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The NLRP3 inflammasome is up-regulated in cardiac fibroblasts and mediates myocardial ischaemia–reperfusion injury

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Aims *N*ucleotide-binding oligomerization domain-*L*ike *R*eceptor with a *P*yrin domain 3 (NLRP3) is considered necessary for initiating a profound sterile inflammatory response. NLRP3 forms multi-protein complexes with *A*poptosis-associated *S*peck-like protein containing a *C*aspase recruitment domain (ASC) and Caspase-1, which activate pro-interleukin-1β (IL-1β) and pro-IL-18. The role of NLRP3 in cardiac cells is not known. Thus, we investigated the expression and function of NLRP3 during myocardial ischaemia.

Methods and results Myocardial infarction (MI) was induced in adult C57BL/6 mice and Wistar rats by ligation of the coronary artery. A marked increase in NLRP3, IL-1β, and IL-18 mRNA expression was found in the left ventricle after MI, primarily located to myocardial fibroblasts. *In vitro* studies in cells from adult mice showed that myocardial fibroblasts released IL-1β and IL-18 when primed with lipopolysaccharide and subsequently exposed to the danger signal adenosine triphosphate, a molecule released after tissue damage during MI. When hearts were isolated from NLRP3-deficient mice, perfused and subjected to global ischaemia and reperfusion, a marked improvement of cardiac function and reduction of hypoxic damage was found compared with wild-type hearts. This was not observed in ASC-deficient hearts, potentially reflecting a protective role of other ASC-dependent inflammasomes or inflammasome-independent effects of NLRP3.

Conclusion This study shows that the NLRP3 inflammasome is up-regulated in myocardial fibroblasts post-MI, and may be a significant contributor to infarct size development during ischaemia–reperfusion.

Keywords Ischaemia–reperfusion • Heart • Myocardial infarction • IL-1β • Inflammasome

1. Introduction

Myocardial infarction (MI) is associated with a sterile inflammatory response.[1] This inflammatory process is a prerequisite for tissue healing, but may also cause excessive damage and maladaptive ventricular remodelling leading to impaired myocardial function and heart failure.[1] Accumulating evidence indicates that the myocardial response to tissue injury is regulated by the innate immune system, involving several families of pattern recognition receptors (PRRs).[2] The best studied PRR family is the Toll-like receptors (TLRs) which in addition to recognize conserved motifs of microbial origin (pathogen-associated molecular patterns),[3] also can be activated by endogenous molecules released by stressed or necrotic cells and by damaged extracellular matrix [danger associated molecular patterns (DAMPs)].[2] During acute MI, DAMP-induced

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activation of TLRs may initiate a signalling cascade leading to enhanced synthesis of inflammatory cytokines.[2]

Of the first up-stream inflammatory cytokines produced, interleukin (IL)-1β is probably the most important signal amplifier due to its potent ability to induce secretion of other cytokines.[4] IL-1β release is tightly regulated, and at least two qualitatively different signals are required: (i) an initial signal leading to pro-IL-1β synthesis; (ii) a secondary signal causing IL-1β activation by proteolytic cleavage of pro-IL-1β to its active form.[5] In MI, the first signal can be provided through activation of TLRs.[2] The second signal could be mediated by activation of *N*ucleotide-binding oligomerization domain-*L*ike *R*eceptor with a *P*yrin-domain (NLRP)3,[5] but at present, there are no reports on regulation of NLRP3 within the myocardium. Activation of NLRP3, for example by extracellular adenosine triphosphate (ATP) and monosodium urate (MSU) crystals,[5] leads to assembly of a multi-protein complex termed the NLRP3-dependent inflammasome, consisting of NLRP3, an adaptor protein termed *A*poptosis-associated *S*peck-like protein containing a *C*aspase-recruitment domain (ASC) and caspase-1. Within this complex, activated caspase-1 cleaves pro-IL-1β into active IL-1β and facilitates release through an unknown mechanism.[5] Similarly, release of IL-18, another member of the IL-1 cytokine family, also depends on caspase-1.[4]

Necrotic cells have been shown to activate NLRP3, and Nlrp3-deficient mice were protected against mortality and renal dysfunction in a renal ischaemic acute tubular necrosis model.[6,7] Kawaguchi *et al.*[8] were the first to address the function of the inflammasome in the heart. They demonstrated inflammasome activation and IL-1β production during myocardial ischaemia–reperfusion (I/R) *in vivo*. When studying ASC-deficient mice during myocardial I/R, they found reduced inflammation and, most importantly, markedly reduced infarct sizes. Notably, based on *in vitro* and bone marrow transplantation experiments, they pointed out the cardiac fibroblast as a central myocardial resident cell for inflammasome activation specifically, and I/R injury in general.[8] However, while the study of Kawaguchi *et al.*[8] focused on the scaffold protein ASC, the role of NLRP3 in the pathology of MI is still unknown. In the present study, we examined the role of NLRP3 during MI by different experimental approaches including the regulation of NLRP3 and related molecules in the left ventricle (LV) in mice subjected to MI, the regulation and function of NLRP3 in myocardial fibroblasts, and the effect of NLRP3 on hypoxic damage using the *ex vivo* I/R Langendorff technique on hearts from NLRP3$^{-/-}$, ASC$^{-/-}$, and WT mice.

2. Methods

See Supplementary material online, for detailed methodology.

2.1 NLRP3$^{-/-}$ and ASC$^{-/-}$ mice

Generation of NLRP3$^{-/-}$ and ASC$^{-/-}$ mice on a C57BL/6 background is described elsewhere.[9,10] Animal experiments were approved by the Norwegian Animal Research Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

2.2 The MI model

MI was surgically induced in male C57BL/6 mice and Wistar rats as described.[11] To provide analgesia, buprenorphine (0.1 mg/kg) was injected subcutaneously before the operation. Anaesthesia was induced with >5% isoflurane and <95% O_2 in a gas chamber, followed by tracheotomy and ventilation with 2% isoflurane and 98% O_2. The depth of anaesthesia was monitored by the respiratory rate and lack of withdrawal reflex upon toe pinching. Three or 7 days after surgery, the mice were anaesthetized as described above and the hearts were extirpated and LV sectioned into visible infarcted area and non-infarcted LV tissue. In separate experiments, adult male Wistar rats were subjected to MI, and 7 days post-surgery, cardiomyocytes and non-cardiomyocytes were isolated as previously described.[12]

2.3 Isolation and culture of cardiac fibroblasts

Adult C57BL/6, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice were anaesthetized with >5% isoflurane gas and <95% O_2 in a gas chamber. The hearts were extirpated and cardiac fibroblasts were isolated by Ca^{2+}-free retrograde perfusion with Tyrode's solution and enzymatic digestion using collagenase type 2.

2.4 Isolation of monocytes

Monocytes were isolated from whole blood from healthy donors by Lymphoprep (Axis-Shield, Oslo, Norway) and plastic adherence.[13] The study was approved by the local ethical committee (Regional ethics committee of Helse Sør-Øst; Permit number S-05172) and conducted according to the ethical guidelines outlined in the Declaration of Helsinki for use of human tissue and subjects.

