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AIM2-driven inflammasome activation in heart failure

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Aims Interleukin-1β (IL-1β) is an important pathogenic factor in cardiovascular diseases including chronic heart failure (HF). The CANTOS trial highlighted that inflammasomes as primary sources of IL-1 β are promising new therapeutic targets in cardiovascular diseases. Therefore, we aimed to assess inflammasome activation in failing hearts to identify activation patterns of inflammasome subtypes as sources of IL-1β.

Methods and results Out of the four major inflammasome sensors tested, expression of the inflammasome protein absent in melanoma 2 (AIM2) and NLR family CARD domain-containing protein 4 (NLRC4) increased in human HF regardless of the aetiology (ischaemic or dilated cardiomyopathy), while the NLRP1/NALP1 and NLRP3 (NLR family, pyrin domain containing 1 and 3) inflammasome showed no change in HF samples. AIM2 expression was primarily detected in monocytes/macrophages of failing hearts. Translational animal models of HF (pressure or volume overload, and permanent coronary artery ligation in rat, as well as ischaemia/reperfusion-induced HF in pigs) demonstrated activation pattern of AIM2 similar to that of observed in end-stages of human HF. *In vitro* AIM2 inflammasome activation in human Tohoku Hospital Pediatrics-1 (THP-1) monocytic cells and human AC16 cells was significantly reduced by pharmacological blockade of pannexin-1 channels by the clinically used uricosuric drug probenecid. Probenecid was also able to reduce pressure overload-induced mortality and restore indices of disease severity in a rat chronic HF model *in vivo*.

Conclusions This is the first report showing that AIM2 and NLRC4 inflammasome activation contribute to chronic inflammation in HF and that probenecid alleviates chronic HF by reducing inflammasome activation. The present translational study suggests the possibility of repositioning probenecid for HF indications.

Keywords Inflammation • Heart failure • Cardiomyopathy • Canakinumab • Probenecid • Drug repurposing

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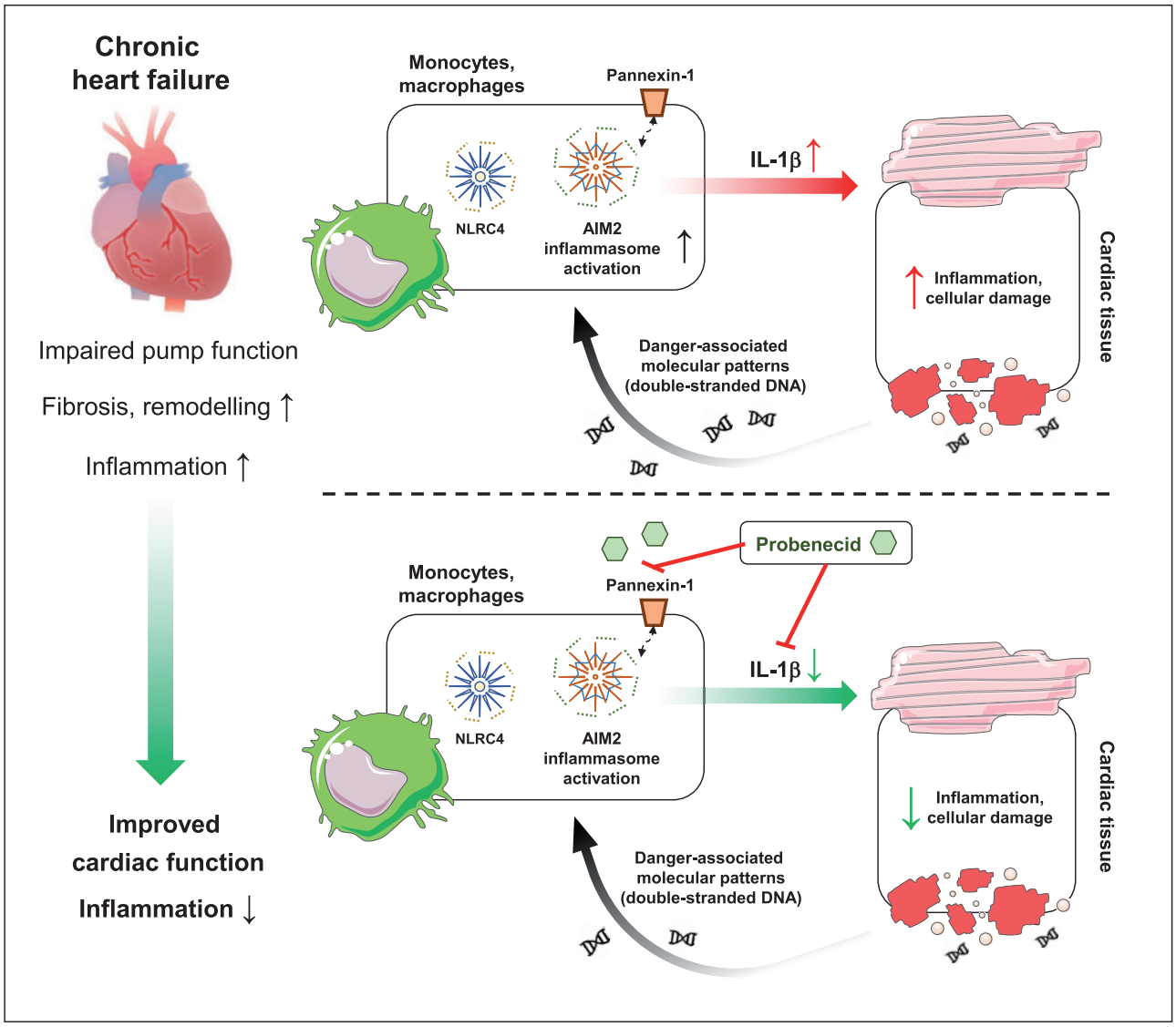

