Finally, no study to date has explored whether CMF secrete IL-1β in a manner similar to CF. We used an ultrapure LPS to activate TLR4, mimicking a bacterial myocarditis and evaluated its effects on pro-IL-1β synthesis and IL-1β secretion after inflammasome assembly and activation in CF and CMF.

2. Materials and methods

2.1. Materials

Trypan blue, α-smooth muscle actin antibody, and inhibitors for ERK (PD98059), JNK (SP600125), p38 (SB202190), and ALK5 (SB431542) were acquired from Sigma Chemical Co. (St. Louis, MO, USA); trypsin/EDTA, prestained molecular weight standard, fetal bovine serum (FBS), and fetal calf serum (FCS) from Gibco BRL (Carlsbad, CA, USA); all organic and inorganic compounds from Merck (Darmstadt, Germany); the enhanced chemo-luminescence (ECL) reagent from Perkin Elmer Life Sciences, Inc. (Boston, MA, USA); sterile plastic material from Costar® (NJ, USA); the primary antibodies for phospho-ERK, phospho-Akt, total-ERK, total-Akt, phospho p65NF-κB, total-NF-κB, and anti-ubiquitin from Cell Signaling Technology (Boston, MA, USA); TLR4, NLRP3, ASC, caspase-1, and goat secondary antibodies from Santa Cruz Biotechnology (CA, USA); IL-1β antibody from Merck Millipore (MA, USA); TAK-242 from InvivoGen; GAPDH, rabbit, and mouse secondary antibodies from Calbiochem (Darmstadt, Germany); TGF-β1 from Millipore (Billerica, USA); the ELISA kit from R&D Systems (MN, USA); and ATP and the Caspase-1 Assay Kit (Fluorometric) from Abcam (Cambridge, UK).

2.2. Animals

Male adult Sprague Dawley rats (250 g) from the animal breeding facility at the University of Chile's Chemical and Pharmaceutical Sciences, were housed in cages (12 h light/dark cycles) with rat chow and water *ad libitum*. All studies complied with the NIH's Guide for the Care and Use of Laboratory Animals, updated in 2011 (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals), and experimental protocols were approved by our Institutional Review Board.

2.3. Cardiac fibroblast isolation

Rats were anesthetized with ketamine-xylazine (66 mg/kg and 1.6 mg/kg i.p., respectively). Adult rat CF were isolated by retrograde aortic perfusion as previously described (Aránguiz-Urroz et al., 2011), with a few modifications. Briefly, the hearts were digested with a collagenase-hyaluronidase (1:1)-containing solution and cells centrifuged at 500 rpm for 1 min. The supernatant, containing mainly CF, was centrifuged at 1000 rpm for 10 min and then resuspended in DMEM F-12 plus 10% FBS and seeded in untreated culture dishes for 2 h. The cells were washed with PBS to eliminate debris and non-adherent cells. CF were used at passage 1 and seeded on plastic dishes at a density of 2×10^4 cells/cm^2. The cells were serum-starved for 24 h before stimulation under the various experimental conditions.

2.4. Differentiation of cardiac fibroblasts into cardiac myofibroblasts

Primary CF cultures were stimulated with TGF-β1 (5 ng/mL) for 96 h and characterized as CMF (α-SMA-positive).

2.5. Western blot of proteins in fibroblast cultures

Protein extracts were separated by 10% polyacrylamide gel electrophoresis, and 50 μg of total proteins were submitted to Western blot analysis. SDS-PAGE was performed at 100 V in 1X electrophoresis buffer (Tris Base 30.25 g, glycine 144 g, SDS 10 g, and water 1 L, for a 10X electrophoresis buffer). Proteins were electrotransferred to a nitrocellulose membrane (BioRad) at 350 mA for 90 min in transference buffer. Membranes were blocked using blocking buffer (nonfat milk 5% (w/v)/1X TBS Tween 0.1%) for 60 min at room temperature. Blocking was followed by three wash cycles using 1X TBS Tween 0.1%. Membranes were incubated with the antibodies overnight at 4 °C in dilutions of 1:200, 1:1000, or 1:5000, depending on antibody type. After incubation, membranes were washed three times with 1X TBS Tween 0.1% for 10 min. The secondary antibody

was incubated for 2 h at room temperature using a 1:5000 dilution. After washing with 1X TBS Tween 0.1%, the membranes were exposed to ECL reagent. Agfa film was used for Western blot imaging, and blots were quantified with densitometry. Results were expressed as the ratio of proteins in the experimental versus control conditions. GAPDH and tubulin were used as loading controls.

2.6. Immunofluorescence

CF were seeded onto coverslips in six-well cell culture dishes then serum-starved by washing twice with PBS, adding starvation media, and incubating for 24 h. The cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then washed three times with PBS and permeabilized with 0.1% Triton X-100, followed by three additional washes. Slides were blocked with 3% bovine serum albumin in PBS for 30 min, washed, and incubated overnight with the primary antibodies anti-TLR4, anti α-SMA, anti NF-κB, or anti-vimentin, diluted with 1% BSA in PBS. Cells were again washed three times with PBS and incubated with Alexa Fluor-conjugated secondary antibody 488 and/or 586 as applicable for 1.5 h, followed by another wash cycle. The nucleus was labeled with Hoechst (blue). Coverslips were dried using a vacuum; mounted on slides using Dako; and then sealed with nail polish. The final images were obtained by confocal microscopy.

2.7. RT-PCR analysis

Total RNA was isolated from CF and CMF with a commercial kit (E.Z.N.A. RNA isolation system®). RNA pellets were suspended in DEPC-treated distilled water, and the concentrations were measured with UV spectroscopy. RNA integrity was assessed according to ethidium bromide staining intensity with 18 and 28S ribosomal RNA after agarose electrophoresis. cDNA synthesis was then performed with the ThermoScript® RT-PCR System in a mixture containing random primers and deoxynucleotides in reverse transcription buffer. Finally, to amplify the resulting cDNA we used Platinum Taq DNA polymerase with the following primers:

IL-1β: Sense 5′-TTCTTTGAGGCTGACAGACC-3′; Antisense 5′-CGTCTTTCATCACACAGGAC-3′ (530 pb); and 18S classic II (AMBION) (315 pb).