2.5 Quantification of mRNA levels

Total RNA was extracted from cardiac tissue or cardiac fibroblasts. Quantification of gene expression was performed using real-time RT–PCR.

2.6 Confocal imaging

Cells were fixed and incubated with antibodies against NLRP3 and ASC. Images were captured with an Axiovert 100-M microscope housing a Zeiss LSM 510 META scanning unit and a 1.4 × 40 plan apochromat objective.

2.7 *Ex vivo* ischaemia–reperfusion with the langendorff technique

Adult C57/BL6, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice were injected intraperitoneally with sodium pentobarbital 60 mg/kg and 500 IU heparin. Adequacy of anaesthesia was assessed by the pedal withdrawal reflex. Hearts were extirpated and perfused retrogradely as described,[14] stabilized for 20 min, and exposed to 35 min global ischaemia and 60 min reperfusion. Details on monitoring of cardiac function and infarct size determination are in Supplementary material online.

2.8 Detection of apoptosis

Apoptosis was visualized using a TdT-mediated dUTP-biotin nick end labelling (TUNEL) fluorometric kit.

2.9 Quantification of cytokines

Mouse IL-1β, tumour necrosis factor (TNF)-α, and fibroblast growth factor (FGF)-2 were quantified with bead-based immune assays from R&D Systems. Mouse IL-18 and Macrophage inflammatory protein (MIP)-2 were quantified with ELISAs (MBL International, Woburn, MA; R&D Systems, respectively). Intracellular IL-1β was detected by immunoblotting of protein homogenates of cardiac fibroblasts and secreted IL-1β was immunoprecipitated from conditioned media.

2.10 Statistical analysis

For comparisons of more than two groups, the Kruskal–Wallis test was used. If a significant difference was found, the Mann–Whitney *U* test was used to determine the differences between a hypothesis-driven selection of pairs of groups. Differences in the recovery of post-ischaemic haemodynamic parameters were tested by using two-way analysis of variance with repeated measures on one factor, taking genotype as an independent factor, and time as a dependent factor. A Tukey's *post hoc* test was used. Probabilities are two-sided and considered to be significant when $P < 0.05$.

3. Results

3.1 NLRP3, IL-1β, and IL-18 mRNA expression are increased in cardiac fibroblasts in post-MI mice

As shown in *Figure 1A–C*, NLRP3, IL-1β, and IL-18 mRNA levels were all significantly increased in tissue from the non-infarcted and in particular the infarcted area in post-MI mice (3 and 7 days) compared with LV tissue from sham-operated mice. In parallel, in a rat model of MI, we investigated whether the up-regulation of these mediators was restricted to a particular cell type within the LV 7 days following MI. Although MI significantly increased NLRP3, IL-1β, and IL-18 gene expression compared with sham-operated mice in both cardiomyocytes and non-cardiomyocytes, the non-cardiomyocyte cell fraction had by far the highest NLRP3, IL-1β, and IL-18 mRNA expression (*Figure 1A–C*, right panels).

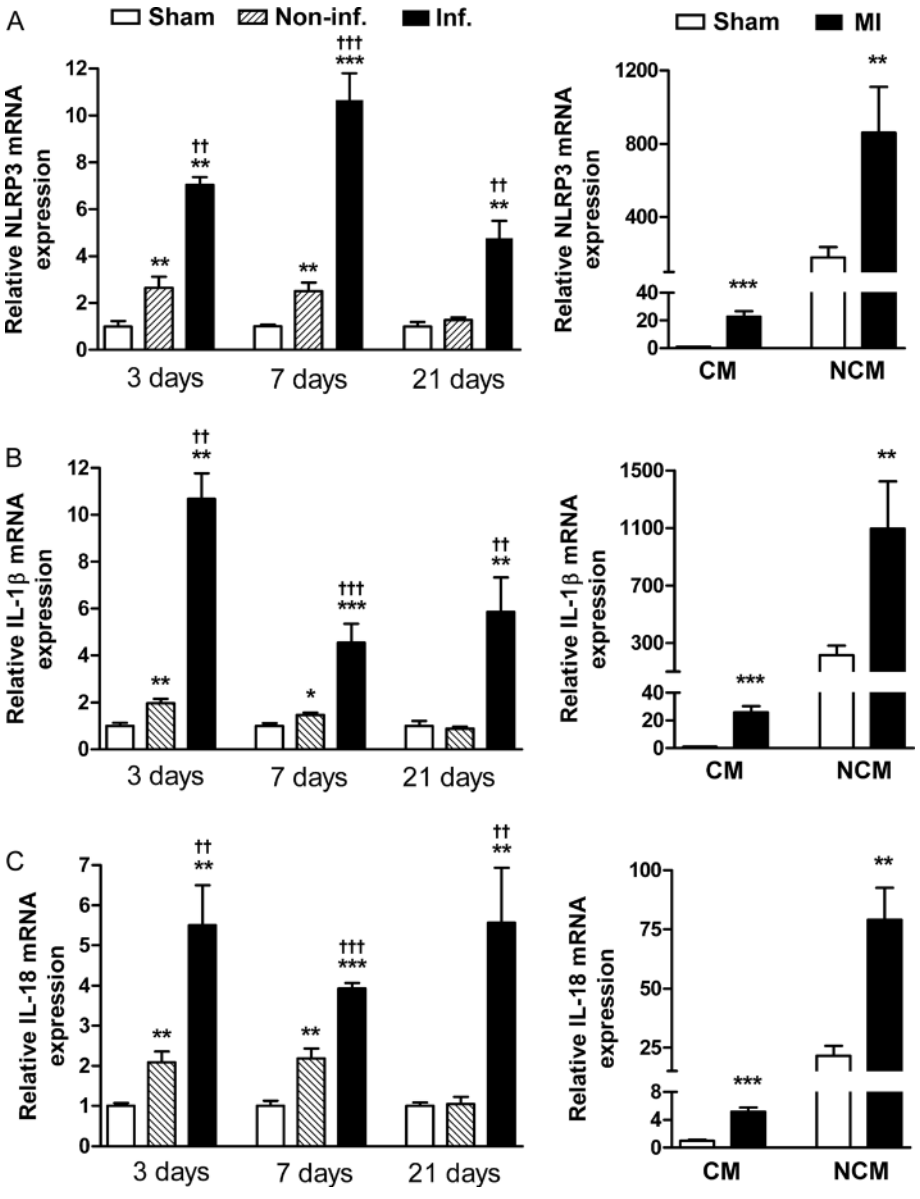

Figure 1 NLRP3, IL-1β, and IL-18 mRNA expression are increased in cardiac tissue post-MI, primarily in the non-cardiomyocyte cell fraction. (*A–C*, left panels) Male mice were sham-operated or MI was induced by ligation of the left anterior descending coronary artery (LAD). Infarcted and non-infarcted areas of the LV were harvested 3 or 7 days post-MI, and relative NLRP3, IL-1β, and IL-18 mRNA expression were quantified with RT–PCR, using GAPDH as the reference gene. (*A–C*, right panels) Male Wistar rats were sham-operated or MI was induced by LAD ligation. After 7 days, the hearts were extirpated, collagenase digested, and the cardiomyocytes separated from the non-cardiomyocyte cell fraction. Relative NLRP3, IL-1β, and IL-18 mRNA expression in the cell fractions were quantified with RT–PCR, using GAPDH as the reference gene. All mRNA expression values are normalized to that of sham-operated animals. Data are mean ± SEM ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. sham and $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ vs. non-inf (Mann–Whitney test). Inf, infarcted areas; non-inf, non-infarcted areas; MI, myocardial infarction; CM, cardiomyocytes; NCM, non-cardiomyocytes.