1. Introduction

Heart failure (HF) with reduced ejection fraction is associated with pathological structural, cellular, and molecular changes of the heart leading to impaired cardiac function. Maladaptive activation of the neurohormonal system ultimately induces detrimental effects on cardiac cells leading to cellular damage, remodelling, fibrosis, and cell death.[1] Current therapies for HF aim the interruption of this maladaptive activation, which resulted in significant improvement in the outcome measures of HF.[2]

Inflammatory mediators such as interleukin-1β (IL-1β), interleukin-6, or tumour necrosis factor alpha (TNFα) has been considered so far as a biomarkers of HF, however, recent studies propose them as prognostic markers as well, raising the question whether inflammation represents a therapeutic target in HF.[3,4] Increased amounts of circulating proinflammatory cytokines have been linked to impaired cardiac function and worse outcomes of patients with HF, suggesting that inflammation might be an important common factor in the pathomechanism of HF.[3] Even though there are promising preclinical studies on targeting inflammation in HF, clinical trials have provided discouraging results so far.[5–8] However, in the recent Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) assessing the efficacy of canakinumab, a monoclonal antibody against IL-1β, promising outcomes for HF patients have been reported; as well as in patients having myocardial infarction or stroke.[9,10]

IL-1β is secreted mainly by immune cells as a part of the inflammatory reaction and acts both via autocrine and paracrine manner. The maturation and release of IL-1β is strictly achieved by inflammasomes, special cytosolic multiprotein complexes. Inflammasome activation is triggered by a series of pathogen- or danger-associated molecular patterns (DAMP) leading to maturation of caspase-1 enzyme which ultimately cleaves pro-IL-1β to its mature form.[11] Additional mechanisms, e.g. the activity of pannexin-1 channel (PANX1) play critical roles both in inflammasome assembly, IL-1β release and even in priming of inflammasomes.[12,13] Recent studies

suggest that inflammasome activation might play a role in various cardiovascular events[14–16]; however, the role of inflammasome activation in chronic heart diseases such as HF remains unknown. Cardiovascular inflammasome research has so far focused mainly on the role of NLRP3, and revealed its activation in models of acute myocardial infarction, in atherosclerosis, in stroke, and in hypoxia and adrenergic stimuli-induced adverse remodelling giving a boost to the development of NLRP3 inhibitors.[15,17,18] However, recent studies pointed out that other inflammasome pathways such as the absent in melanoma 2 (AIM2) and/or NLR family CARD domain-containing protein 4 (NLRC4) inflammasome may also play central role in disease development in stroke, atherosclerosis, and in diabetic cardiomyopathy.[19–22]