After PCR, the amplification products were fractionated on a 1.5% (w/w) agarose gel and visualized with ethidium bromide staining. Band intensities were quantified with computerized densitometry and normalized with respect to 18 s mRNA.

2.8. IL-1β ELISA and caspase-1 activity kit

Cell culture medium was collected and analyzed using the IL-1β ELISA kit according to protocol. Cell lysate was collected and analyzed with caspase-1 activity fluorometric assay according to protocol.

2.9. Statistical analysis

Data are expressed as the mean ± SD of *n* independent experiments. Statistical analysis consisted of one-way ANOVA followed by Tukey's multiple comparisons test using Graph Pad Prism Software. $p < 0.05$ was considered significant.

3. Results

3.1. TGF-β1 induces TLR4 expression in cardiac fibroblasts

CF are known to express TLR4, which initiates non-sterile cardiac inflammation after engaging with PAMPs. However, it was not clear whether TLR4 expression was associated with cytokines like TGF-β1 that play crucial roles in CF activity and differentiation. Specifically, there was no evidence demonstrating that TGF-β1 regulates TLR4 in CF or that TGF-β1-induced CF-to-CMF differentiation is associated with altered TLR4 expression. Therefore, our aim was to determine the presence and distribution of TLR4 in these cells. Fig. 1A shows that stimulating CF with TGF-β1 (5 ng/mL) induced a time-dependent increase in TLR4 expression levels, reaching statistical significance at 48 h (2.4-fold over control) and peaking at 96 h (5.8 fold over control). We then performed experiments to measure the concentration-dependent increase in TLR4 expression levels at 48 h. Fig. 1B shows that TLR4 expression levels were again increased with 5 ng/mL of TGF-β1 (2.7-fold over control in this run, very similar to the above trial), peaking at 10 ng/mL (5.2-fold over control). The increases in TLR4 expression were accompanied by parallel increases α-SMA protein, a hallmark of CF-to-CMF differentiation. By immunofluorescence staining we also confirming the presence of TLR4 in CF, and when we repeated these experiments in the CMF; we found similar findings of increased TLR4 expression but with higher immunofluorescence intensity in the TGF-β1-treated CMF versus the CF. The CMF also increased in size and α-SMA content after treatment (Fig. 1C). Collectively, these data indicate that both CF and CMF express TLR4, with higher expression levels in CMF. Our next step was to determine which of the TGF-β1-activated signaling pathways are responsible for regulating TLR4 expression Fig. 2.

3.2. TGF-β1 induces TLR4 overexpression in cardiac fibroblasts through the TGFβRI and p38 signaling pathways

The effects of TGF-β1 are mediated by TGF-β1 type I and type II membrane receptors. Activating these receptors induces canonical serine phosphorylation of SMAD2/3 and non-canonical signaling pathways involving ERK1/2, p38, PI3K-Akt, and JNK. To determine whether these signaling pathways are implicated in TLR4 expression, we evaluated the effects of TGF-β1 on TLR4 expression in the presence of several chemical inhibitors. Fig. 1D shows that TGF-β1 (5 ng/mL) significantly induced TLR4 expression in CF (3.6-fold over control). Both the TGF-β type I receptor blocker (SB431542) and the p38 signaling pathway inhibitor (SB202190) prevented TGF-β1′s effect on TLR4 expression. However, PI3K-Akt (LY294002), MEK (PD98059), and JNK (SP600125) signaling pathway inhibitors were not able to block the effects of TGF-β1. Furthermore, only the TGF-β type I receptor blocker counteracted the TGF-β1-mediated increases in α-SMA level. These data mean that both canonical and non-canonical TGF-β1-activated signaling pathways regulate TLR4 expression in CF. After characterizing TLR4 expression, our next step was to evaluate the signaling pathways activated by this receptor, which was performed by LPS.

3.3. TLR4 activation in cardiac fibroblasts and myofibroblasts triggers ERK1/2, PI3K-Akt, and NF-κB signaling pathways

Our previous data confirmed that both CF and CMF express TLR4; however, it is also important to recognize the signaling pathways triggered by LPS in both cell types. TLR4 activation triggers various MyD88-dependent and −independent signaling pathways, including the NF-κB and MAPK signaling pathways; therefore, we stimulated CF and CMF with ultrapure LPS (a well-known TLR4 stimulus) and evaluated the classical TLR4-activated signaling pathways. First, we studied whether LPS produces time-dependent increases in phosphorylated ERK1/2, Akt, and NF-κB protein levels in CF. As shown in Supplementary Fig. S1 in the online version at DOI: 10.1016/j.molimm.2016.05.001, exposing CF to LPS (1 μg/mL) induced p65 NF-κB phosphorylation at 5 min, peaking at 30 min (Supplementary Fig. S1A in the online version at DOI: 10.1016/j.molimm.2016.05.001), as well as ERK1/2 (Supplementary Fig. S1B