3.2 TLR signalling induces both NLRP3 and IL-1β mRNA expression in cardiac fibroblasts in an NFκB-dependent manner

Data presented in *Figure 1* indicate that the non-cardiomyocyte fraction of cardiac cells was the most important for inflammasome regulation. Since the fibroblast is by far the most abundant cell within this fraction (data not shown), we further focused on these cells. Cardiac tissue express TLRs,[2] which are activated during acute MI, although proposed endogenous ligands are poorly characterized. When cardiac fibroblasts were stimulated with LPS, a specific ligand for TLR4, we found a dose-dependent increase in NLRP3 and IL-1β mRNA (*Figure 2A* and *B*). While NLRP3 mRNA induction peaked at 2 h, IL-1β mRNA expression increased and persisted throughout the 24 h time period (*Figure 2C* and *D*). Pam3Cys, poly(I:C), and ssDNA from *E. Coli*, specific ligands for TLR2, TLR3, and TLR9, respectively, all increased NLRP3 and IL-1β mRNA levels, although the effects were more modest than for LPS (*Figure 2E* and *F*). HSP60, an endogenous ligand shown to activate both TLR2 and TLR4,[15] induced a modest, but significant induction of NLRP3 and IL-1β mRNA (*Figure 2E* and *F*). In contrast, the proposed danger signals HMGB1[16] and biglycan, a small leucine-rich proteoglycan with relation to myocardial extracellular matrix remodelling,[17,18] showed no effect on either NLRP3 or IL-1β (*Figure 2E* and *F* and data not shown). Finally, both the induction of NLRP3 and IL-1β mRNA by TLR ligands could be blocked by adding a specific NFκB inhibitor, Bay 11-7082, 30 min prior to stimulation (*Figure 2G* and *H*). In contrast, IL-18 mRNA expression was not increased by any of the above-mentioned ligands (Supplementary material online, *Figure S1*).

3.3 Extracellular ATP leads to assembly of the NLRP3 inflammasome in cardiac fibroblasts

To see if cardiac fibroblasts were able to assemble and activate the NLRP3 inflammasome, we pre-treated cells with medium only or LPS (10 ng/mL) for 18 h (signal 1) and then further incubated the cells with medium only or extracellular ATP (3 mM), a known inflammasome activator, for 1 h (signal 2). The spatial distribution of NLRP3 and ASC proteins was then visualized with laser scanning confocal microscopy. LPS priming increased both NLRP3 expression and NLRP3/ASC co-localization compared with non-stimulated control cells (*Figure 3A*). As expected, LPS-primed cardiac fibroblasts stimulated with ATP showed NLRP3/ASC co-localization most prominently. However, co-localization of ASC and NLRP3 was also induced by ATP in cells not primed with LPS (*Figure 3A*), indicating that NFκB activation is not a prerequisite for inflammasome assembly in cardiac fibroblasts. Using the Z-stack technique at high magnification, we also demonstrated that the NLRP3-dependent inflammasome is specifically located in the perinuclear area of the cells (*Figure 3B*). The effect of ATP was also translated into a profound release of active IL-1β from LPS-primed cardiac fibroblasts (*Figure 3C*). Little IL-1β release was induced by LPS only, and although ATP induced localization of ASC and NLRP3 without LPS priming, it did not induce any IL-1β release when given alone. While MSU crystals were an equally potent inducer of IL-1β release from LPS-primed monocytes as ATP, it failed to induce IL-1β release from cardiac fibroblasts, indicating that the route of inflammasome activation may be somewhat different between these two cells (*Figure 3D*). To examine whether other activators of NFκB than LPS could prime the inflammasome, we stimulated cardiac fibroblasts with TNFα as a 'signal 1'. When 'signal 2' (i.e. ATP) was added, we found a minor but consistent release of IL-1β. While TNFα markedly increased NLRP3 mRNA levels, it had a more modest effect on IL-1β mRNA, potentially explaining the low levels of IL-1β release (Supplementary material online, *Figure S2*).

3.4 ATP-induced IL-1β release from cardiac fibroblasts depends on caspase-1 and K^+ efflux

When cells primed with LPS for 18 h were incubated with the caspase-1-specific inhibitor Z-WEHD-FMK 30 min prior to ATP stimulation, the release of IL-1β was markedly attenuated (*Figure 4A*). ATP induction of K^+ efflux through activation of the purinergic receptor P2X7 is a potent activator of NLRP3 inflammasome, and pre-incubation (30 min) of LPS-primed cells with glibenclamide, known to inhibit ATP-induced K^+ efflux, effectively reduced ATP-induced IL-1β release (*Figure 4A*). Inhibition of reactive oxygen species has previously been shown to attenuate inflammasome activation in macrophages.[5] However, in cardiac fibroblasts, addition of the antioxidants N-aceylcysteine and mitoTEMPO prior to ATP stimulation did not affect the release of IL-1β (Supplementary material online, *Figure S3*)

3.5 ATP-induced IL-1β and IL-18 release from cardiac fibroblasts depends on NLRP3 and ASC

When cardiac fibroblasts from WT, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice were primed with medium only or 10 ng/mL LPS for 18 h and cultured for additional 1 h with or without 3 mM ATP, IL-1β release was almost completely abolished as assessed by bead-based immunoassays in the absence of NLRP3$^{-/-}$ and ASC$^{-/-}$ (*Figure 4B*). IL-1β from the same supernatants was also analysed by immunoprecipitation, and 17 kDa band indicating active IL-1β was only detected in supernatants from WT fibroblasts (*Figure 4C*). In contrast, deletion of NLRP3 and ASC had no effect on the TLR-induced IL-1β mRNA level (Supplementary material online, *Figure S4*). Similarly, ATP induced IL-18 secretion from WT fibroblasts primed with LPS, but not from LPS primed fibroblasts deleted of NLRP3 or ASC (*Figure 4D*). Notably, however, while WT and NLRP3$^{-/-}$ fibroblasts spontaneously secreted small amounts of IL-18, no IL-18 could be detected in the supernatants from ASC$^{-/-}$ fibroblasts (*Figure 4D*). Finally, the secretion of TNF-α and MIP-2 are NFκB-dependent and inflammasome-independent,[19] and secretion of these cytokines was not increased when ATP was added to LPS-primed cells, and not impaired in ASC$^{-/-}$ or NLRP3$^{-/-}$ fibroblasts (*Figure 4E* and *F*).