In this study, we intended to investigate activation of four major inflammasome types in human chronic HF. Additionally, to prove our concept in preclinical models, we examined failing hearts from rat and pig models to identify relevant translational models for HF with inflammasome activation that reflects the human condition. Furthermore, we induced inflammasome activation in human monocytic THP-1 cells as well as in human AC16 cardiac cells to examine their interactions, as well as the pharmacological inhibition of PANX1 (with the clinically used uricosuric drug, probenecid). In addition, we studied the therapeutic potential of probenecid *in vivo* in a pressure overload-induced chronic HF model.

2. Materials and methods

The extended version of all the materials and experimental methods is described in the Supplementary material online.

2.1 Ethical approval

All experimental procedures were done in accordance with the ethical standards of the responsible institutional and national committee on human experimentation, adhering to the Helsinki Declaration (1975). Written informed consent was obtained from all patients involved in the study according to the protocol approved by the Local Ethics Committees of the Institute of Cardiology, Warszawa, Poland (IK-NP-0021-24/1426/14).

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), to the EU Directive (2010/63/EU) and was approved by the animal ethics committee of the Semmelweis University, Budapest, Hungary (PE/EA/1784-7/2017, and PEI/001/2374-4/2015).

2.2 Human heart tissue collection

Human heart samples ($n = 11–12$) were collected in Department of Heart Failure and Transplantology, Cardinal Stefan Wyszyński National Institute of Cardiology, Warszawa, Poland, as previously described.[23] Details on patients are summarized in Supplementary material online, *Table S1*.

2.3 Chronic heart failure animal models, echocardiography, and tissue collection

Animals were randomly assigned to the experimental groups, and the analysis of data was performed blinded by one to three experimenters. Animals that died during or immediately after the surgery due to technical reasons (e.g. excessive bleeding) or severe complications (e.g. ventricular arrhythmia, acute HF) were excluded from experiments.

Transverse aortic constriction (TAC), left artery descending (post-infarction rat model) (LAD), infrarenal arterio-venous shunt (AVS), and porcine models were performed according to the previously described protocols with slight modifications.[24–27] Surgical procedures and echocardiographic measurements were performed under general anaesthesia induced by inhalation of 5% isoflurane and maintained with 1.5–2% isoflurane mixed with 100% O_2 in rat experiments. After completion of the echocardiographic measurement, the abdominal aorta of the animals was cannulated and arterial blood was subsequently collected to euthanize the animals.

In porcine study,[27,28] anaesthesia was induced with an intramuscular injection of ketamine hydrochloride, xylazine, and atropine (12 mg/kg, 1 mg/kg and 0.04 mg/kg, respectively), then maintained with isoflurane oxygen mix (2–2.5 vol% and 3 L/min). After the procedure, animals were administered by an antibiotic cocktail containing 100 mg benzathine benzylpenicillin, 100 mg procaine benzylpenicillin, 200 mg dihydrostreptomycin-sulphate before recovery, and intramuscular injections of 1 g metamizole for analgesia. Animals were euthanized under general anaesthesia induced by intramuscular injection of ketamine hydrochloride, xylazine, and atropine (12 mg/kg, 1 mg/kg and 0.04 mg/kg, respectively) with an intravenous injection of 10% potassium chloride solution.

2.4 Data analysis

All data are expressed as *mean ± SEM* except in Supplementary material online, *Table S1*, where the *mean ± ranges* are shown. Comparisons of two groups were performed using *unpaired Student's t-test*. Experiments with more than two groups were evaluated by *one-way Analysis of variance (ANOVA)* followed by *Tukey's multiple comparisons test* or *two-way ANOVA followed by Bonferroni multiple comparisons test*. Overall mortality was assessed by *Kaplan-Meier survival curves* and *log-rank (Mantel-Cox) test*. $P < 0.05$ were considered statistically significant. Statistical analysis was performed with GraphPad Prism 8 (GraphPad Software Inc.).