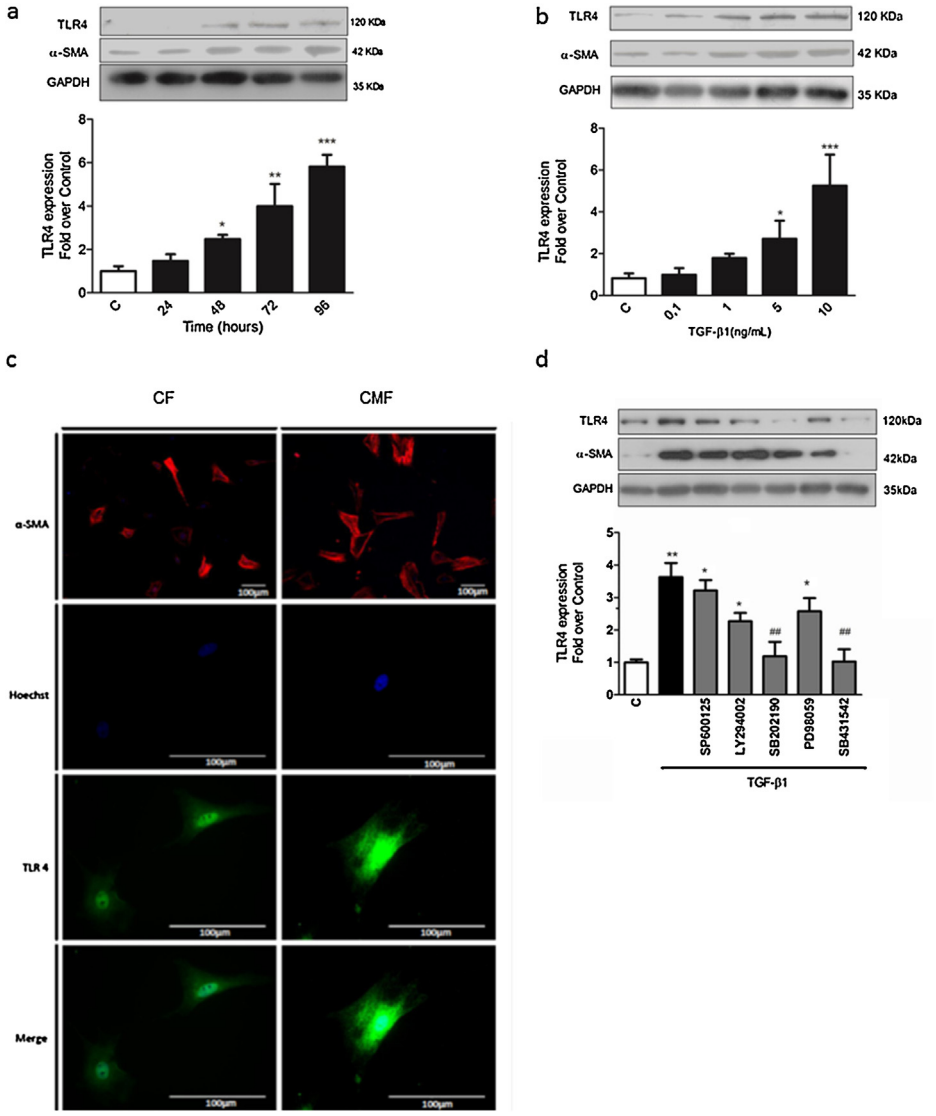

Fig. 1. TGF-β1 regulates TLR4 expression in cardiac fibroblasts. (a) TGF-β1 (5 ng/mL) in a time-dependent manner, and (b) TGF-β1 (0.1-10.1161/JAHA.115.001993. ng/mL) increases TLR4 expression in CFs. In each experiment, protein levels were determined by westernblot; a representative immunoblot and a bar graph are showed. The results represent mean (±S.E.M.) of 6 independent experiments (*$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$ vs. control). (c) TLR4 immunocytochemical detection. CFs or CMFs were incubated with α-SMA or TLR4 antibodies and the nucleus was labeled with Hoechst. Merged images indicate that TLR4 is present in cell nuclei and cytoplasm. A representative confocal photograph, from four independent experiments, is showed. (d) CFs were treated with TGF-β1 (5 ng/mL) by 48 h, in the presence of PD 98059 (ERK1/2, inhibitor), LY294002 (Akt, inhibitor), SB 600125 (JNK, inhibitor), SB431542 (TGF-β1R blocker) or SB202190 (p38 inhibitor). Protein levels were determined by westernblot; GAPDH was used as load control. A representative immunoblot and a bar graph analysis are showed. The results represent means (± S.E.M.) of 6 independent experiments (*$p < 0.05$; **$p < 0.01$ vs. control and ##$p < 0.01$ vs. TGF-β1).

in the online version at DOI: 10.1016/j.molimm.2016.05.001) and PI3K-Akt (Supplementary Fig. S1C in the online version at DOI: 10.1016/j.molimm.2016.05.001) phosphorylation, reaching significance at 60 min. Furthermore, LPS induced p65 NF-κB nuclear translocation, reaching maximum immune-reactive detection at 60 min (Supplementary Fig. S1D in the online version at DOI: 10.1016/j.molimm.2016.05.001).

3.4. LPS increases pro-IL-1β mRNA and protein levels in cardiac fibroblasts and myofibroblasts

Once TLR4 binds with its ligands, various signaling pathways are activated, leading to the expression and synthesis of cytokines such as IL-1β. These cytokines may affect surrounding cells in cardiac tissue, but their expression in CMF had never been measured. To determine whether TLR4 activation by LPS is associated with cytokine gene expression, we measured pro-IL-1β mRNA expression levels in CF using RT-PCR. Supplementary Fig. S2A in the online version at DOI: 10.1016/j.molimm.2016.05.001 shows that LPS (1 μg/mL) induced pro-IL-1β mRNA expression at 3 h, with levels decreasing back to baseline by 12 h. Furthermore, pro-IL-1β protein levels showed time-dependent increases, peaking at 8 h (Supplementary Fig. S2B in the online version at DOI: 10.1016/j.molimm.2016.05.001).