3.6 Contractile function is preserved and infarct size is reduced in hearts from NLRP3$^{-/-}$ mice after *ex vivo* I/R

We hypothesized that the NLRP3 inflammasome in cardiac fibroblasts contributes to tissue damage during MI and examined the ability of NLRP3 to modulate myocardial damage following I/R. Hearts from WT, NLRP3$^{-/-}$, and ASC$^{-/-}$ mice were extirpated and perfused retrogradely using the Langendorff technique. Neutrophil-derived enzymes can process the IL-1β precursor,[20] but in this model IL-1β release will solely depend on myocardial cells. Hearts from NLRP3$^{-/-}$ mice showed significantly higher LVDP, lower LVEDP, and preserved coronary flow, compared with WT hearts after 60 min of re-perfusion (*Figure 5A–C*). TTC staining confirmed the functional data with less ischaemic damage in hearts from NLRP3$^{-/-}$ mice compared with WT

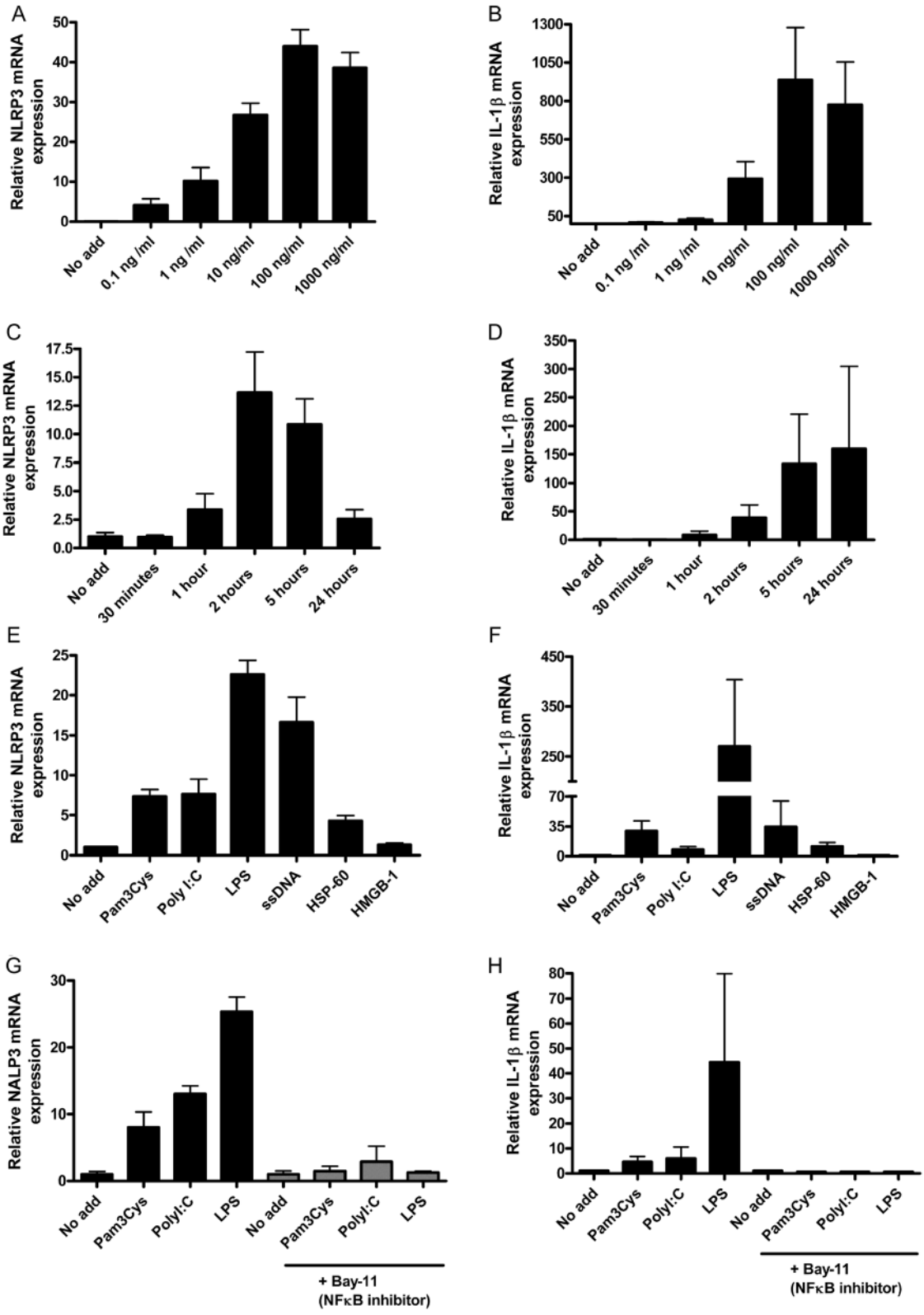

Figure 2 NFκB-dependent induction of NLRP3 and IL-1β mRNA expression in cardiac fibroblasts by TLR ligands. (*A*–*D*) LPS induced NLRP3 and IL-1β mRNA expression in a dose- and time-dependent manner. Cardiac fibroblasts were stimulated with LPS for 2 h with doses as indicated (*A* and *B*), or with 10 ng/mL LPS for the indicated time (*C and D*). (*E* and *F*) cardiac fibroblasts were stimulated with medium or Pam3Cys (10 ng/mL), Poly(I:C) (5 μg/mL), LPS (10 ng/mL), ssDNA from *E. coli* (10 μg/mL), HSP60 (1 μg/mL), or HMGB1 (10 μg/mL) for 2 h. (*G* and *H*) Cardiac fibroblasts were pre-incubated with the NFκB inhibitor, Bay 11-7082 (12 μM) 30 min prior to TLR ligand stimulation for 2 h (concentrations as *E*–*F*). Relative NLRP3 and IL-1β mRNA expression were quantified with RT–PCR, using GAPDH as the reference gene. mRNA expression values are normalized to that of cells stimulated with medium only. Data are mean ± SEM ($n = 3$–6).