3. Results

3.1 Expression of AIM2 and NLRC4 inflammasome sensors increases in human failing hearts

Although the role of NLRP3 inflammasomes has been described in early-stage HF,[29] the expression of inflammasome components in the late-stage and in cases with different aetiologies of HF in humans has not been investigated so far. Therefore, the well-characterized inflammasome sensors (NLRP3, NLRC4, AIM2 and NOD, LRR, FIIND, CARD domain and PYD domains-containing protein 1 aka, NALP1) were detected in left ventricular tissue ($n = 11–12$) harvested from healthy donor patients (CON) as well as from HF patients with history of ischaemic cardiomyopathy (ICM) or non-ischaemic cardiomyopathy (DCM) (see Supplementary material online, *Table S1* for patient characteristics). Interestingly, there was no difference in NLRP3 protein expression in the HF groups compared to control (*Figure 1A and B*).

In contrast, the expression of AIM2 markedly increased both in ICM and DCM groups (*Figure 1A and B*), and we also found a significant increase of NLRC4 protein level in left ventricular tissue of HF patients (*Figure 1A and B*), This increased AIM2 expression was also observed among patients with hypertrophic cardiomyopathy (HCM; $n = 5$), but NLRC4 expression showed only a tendency towards increase in HCM patients (Supplementary material online, *Figure S1*). The expression of NALP1 protein was not altered in HF induced by any forms of