At the peak of protein expression (8 h), we measured and compared pro-IL-1β levels in the CF and CMF. Fig. 3A shows that LPS triggered greater pro-IL-1β elevations in CF than in CMF. Fig. 3B and C show that preincubation with TAK-242 and the inhibitors for ERK1/2 (PD98059), PI3K-Akt (LY294002), and NF-κB (Bay-117082) blocked the LPS-induced pro-IL-1β protein expression in both CF (Fig. 3B) and CMF (Fig. 3C). This result indicates that TLR4 activation increases IL-1β expression and synthesis; however, this

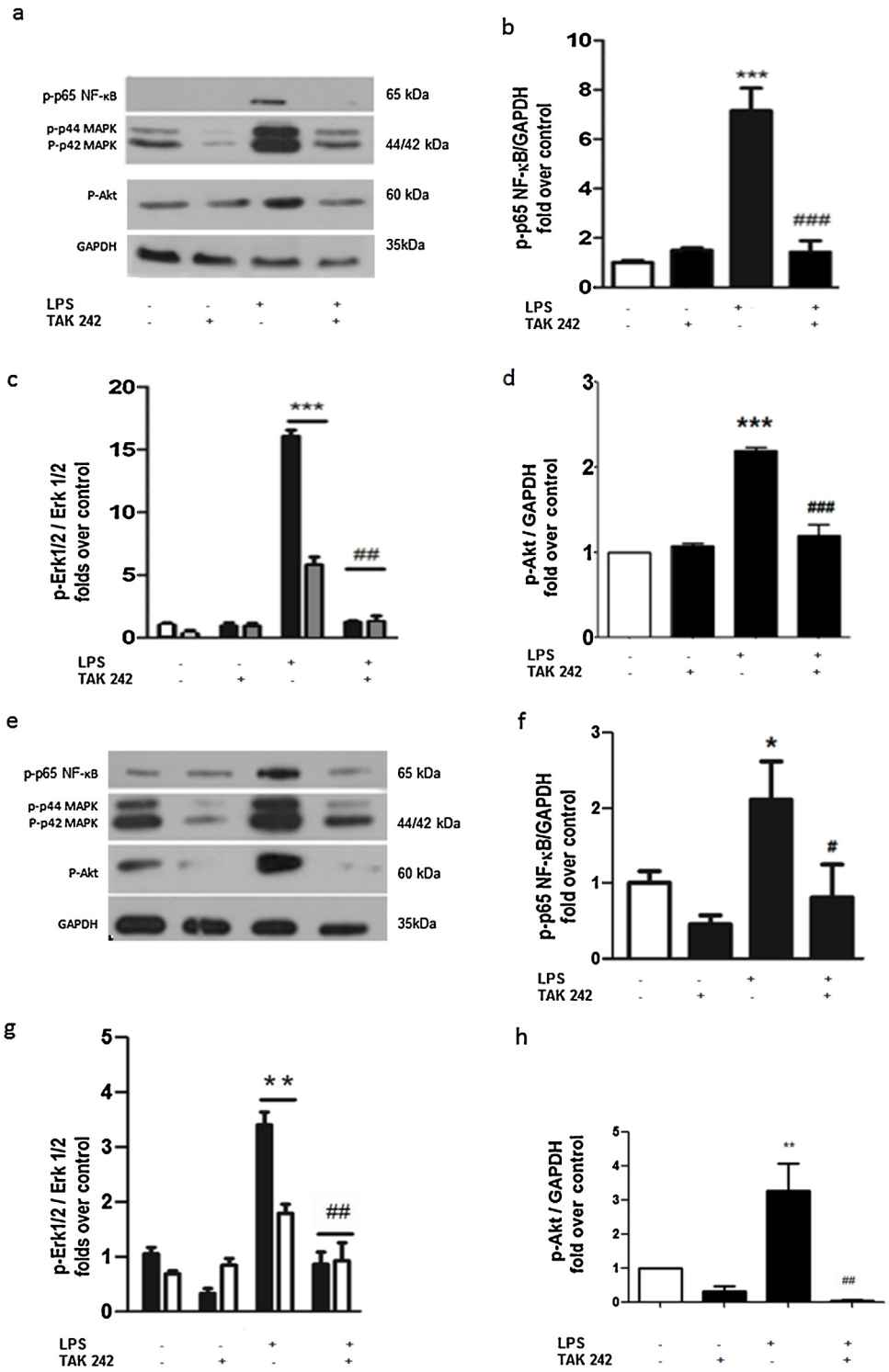

Fig. 2. LPS through TLR4 activates NF-κB, ERK1/2 and Akt signaling pathways in cardiac fibroblast and myofibroblast. Cells were treated with LPS (1 μg/mL) during 30 min for pNF-kB and 60 min for pERK1/2 and pAkt in presence or absence or TAK-242, and protein levels were analyzed. (a and e). Representative immunoblot of p65-NF-κB, p-ERK1/2, p-Akt and GAPDH as load control for CFs and CMFs respectively, are showed. (b and f) graphi graphic analysis of p65-NF-κB for CFs and CMFs; (c and g) graphic analysis of p-ERK1/2 for CFs and CMFs; and (d and h) graphic analysis of p-Akt for CFs and CMFs. The results represent means (±S.E.M.) of 6 independent experiments ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs control and $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ vs LPS).

cytokine must be fragmented by inflammasomes before it can exert its actions on resident immune cells or cardiac cells.

3.5. ATP activates the inflammasome complex and triggers the release of IL-1β in cardiac fibroblasts and myofibroblasts

The inflammasome is a molecular platform that is activated upon cellular stress, triggering the IL-1β maturation (activation) and secretion, which in turn engages the inflammatory response. To determine whether treatment with ATP (a classical inflammasome activator) induces IL-1β processing and secretion in CF and CMF, we measured intracellular ASC, NLRP3, and pro-caspase-1 levels and caspase-1 activity. We evaluated and compared inflammasome protein levels in CF and CMF after 8 h of stimulation with LPS (the peak of pro-IL-1β expression). NLRP3 and ASC levels were similar