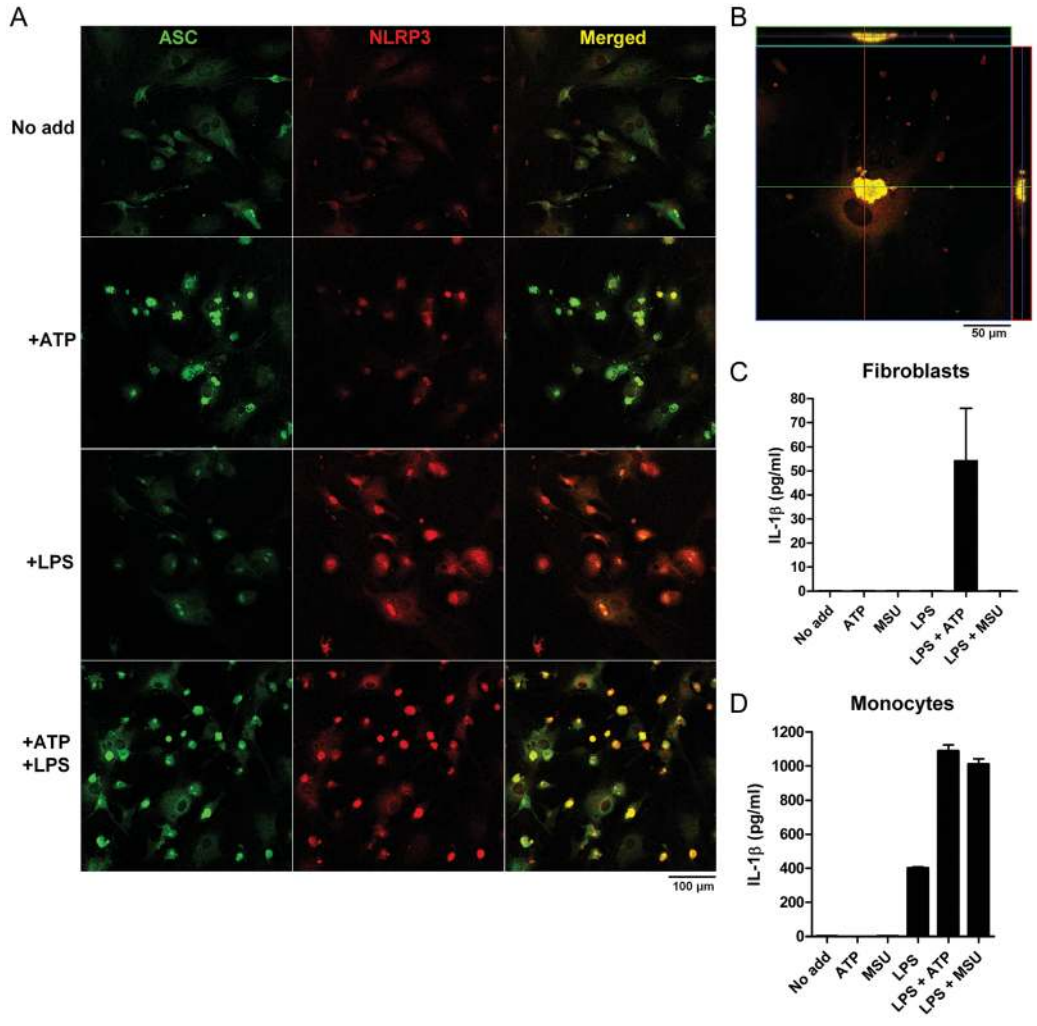

Figure 3 ATP induce assembly of the NLRP3 inflammasome, but TLR signalling is prerequisite for ATP-induced IL-1β release. (*A*) Confocal microscopy visualizing NLRP3 and ASC in cardiac fibroblasts. (*B*) High magnification z-stack image of the NLRP3 inflammasome. Cardiac fibroblasts were stimulated with medium or LPS (10 ng/mL) for 18 h, then incubated with ATP (3 mM) for 1 h. Cells were indirectly labelled with anti-NLRP3 (NLRP3/NALP3 mAb Cryo 2; anti rabbit-Alexa633) and anti-ASC (ASC pAb AL177; anti mouse-Alexa488). Images are representatives from three independent experiments. (*C*) IL-1β release from cardiac fibroblasts quantified with a bead-based immunoassay. Cardiac fibroblasts were stimulated with LPS (10 ng/mL) for 18 h, then incubated with ATP (3 mM) or MSU (10 μg/mL) for 1 h. (*D*) IL-1β release from monocytes quantified with ELISA. Human monocytes were stimulated with LPS (10 ng/mL) for 5 h, then incubated ATP (3 mM) or MSU (10 μg/mL) for 1 h. Data are mean ± SEM ($n = 3–6$). MSU, monosodium urate.

mice (*Figure 5D–E*). ASC$^{-/-}$ mice had similar LVDP as NLRP3$^{-/-}$ mice, indicating preserved contractile function, and tended to have preserved coronary flow. However, there was no significant difference in LVEDP or ischaemic tissue damage between hearts from ASC$^{-/-}$ and WT mice (*Figure 5*). LVSP, HR, and RPP were not statistically significant different between WT, NLRP3$^{-/-}$, and ASC$^{-/-}$ hearts (Supplementary material online, *Figure S5A–C*).

3.7 Decreased apoptosis in hearts from NLRP3$^{-/-}$ mice compared with WT-mice after *ex vivo* I/R

Enhanced apoptosis contributes to myocardial damage during I/R.[21] To elucidate the difference between NLRP3$^{-/-}$ and ASC$^{-/-}$ mice in the Langendorff model, we examined if apoptosis was modulated differently by deletion of NLRP3 and ASC in this model. Myocardial apoptosis was significantly decreased in NLRP3$^{-/-}$, but not ASC$^{-/-}$ mice, when compared with WT mice after 60 min of reperfusion, as assessed by detection of histone-associated DNA fragments (TUNEL assay; Supplementary material online, *Figure S6*).

3.8 Altered release of caspase-1-dependent and -independent mediators in hearts from NLRP3$^{-/-}$ and ASC$^{-/-}$ during I/R

We next examined the effect of NLRP3 and ASC deficiency on levels of caspase-1-dependent and caspase-1-independent mediators in myocardial homogenates and perfusates obtained after 35 min of ischaemia and 60 min reperfusion. As depicted in *Figure 6A*, *B*, and *E*, IL-1β (homogenate) and IL-18 levels (perfusate) were decreased in NLRP3$^{-/-}$ and ASC$^{-/-}$ mice when compared with WT, with the most prominent effects in ASC$^{-/-}$ mice. IL-1β\xEF\x80 was not detectable in the