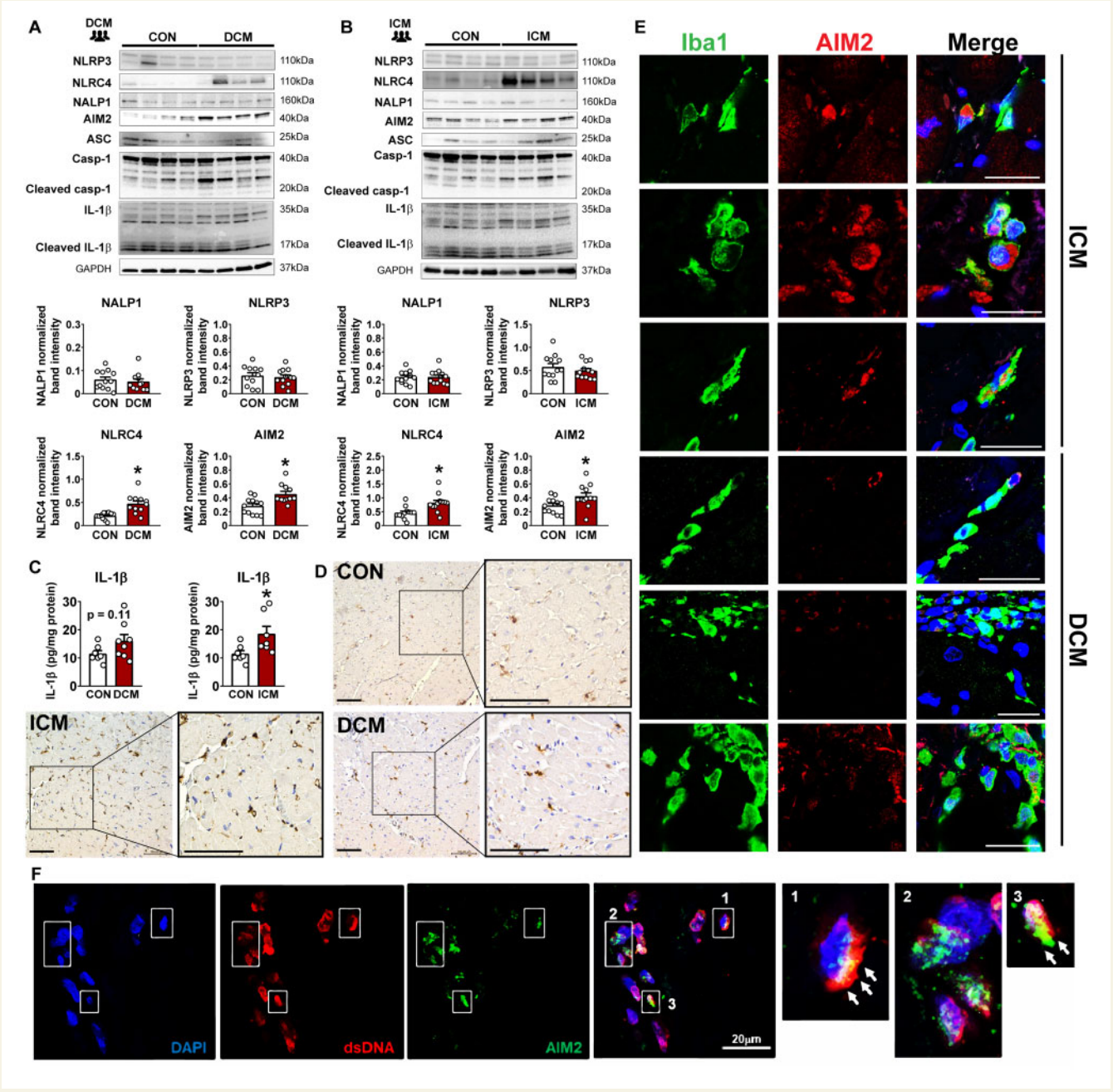

Figure 1 AIM2 and NLRC4 are the major inflammasome components expressed in human failing hearts. Western blot analysis of the inflammasome sensors (NLRP3, AIM2, NLRC4, and NALP1) and downstream signalling (ASC, caspase-1, IL-1β) in left ventricle of patients with dilated (DCM, *A*) or ischaemic cardiomyopathy (ICM, *B*). *$P < 0.05$ vs. CON, Student's *t*-test; $n = 11–12$. (*C*) Quantification of IL-1β content in human left ventricular tissue by ELISA. *$P < 0.05$ vs. CON, Student's *t*-test; $n = 7–8$. (*D*) Identification of monocytes/macrophages in human heart tissue by immunohistochemical detection of Iba1. Scale bar: 100 μm. (*E*) Representative images of immunofluorescence detection of AIM2 (red) and Iba1 (green) proteins in failing heart harvested from ICM and DCM patients. DAPI (blue) was used for counterstain. Scale bar: 30 μm. (*F*) Representative images of immunofluorescence detection of double-stranded DNA (dsDNA, red) and AIM2 (green) protein in a failing heart harvested from a DCM patient. DAPI (blue) was used for counterstain. Scale bar: 20 μm.

cardiomyopathies examined (*Figure 1A and B*). Inflammasome activation was further confirmed by detection of cleaved fragments of caspase-1 and IL-1β and by the detection of elevated IL-1β levels by ELISA in failing hearts (*Figure 1A–C*).

Inflammasomes are predominantly but not exclusively expressed and activated in the innate immune system e.g. in monocytes/macrophages or granulocytes.[30] It is well known that adverse remodelling both on an ischaemic or non-ischaemic background is associated with chronic expansion of macrophage populations and with IL-1β secretion in the myocardium.[31–33] To assess the presence of macrophages in human failing hearts, immunohistochemistry was performed to stain ionized calcium binding adaptor molecule 1 (Iba1) and CD68, general markers of