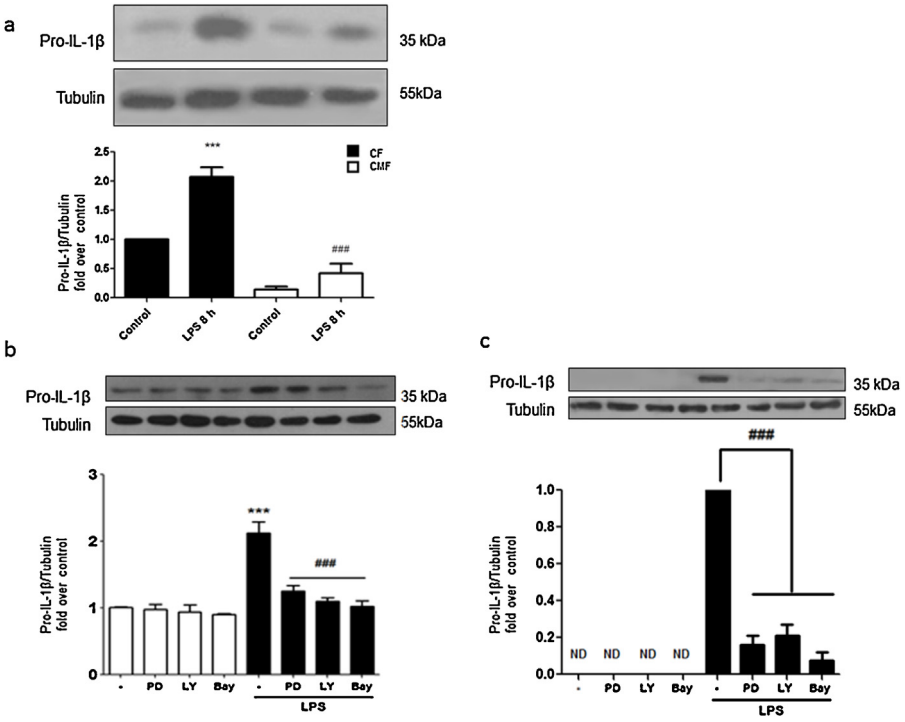

Fig. 3. LPS through NF-κB, ERK1/2 and Akt signaling pathways increases pro-IL-1β expression in cardiac fibroblast and myofibroblast. (a) CFs and CMFs were stimulated with LPS (1 μg/mL) for 8 h, pro-IL-1β and protein levels were analyzed by westernblot. The results represent means (±S.E.M.) of 5 independent experiments (***$p < 0.001$ vs. CF control and ###$p < 0.001$ vs. CF LPS) (b) CFs and (c) CMFs stimulated with/without LPS (1 μg/mL) for 8 h, in presence or absence of Bay (NF-κB, inhibitor), PD (ERK1/2, inhibitor) and LY (Akt, inhibitor); and pro-IL-1β protein levels were determined by westernblot. Tubulin was used as load control. A representative immunoblot and graphic analysis are showed. The results represent means (±S.E.M.) of 5 independent experiments (***$p < 0.001$ vs. control and ###$p < 0.001$ vs. LPS).

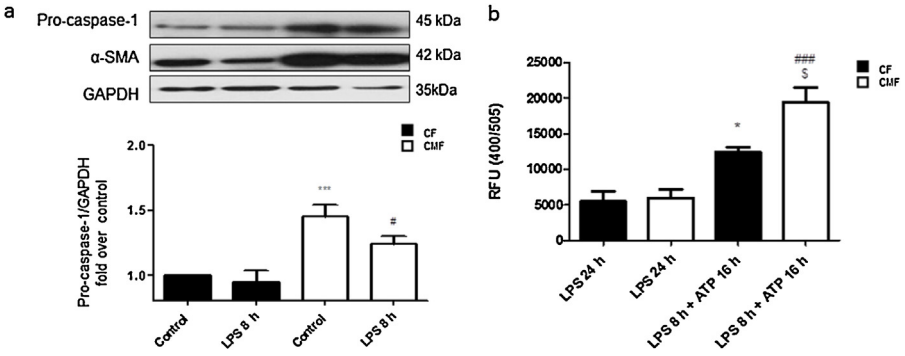

Fig. 4. Pro-caspase-1 protein levels and activity in cardiac fibroblast and myofibroblast. CFs and CMFs were stimulated with LPS (1 μg/mL). (a) pro-caspase-1 protein levels were analyzed at 8 h of LPS. Protein levels were determined by westernblot. α-SMA was used as marker of CMFs. GAPDH was used as load control. A representative immunoblot image and a bar graph analysis are showed (b) Caspase-1 activity was analyzed after 24 h LPS and after 8 h LPS + 16 h ATP (total 24 h). Caspase-1 activity was analyzed as described in methods section. The results represent means (±S.E.M.) of 5 independent experiments (*$p < 0.05$CF LPS 24 h; ***$p < 0.001$ vs. CF control; #$p < 0.05$ vs CF LPS 8 h; ###$p < 0.001$ vs. CMF LPS 24 h and \$$p < 0.05$CF LPS 8 h + ATP 16 h).

in the two cells types, and treatment with LPS did not affect protein levels (Supplementary Fig. S3A and B in the online version at DOI: 10.1016/j.molimm.2016.05.001). Baseline pro-caspase-1 levels were higher in CMF than CF, but LPS did not affect pro-caspase-1 protein expression (Fig. 4A). We then measured caspase-1 activity as a proxy for inflammasome activation after determining the ATP exposure time necessary to induce inflammasome assembly and activation in CF. CF were stimulated with LPS for 8 h and then with ATP for 1, 4, or 16 h, and then IL-1β levels were measured using ELISA. LPS 8 h + ATP 16 h significantly induced the release of IL-1β into the culture media. Interestingly, neither LPS nor ATP alone induced the release of IL-1β (Supplementary Fig. S4 in the online version at DOI: 10.1016/j.molimm.2016.05.001). Thus, we assessed capase-1 activity after the same time periods, finding that LPS + ATP significantly increased caspase-1 activity in CF and CMF (Fig. 4B).

Taken together, these data suggest that both LPS and ATP are necessary for CF and CMF to release IL-1β. Additionally, caspase-1 protein and activity levels were higher in CMF versus CF. In order to further validate this finding we measured the levels of released IL-1β.