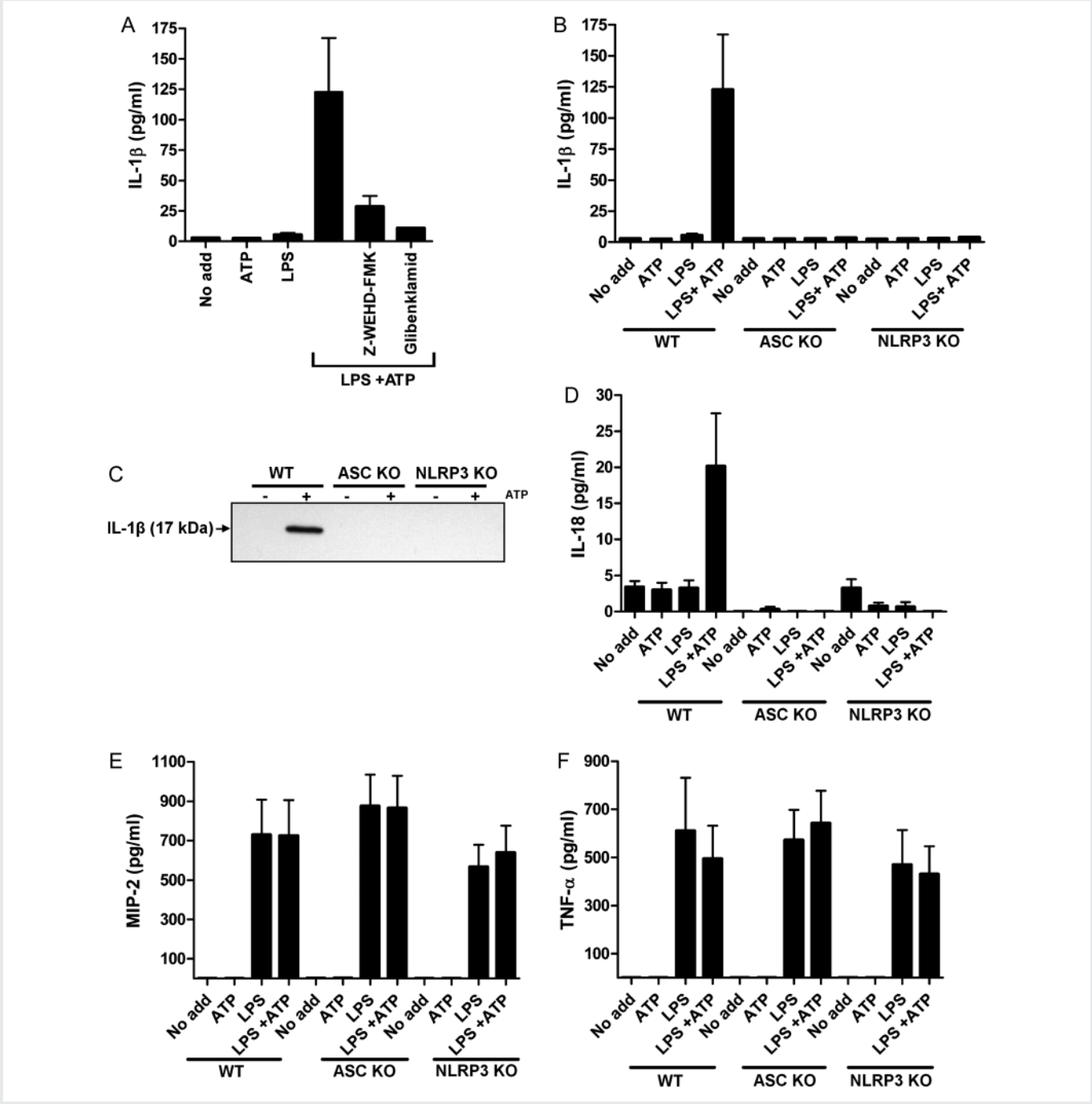

Figure 4 ATP-induced IL-1β release from cardiac fibroblasts depends on Caspase-1, K^+ efflux, ASC, and NLRP3. (*A*) Inhibition of IL-1β release from cardiac fibroblasts by glibenclamide and a caspase-1-specific inhibitor (Z-WHED-FMK). (*B*) Comparison of IL-1β release from WT, NLRP3 knockout (KO) and ASC KO cardiac fibroblasts. (*C*) Western blot of IL-1β released into conditioned medium. (*D*–*F*) Release of IL-18, MIP-2, and TNF-α from WT, NLRP3 KO, and ASC KO cardiac fibroblasts. Cardiac fibroblasts were stimulated with LPS (10 ng/mL) for 18 h, then incubated with ATP (3 mM) for 1 h. IL-1β was quantified in conditioned media with a bead-based immunoassay (*A* and *B*), or immunoprecipitated for western blot analysis (*C*). IL-18, MIP-2, and TNFα were quantified with ELISA (*D*–*F*). Data are mean ± SEM ($n = 3$–6).

perfusate from either WT, NLRP3$^{-/-}$, or ASC$^{-/-}$ hearts (data not shown). While there was no effect on the caspase-1-independent cytokine TNFα in the homogenate from NLRP3$^{-/-}$ when compared with WT heart, TNFα markedly decreased in ASC$^{-/-}$ heart (*Figure 6C*), with no detectable levels in the perfusate in either of the mice (data not shown). FGF-2, which may have cardioprotective features and is released in a caspase-1-dependent manner,[22,23] showed a different pattern in homogenate and perfusate. While ASC$^{-/-}$ showed increased levels in the homogenate, with modestly decreased levels in NLRP3$^{-/-}$ hearts, a rather opposite pattern was seen in the perfusate with markedly increased levels in NLRP3$^{-/-}$ hearts (*Figure 6D* and *F*), potentially reflecting increased release of FGF2 during I/R in the NLRP3$^{-/-}$ heart.