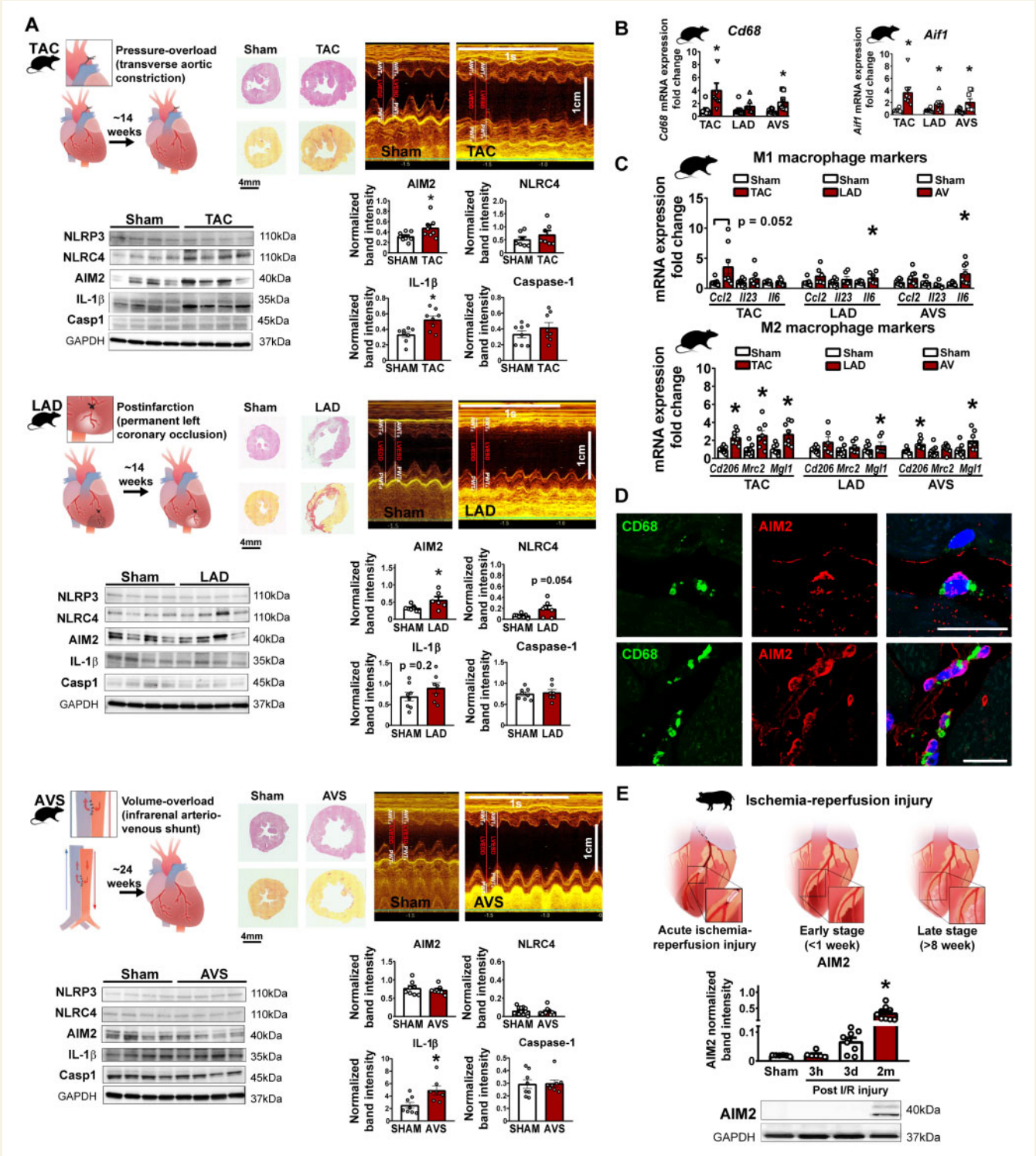

Figure 2 AIM2 inflammasome expression increased in the late phase of chronic heart failure in rat and pig models. (*A*) Pressure-overload, post-infarction, and volume-overload-induced rat models of chronic heart failure with representative histology (haematoxylin eosin, picrosirius red) and M-mode echocardiographic images, Western blot analysis of the inflammasome sensors and downstream signalling. Scale bar (echocardiography): 1 cm, timestamp: 1 s; scale bar (histology): 4 mm. *$P < 0.05$ vs. corresponding Sham, Student's *t*-test; $n = 6–8$. (*B*) Analysis of mRNA expression of macrophage marker *Cd68* and *Aif1* by qRT-PCR. *$P < 0.05$ vs. corresponding Sham, Student's *t*-test; $n = 6–8$. (*C*) Analysis of mRNA expression of the M1 and M2 macrophage markers (*Ccl2, Il23, Il6* and *Cd206, Mrc2, Mgl1*, respectively) by qRT-PCR. *$P < 0.05$ vs. corresponding Sham, Student's *t*-test; $n = 6–8$. (*D*) Representative images of immunofluorescence detection of AIM2 (red) and CD68 (green) proteins in a failing heart harvested from a TAC animal. DAPI (blue) was used for counterstain. Scale bar: 20 μm. (*E*) Chronic ischaemia/reperfusion-induced pig heart failure model with western blot analysis of time-dependent AIM2 protein expression. *$P < 0.05$ vs. Sham, one-way ANOVA; $n = 6–8$.