3.6. *Release of IL-1β in cardiac fibroblasts and myofibroblasts*

To evaluate whether the different pro-IL-1β and pro-caspase-1 levels and activity found in CF versus CMF are also reflected in secreted cytokine levels, we measured the IL-1β protein levels in the culture media. Our results show that at LPS 8 h + ATP 16 h, CF and CMF secreted similar amounts of IL-1β (Fig. 5A and B). This

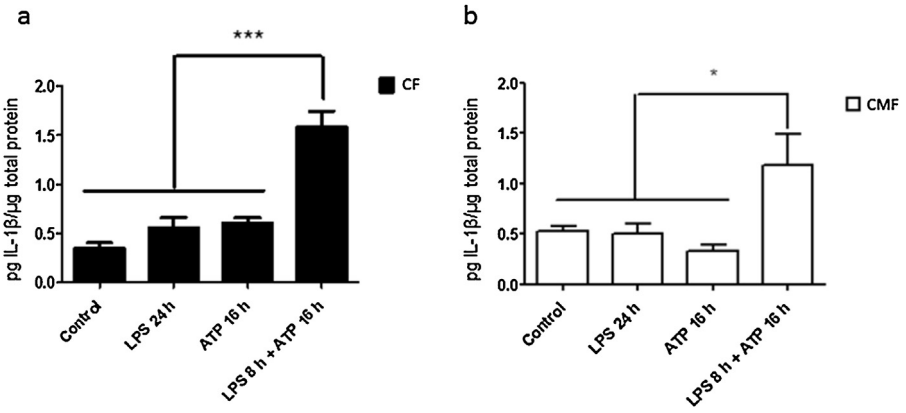

Fig. 5. IL-1β secretion in cardiac fibroblast and myofibroblast. Cells were stimulated with LPS (1 μg/mL) and then IL-1β was quantified in the culture medium. (a) pg IL-1β/μg total protein secreted by CFs; (b) pg IL-1β/μg total protein by CMFs. IL-1β levels were analyzed by ELISA as described in methods section. The results represent means (±S.E.M.) of 5 independent experiments (*$p < 0.05$; and ***$p < 0.001$ vs. controls).

result was surprising, since CF have higher levels of pro-IL-1β and we therefore expected to find higher secretion levels. This result prompted us to study IL-1β degradation mechanisms.

3.7. Intracellular pro-IL-1β is degraded by autophagy

We previously showed that CF expresses higher levels of intracellular pro-IL-1β than CMF. It is well-known that unsecreted intracellular pro-IL-1β can be degraded by proteasomal and autophagic mechanisms, so we used proteasome inhibitors to study degradation. However, because this chemical also affects IL-1β synthesis, proteasome pathways could not be evaluated in this experiment. Therefore, we performed another experiment using rapamycin to induce autophagy so that we could evaluate the involvement of this mechanism. Fig. 6A shows that in CF treated with LPS, pro-IL-1β peaked at 8 h and then decreased in a time-dependent manner. Fig. 6B shows that at 48 h of LPS exposure, the presence of rapamycin strongly decreases pro-IL-1β levels as compared to the control and to LPS without rapamycin.

4. Discussion

Myocardial dysfunction is consequence of an exaggerated inflammatory response in severe sepsis and septic shock, in which NLPR3 inflammasome/caspase-1/IL-1β pathway are involved (Romero-Bermejo et al., 2011). Mice deficient in components of the inflammasome complex (ASC or NLPR3) are more resistant to the lethal effects of endotoxin (Mariathasan et al., 2004, 2006), highlighting the role of inflammasome; however, the role of CF in the development of the LPS-induced inflammatory response are less known. In the present study we highlight the participation of CF in the inflammatory response triggered by LPS in a similar manner to occur in cardiac sepsis.

4.1. TGF-β1 induces and regulates TLR4 expression in cardiac fibroblasts

The role of TLR4 in cardiac fibrosis has been clearly demonstrated over the last decade (Timmers et al., 2008; Dong et al., 2015). It appears that this receptor's presence in CF could be a critical factor in myocardial fibrosis and dysfunction (Zhang et al., 2014; Zhang et al., 2015). Since adverse remodeling is largely attributable to CF, and in particular their differentiation to CMF, it is important to know whether CF-to-CMF differentiation affects TLR4 expression and how regulation of this process might occur. To date no study had conclusively linked TGF-β1 to TLR4 expression in this cell type. This is the first report demonstrating that this growth factor triggers a significant increase in TLR4 expression. According to our data, the molecular weight of the TLR4 found in CF and CMF indicates a mature isoform capable of anchoring to the plasma membrane and producing biological activity (da Silva Correia and Ulevitch, 2002). In cardiac tissue, TLR4 is expressed in cardiomyocytes (Frantz et al., 1999; Tian et al., 2013), endothelial cells (Zeuke et al., 2002), and vascular smooth muscle cells (Yang et al., 2005; Jimenez et al., 2005). Our findings show that TLR4 expression enables CF and CMF to respond to pro-inflammatory stimuli, strengthening the concept of CF as sentinel cells that detect danger and trigger an appropriate inflammatory response.