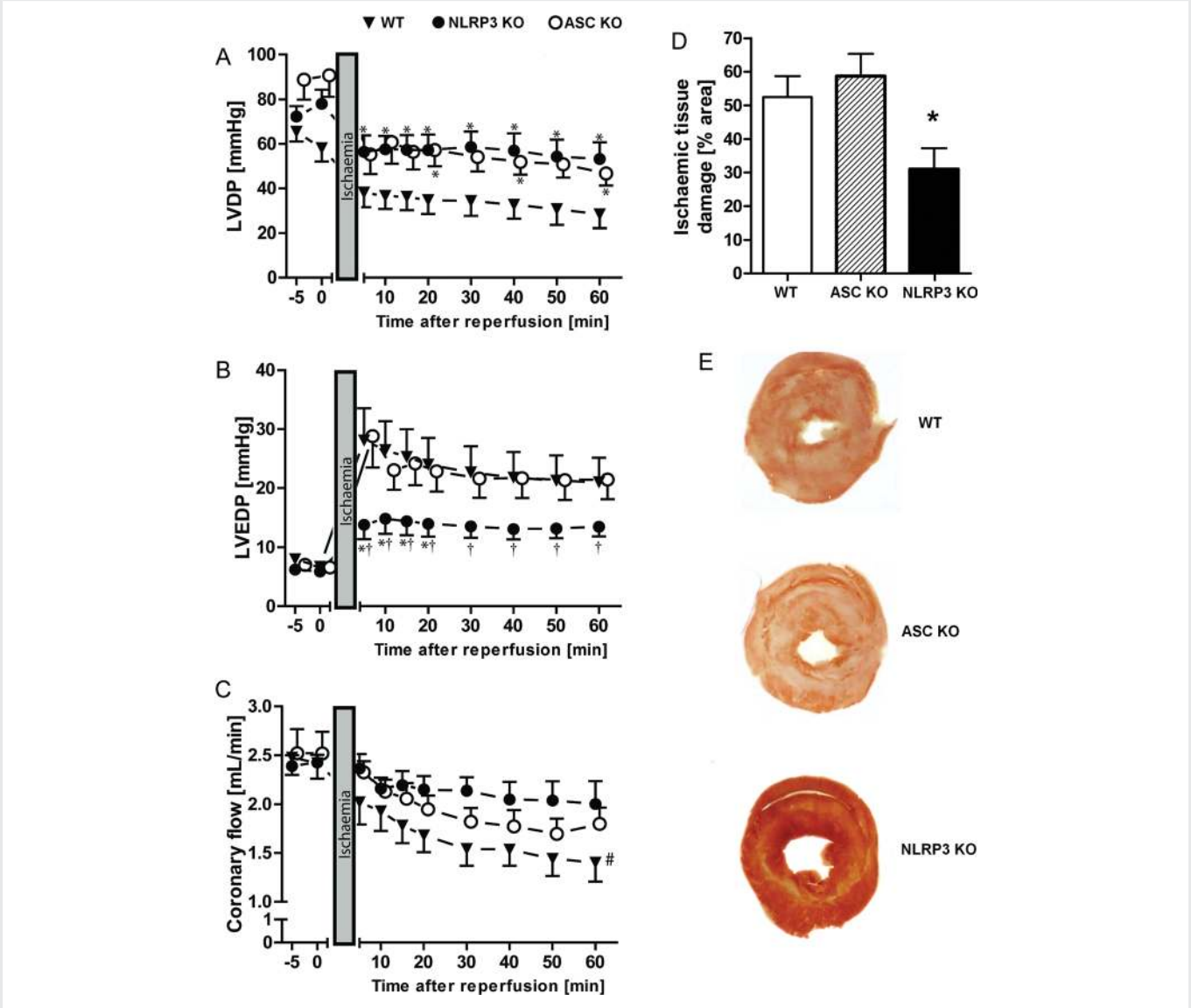

Figure 5 Preserved function and reduced infarct size in NLRP3-deficient hearts in *ex vivo* I/R. (*A*) LV developed pressure (LVDP). (*B*) LV end-diastolic pressure (LVEDP). (*C*) Coronary flow before 35 min of global ischaemia and during 60 min reperfusion. (*D*) Area of ischaemic damage determined by TTC staining. (*E*) Representative TTC stained heart sections. WT, NLRP3$^{-/-}$, and ASC$^{-/-}$ hearts were perfused *ex vivo* (Langendorff technique). Data are mean ± SEM. (*A*–*C*); multivariate analysis of variance, $P < 0.001$. Inverted triangle, WT, $n = 8$; circle, NLRP3 knockout (KO), $n = 10$; ○, ASC KO, $n = 6$. $^{*}P < 0.05$ vs. WT mice; $^{\dagger}P < 0.05$ vs. ASC KO; $^{\#}P < 0.05$ vs. NLRP3 KO and ASC KO.

4. Discussion

In this study, we have addressed the expression and function of a myocardial NLRP3 inflammasome. We show that NLRP3 is up-regulated within the myocardium following MI, primarily located in non-cardiomyocytes (i.e. fibroblasts). Our *in vitro* data show that myocardial fibroblasts release IL-1β and IL-18 when primed with LPS (signal 1) and subsequently exposed to the danger signal ATP (signal 2), a molecule that is released in relation to tissue damage during MI. Finally, we show that NLRP3 deficiency markedly improves myocardial function and reduces infarct size after *ex vivo* I/R. Our findings suggest that the NLRP3 inflammasome is up-regulated in myocardial fibroblasts following MI, potentially contributing to infarct size after I/R.

Cardiac fibroblasts are the most abundant cell type in the adult human heart, accounting for two-thirds of total myocardial cell number. Recent studies that cardiac fibroblasts serve as sentinel cells that sense injury and activate inflammatory as well as matrix regulating responses following MI, being involved in I/R injury as well in infarct repair and scar formation.[1,24] However, although innate immune responses have been shown to be important in the heart's response to injury,[2] very few studies have examined how cardiac fibroblasts respond to activators of innate immunity. Our findings underscore a central role of cardiac fibroblasts as sentinel cells that activate inflammation in response to danger signals that are released during cardiac stress or injury. We show that myocardial fibroblasts express at least TLR1-4, and 9 at a functional level by their ability to enhance IL-1β and NLRP3 mRNA