4.2. TGF-β1 induces TLR4 expression in cardiac fibroblasts through the TGFβRI and p38 signaling pathways

The cytokine TGF-β1 is involved in cardiac fibrosis (Frangogiannis, 2008), inducing CF-to-CMF differentiation and increasing collagen secretion in CF. We have previously reported that TGF-β1 activates canonical (Smad) and noncanonical (MAPK and PI3K-Akt) signaling pathways (Vivar et al., 2013). Furthermore, in parallel to the cell differentiation process, TGF-β1 increases levels of the B1 kinin receptor, which mediates important inflammatory responses (Catalán et al., 2012). In this work, we show that TGF-β1 induces TLR4 expression, suggesting that CF play a key role in the inflammatory response. To our knowledge, there is no previous data indicating that TGF-β1 controls TLR4 expression in mesenchymal cells. TGF-β1 modulates monocyte TLR4 expression in the peripheral blood of septic rats (Zhang and Xing, 2011), and both sterile and pathogenic insults like high glucose concentrations (Kaur et al., 2012) and bacterial infections (Wong et al., 2011) induce TLR4 expression. Interestingly, our findings demonstrate that TGF-β1-induced TLR4 expression is mediated by the TGFβRI and p38 signaling pathways, but not the PI3K-Akt, ERK1/2, or JNK signaling pathways. Consistent with our results, murine macrophages respond to LPS, IL-1β, and TNF-α through the NF-κB and p38 intracellular signaling pathways, promoting TLR2 but not TLR4 expression (Oshikawa and Sugiyama, 2003). The canonical TGF-β1 pathway is controlled by Smad proteins. However, *in silico* analysis of the TLR4 promoter region did not reveal Smad binding sites, although c-ETS and GATA binding sites are present in the regulatory gene region and negatively control TLR4 expression. Both transcription factors are negatively controlled by TGF-β1 through the Smad signaling pathway, suggesting a possible regulatory mechanism for TGF-β1-induced TLR4 expression (Roger et al., 2005). The finding that TGFβRI modulates TLR4 leads us

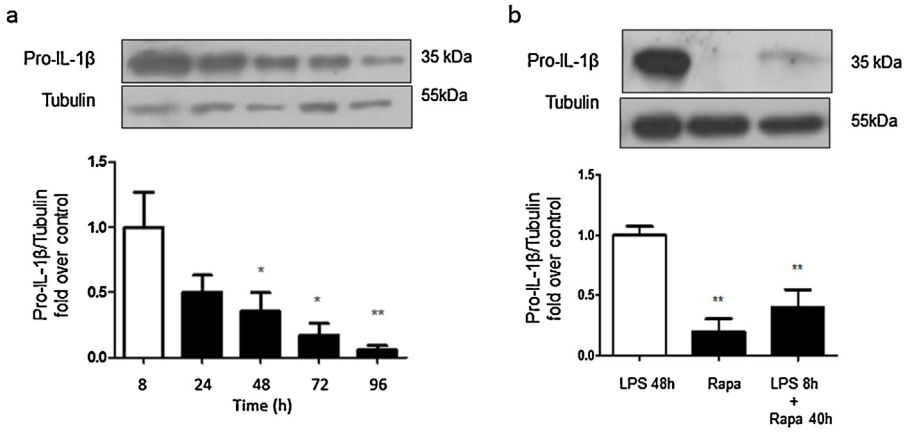

Fig. 6. pro-IL-1β levels are degraded by autophagy in cardiac fibroblast. (a) CFs were stimulated with LPS (1 μg/mL) for 8–96 h and pro-IL-1β levels were analyzed. The results represent means (±S.E.M.) of 5 independent experiments (*$p < 0.05$; **$p < 0.01$ vs. LPS 8 h). (b) CFs were stimulated with LPS (1 μg/mL) for 48 (control) and preincubated with LPS 8 h and then rapamycin for 40 h (rapamycin control for 40 h). pro-IL-1β levels were analyzed. Tubulin was used as load control. A representative immunoblot image and a bar graph analysis are showed. The results represent means (±S.E.M.) of 4 independent experiments (**$p < 0.01$ vs. LPS 48 h).

to propose that TLR4 expression and CF-to-CMF differentiation occur simultaneously but independently of one other, mainly because the p38 inhibitor affects TLR4 but not α-SMA expression, whereas inhibiting the canonical pathway prevents both TLR4 and α-SMA expression. In summary, these data indicate that there are multiple regulatory mechanisms for TLR4 expression and CF-to-CMF differentiation (evaluated here as α-SMA expression) and that TGF-β1-activated canonical signal transduction plays a role in controlling both TLR4 and α-SMA expression, whereas the non-canonical pathway p38 only controls TLR4 expression.

4.3. *In cardiac fibroblasts and myofibroblasts, LPS activates the ERK1/2, Akt, and NF-κB signaling pathways through TLR4*

To analyze TLR4 signaling pathways, we added its classic ligand LPS, to the cells at a commonly-used concentration and measured activation of three signaling pathways, p65-NF-κB, ERK1/2, and PI3K-Akt. In CF, LPS activated NF-κB, ERK1/2, and PI3K-Akt with activation kinetics comparable to those described in the literature (Laird et al., 2009; Stawowy et al., 2003). Moreover, in CF and CMF, inhibiting TLR4 with TAK-242 reduced even baseline protein levels. A likely explanation for this finding is that some factor that was either present in the culture medium or secreted by the cells is responsible for baseline protein expression. We hypothesize that this inductor molecule might be fibronectin ED-A, a component of the ECM, which has been described as an endogenous TLR4 ligand (Okamura et al., 2001). Finally, LPS increased ERK1/2 and NF-κB activation, an effect that was inhibited by TAK-242. Thus, we conclude that LPS triggers these signaling pathways in CF and CMF through TLR4, showing that this receptor is active and that its respective ligands could be able to trigger the expression of proinflammatory cytokines.

4.4. *LPS increases pro-IL-1β mRNA and protein levels in cardiac fibroblasts*

In human CF, LPS increases mRNA and leads to the synthesis and release of proinflammatory cytokines, and therefore these cells are of pathogenetic importance in inflammation and fibrosis in the heart during sepsis, leading to cardiac dysfunction that would affect the outcome of sepsis syndrome (Tomita et al., 2015). We demonstrated here that in CF, LPS provoked a time-dependent increase in pro-IL-1β protein and mRNA levels by engaging with TLR4. LPS also increased pro-IL-1β levels in CMF, although less dramatically than in CF. This result was unexpected, because CMF express higher levels of TLR4 than CF; moreover, stimulation with LPS did not increase pro-IL-1β levels in CMF beyond than those found in CF. This means that the higher TLR4 expression levels in the CMF did not result in a major increase in pro-IL1-β levels. It is well known that CMF play a more profibrotic than proinflammatory role and that TGF-β1 is an anti-inflammatory growth factor; a possible explanation for this finding, therefore, is that although CMF express higher TLR4 levels than CF, the effect of LPS on CMF may have been minimized by pre-treatment with TGF-β1. This explanation is consistent with the literature indicating that TGF-β1 has a disruptive effect on LPS-triggered signaling pathways in terms of cytokine production (Le et al., 2004) and that LPS antagonizes the TGF-β1 signaling pathway (Kim and Kim, 2011). Finally, our data show that LPS activates similar signaling pathways in CF and CMF, increasing pro-IL-1β levels in both cell types. This finding is expected given that CMF are derived from CF, so it is certainly reasonable to imagine that some signaling pathways are maintained in the differentiation process although we have previously shown that CF and CMF also have distinct signaling pathways (Olmedo et al., 2013; Catalán et al., 2012; Vivar et al., 2013). Collectively, these results suggest that the TGF-β1-induced increases in TLR4 levels found in CMF could be involved in the differentiation process. Differentiation of CF to CMF would increase the inflammatory response in wounded areas, where CMF are known to be abundant, participating in the scarring process.

4.5. *ATP activates the NLRP3 inflammasome complex and triggers the release of IL-1β in cardiac fibroblasts and myofibroblasts*

The NLRP3 inflammasome is well-characterized as an exceptional sensor protein that responds to diverse physical and chemical stimuli. It is well-known that LPS induce the expression of NLRP3 and pro-IL-1β (Hsu and Wen, 2002). In the heart, the NLRP3 inflammasome is predominantly expressed in CF and plays a key role in tissue repair (Kawaguchi et al., 2011; Sandanger et al., 2013). Here we show that LPS did not increase NLRP3, ASC, or pro-caspase-1 levels in either CF or CMF. However, CMF had higher pro-caspase-1 and caspase-1 activity levels. Our study provides evidence that the NLRP3 inflammasome complex is not activated by LPS in CF or CMF. In contradiction, Zhang et al., (2014) reported that LPS activated the inflammasome and triggered the release of IL-1β into the culture medium. A similar result was found in human CF, where LPS increased IL-1β levels in the culture medium, although inflammasome activity was not studied (Tomita et al., 2015). However, in most cell types, inflammasome activation requires an

additional signal independent of LPS. Signal 1 induces pro-IL-1β synthesis, while signal 2 facilitates inflammasome assembly and activation, culminating with the cleaving of inactive pro-IL-1β to mature IL-1β and finally secretion. Our results show that ATP induces caspase-1 activation and IL-1β release· Zhang et al. (2014) reported that secreted IL-1β can impact cardiomyocyte function, impairing its contractile function, and inappropriate activation of the NLRP3 inflammasome in CF has been implicated in ischemia/reperfusion-induced myocardial injury (Kawaguchi et al., 2011).

CMF had higher baseline pro-caspase-1 levels, a finding consistent with their superior ability to cleave pro-IL-1β (Tsung et al., 2005; Harris et al., 2011; Stawowy et al., 2003), whereas pro-IL-1β protein levels were lower in CMF vs. CF. However, IL-1β secretion was almost identical between the two cell types. It is possible that the heightened NLRP3 inflammasome activity in CMF accelerates fragmentation of pro-IL-1β into IL-1β, while low inflammasome activity levels in CF lead to low rates of IL-1β secretion despite high pro-IL-1β levels, ultimately leading to similar IL-1β levels in the two cell types.

Caspase-1 activity, on the other hand, has been associated with various cellular processes, such as pyroptosis (Takahashi, 2014) and cell survival mechanisms (Sun et al., 2013). We did not observe CF or CMF death attributable to LPS treatment in this study (data not shown); however, we have previously reported that CMF are more resistant than CF to various cell death stimuli, possibly indicating that caspase-1 has a cytoprotective effect similar to that of TGF-β1 (Copaja et al., 2011; Vivar et al., 2013). In epithelial and immune cells, this enzyme has substrates other than pro-IL-1β and pro-IL-18 (pro-IL-37, RIP2, SREBP2), whose activation leads to anti-inflammatory consequences, suggesting potential involvement with cell survival processes (Winkler and Rösen-Wolff, 2015).

4.6. In cardiac fibroblasts, pro-IL-1β is degraded by autophagy

Unprocessed and inactive pro-IL-1β levels in the CF decreased in a time-dependent manner. Importantly, this degradation was not abrupt, but rather subtle and continuous. We propose that this degradation process may be a regulatory mechanism, possibly conducted by autophagy, because rapamycin strongly decreases intracellular pro-IL-1β levels. Several authors have suggested that autophagy may be a mechanism to regulate pro-IL-1β levels (Harris et al., 2011), and abundant data in the literature indicate that either the proteosomal or the autophagic pathway could degrade this protein. However, we could not demonstrate that pro-IL-1β is degraded by proteasomes, because proteasome inhibitors (i.e. MG-132) also inhibit pro-IL-1β synthesis (data not shown).

5. Conclusion

Collectively, our results indicate that TLR4 expression allows CF and CMF to mediate inflammation in cardiac tissue, as occur in cardiac sepsis or septic shock. Stimulating TLR4 with LPS differentially triggers and modulates the release of proinflammatory cytokines, potentially affecting tissue function and repair. CF have a proinflammatory role, whereas CMF have a profibrotic role; however, both cell types participate in the inflammatory process. IL-1β secreted by CF initiates the inflammatory process, attracting proinflammatory immune cells, while IL-1β secreted by CMF may maintain the inflammatory process through the later stages of wound healing. The prolonged inflammation attributable to the actions of CMF may result in fibrotic scarring and ultimately cardiac dysfunction induced by sepsis.

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