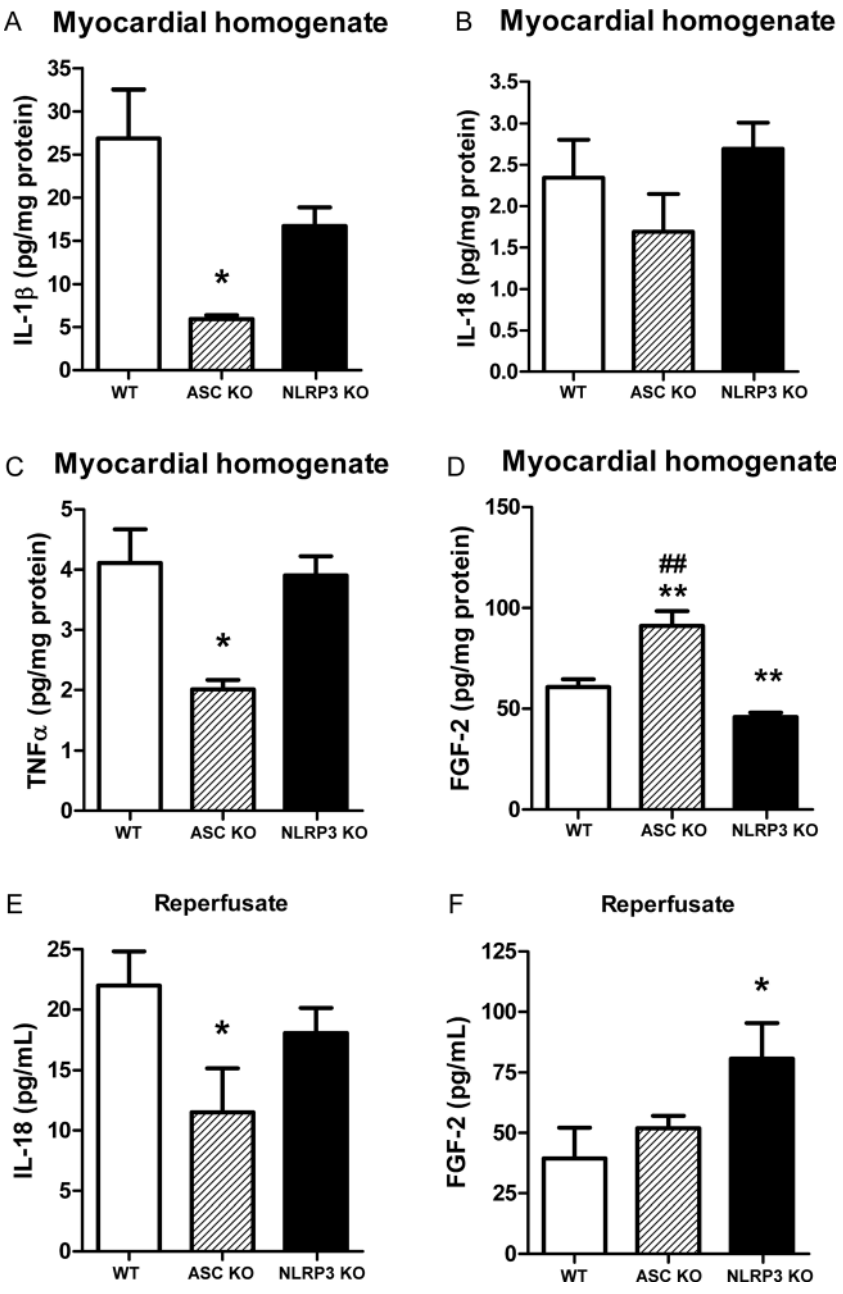

Figure 6 Altered levels of caspase-1-dependent and -independent mediators in hearts from NLRP3$^{-/-}$ and ASC$^{-/-}$ mice during *ex vivo* I/R. (*A*–*D*) IL-1β, IL-18, TNF-α, and FGF-2 quantified in myocardial homogenates. (*E* and *F*) IL-18 and FGF-2 quantified in reperfusates. Hearts from WT, NLRP3 knockout (KO), and ASC KO mice were extirpated and perfused *ex vivo*. Global ischaemia was inflicted for 35 min, followed by 10 min reperfusion. *$P < 0.05$ vs. WT; **$P < 0.01$ vs. WT; ##$P < 0.01$ vs. NLRP3 KO.

expression in response to specific TLR agonists in an NFκB-dependent manner. Most importantly, we visualized the NLRP3 inflammasome in LPS-primed cardiac fibroblasts stimulated with ATP. These LPS-primed myocardial fibroblasts exposed to ATP released significant amounts of IL-1β in a caspase-1- and NLRP3/ASC-dependent manner. The NLRP3 inflammasome drives inflammation in response to a wide variety of molecular patterns, and here we show that this important mediator of innate immune responses is functional within myocardial fibroblasts and up-regulated during MI.

In the present study, we used LPS as a 'signal 1' activator of NLRP3 inflammasome. Although LPS as a prototypical activator of TLR4 is often used in 'proof of concept' studies instead of the more newly discovered endogenous TLR4 ligands, low-grade endotoxinemia can indeed be relevant in patients with MI even in the absence of overt bacterial infections for example as a consequence of low-grade gut-leakage mechanisms.[25] Moreover, a range of chemically diverse molecules have been reported to function as a 'signal 2' in NLRP3 activation.[5] However, among the proposed NLRP3 ligands, ATP and MSU are of particular interest in sterile inflammation.[26] All living cells contain ATP and uric acid, and following necrosis, they will be released extracellularly. Notably, the heart and cardiac resident cells, including cardiomyocytes, have been shown to release ATP to the extracellular space during

hypoxia, ischaemia, and haemodynamic stress.[27] We show that LPS-primed cardiac fibroblasts release IL-1β after stimulation with ATP, but not after stimulation with MSU crystals, even though both ATP and MSU crystals induced profound IL-1β release from LPS-primed monocytes. While ATP triggers P2X7-dependent pore formation by the pannexin-1 hemichannel, inducing K^+ efflux and subsequent NLRP3 activation, MSU and other crystals are dependent of engulfment or phagocytosis for NLRP3 activation. It is therefore possible that the absence of MSU-mediated NLRP3 response in cardiac fibroblasts is due to lack of phagocytosis. Silencing P2X7 expression with siRNA *in vivo* in mice has been shown to reduce infarct size 7 days after permanent MI, suggesting that ATP is an important DAMP mediating collateral damage in cardiac pathology.[28] Glibenclamide has been reported to inhibit ATP-induced IL-1β release in LPS-primed monocytes. Herein we demonstrate that similar mechanisms are operating in myocardial fibroblasts. Glibenclamide has been shown to protect against I/R injury in the kidney and the brain,[29,30] and these effects could potentially involve modulation of NLRP3 activity. ROS have been suggested to be involved in the activation of the NLRP3 inflammasome, but somewhat surprisingly, we did not find any effect of anti-oxidants on the release of activated IL-1β in cardiac fibroblast. ROS inhibition has previously been shown to attenuate inflammasome activation in macrophages.[5] However, we were not able to use the doses used in those studies due to toxic effects of the antioxidants, potentially reflecting cellular differences (macrophages vs. cardiac fibroblasts) in tolerating these compounds. It is also possible that the role of ROS in activation of NLRP3 inflammasomes may differ between different cell types.

In the present study, we show for the first time that hearts from NLRP3$^{-/-}$ mice featured smaller infarct size and preserved contractile function and coronary flow compared with hearts from WT mice after *ex vivo* I/R. However, there was an unexpected discrepancy in infarct size between NLRP3$^{-/-}$ and ASC$^{-/-}$ hearts. Although ASC-deficient hearts showed improved systolic function, their infarct size was not significantly different from WT mice. Kawaguchi *et al.*[8] recently demonstrated that ASC- and caspase-1-deficient mice developed reduced infarct sizes in a model of *in vivo* myocardial I/R, findings which apparently may seen at variance with the present study. However, while leucocytes, complement, or other blood derived components are operating during I/R *in vivo*, these components were excluded in the present *ex vivo* model reflecting solely the influence of NLRP3 and ASC in cardiac cells. Moreover, while the function of NLRP3 is restricted to one inflammasome, ASC acts as an adaptor protein in at least five inflammasomes (i.e. NLRP1, NLRC4, NLRP6, Aim2, and NLRP3 inflammasomes),[5] and the net effect of blocking all these activators is unclear. Inflammatory cytokines such as IL-1β and IL-18 as well as TNFα are not only detrimental, but may also possess cardioprotective properties providing short-term adaptive responses to tissue injury.[31] Herein we noted that while both WT and NLRP3$^{-/-}$ fibroblasts cultured *in vitro* secreted small amounts of IL-18 prior to any stimulation, no IL-18 could be detected in the supernatants from ASC$^{-/-}$ fibroblasts. In accordance with this, IL-18 release from extirpated ASC$^{-/-}$ hearts, but not NLRP3$^{-/-}$ hearts, was significantly reduced compared with that of explanted WT hearts. It is tempting to hypothesize that while a moderate decrease in these mediators may be beneficial, a totally abolished response may be detrimental. Furthermore, we detected higher levels of FGF-2 in the collected reperfusion buffers from NLRP3$^{-/-}$ mice compared with perfusates from WT and ASC$^{-/-}$ mice. FGF-2 has been reported to increase contractile function and reduce infarct size during I/R,[23] and it is possible that the increased FGF-2 release could have contributed to reduced infarct size in NLRP3$^{-/-}$ hearts. Finally, while NLRP3-deficient mice showed decreased apoptosis during I/R, this was not seen in ASC-deficient mice. Although the contribution of necrosis to myocardial cell loss by far exceeds that of apoptosis, this finding could have contributed to the decreased infarct size in the NLRP3-deficient heart.[21]

Our findings suggest that the NLRP3 inflammasome is activated within the myocardium during MI, in particular in myocardial fibroblasts. The results from the *ex vivo* model of myocardial I/R injury show that NLRP3 activation could contribute to tissue damage during this process. Further studies are needed to clarify the exact mechanisms for this protective effect and in particular the mechanisms for the surprisingly different effect of NLRP3 and ASC deficiency in this model.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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