monocyte-macrophage lineage (*Figure 1D*, Supplementary material online, *Figure S2A*),[34] and the number of cells were counted. We observed a mild but not significant increase in the total number of monocytes/macrophages in failings hearts (Supplementary material online, *Figure S2B*). Despite of the growing interest regarding the role of inflammasomes in heart diseases, there is a general lack of reliable evidence on the cell-type specificity of inflammasome sensors in human hearts, and it is not known, whether resident myocardial cells are capable to express inflammasome components. To assess cell-type specificity of AIM2, indirect immunofluorescence staining was used to confirm the localization of AIM2 inflammasomes by detecting AIM2 in combination with monocyte/macrophage-specific markers Iba1 (*Figure 1E*, Supplementary material online, *Figure S2C*). Immunofluorescence staining showed that AIM2 is localized predominantly in Iba1 positive cells, though weaker AIM2 signals can be found in other cell types, suggesting that primarily monocytes/macrophages might be key players in the enhanced inflammasome activity but their interactions with the surrounding non-myeloid cells might be also important in the development of the proinflammatory milieu in failing hearts (*Figure 1E*). In addition, immunofluorescence assay revealed that not all Iba1 positive cells are characterized by increased AIM2 expression indicating the presence of a heterogeneous macrophage population in the cardiac tissue during HF (*Figure 1E*).

Controlled cell death may eventually lead to the release of nuclear double stranded DNA (dsDNA) to the cytosol that can be identified by the AIM2 inflammasome leading to the release of IL-1β and interleukin-18 (IL-18). We performed co-staining of dsDNA and AIM2 in sections from failing human hearts, and found that extranuclear dsDNA (*Figure 1F*, Supplementary material online, *Figure S2C*, red signal) shows tight co-localization with the AIM2 signal (*Figure 1F*, Supplementary material online, *Figure S2C*, green signal).

3.2 Inflammasome activation in animal models of chronic heart failure

It was previously demonstrated that in animal models of early-stage HF, NLRP3 inflammasome activation might play a significant role in initiating inflammatory reactions.[15,29,35] However, there is no data on the activation of other inflammasome types, especially in a later stage of HF. To find suitable reverse translational animal models to study inflammasome activation, we assessed three pathologically different models of HF i.e. pressure-overload (TAC), volume-overload (AVS), and the post-infarction HF rat model (LAD), as described previously (*Figure 2A*).[24–26] The detailed phenotypic and functional characterization of each model with transthoracic echocardiography is shown in Supplementary material online, *Table S2*. Increased lung mass and mRNA levels of failing markers [natriuretic peptide A (*Nppa*), natriuretic peptide B (*Nppb*)] in Supplementary material online, *Figure S3* indicated chronic HF at the primary endpoint; however, marked differences were found in morphology and function. Pressure-overload-induced excessive myocardial hypertrophy and fibrosis in TAC animals,[24] while volume-overload and ischaemic conditions promoted severe dilation as shown by the left ventricular dimensions and relative wall thicknesses (Supplementary material online, *Table S2* and *Figure 2A*). Despite the observed morphological differences between the animal models, expression of NLRP3 did not increase in any of the HF groups as compared to corresponding sham groups, whereas the expression of AIM2 increased significantly in TAC and LAD, but not in AVS rats (*Figure 2A*). In addition, a tendency towards elevation in the level of NLRC4 was observed in TAC and LAD animals (*Figure 2A*). In accordance with the elevation in the expression levels of inflammasome sensors, the tissue level of IL-1β increased in TAC animals and in AVS animals (*Figure 2A*). Similarly, we found enhanced monocyte/macrophage presence in rat failing hearts by assessing allograft inflammatory factor 1 (*Aif1*) and *Cd68* mRNA expression with qPCR analysis (*Figure 2B*) and by detecting Iba1 protein (encoded by the *Aif1* gene) with immunohistochemistry as well (Supplementary material online, *Figure S2D*). Interestingly, detection of chemokine (C-C motif) ligand 2 (*Ccl2*), interleukin 23 (*Il23*), interleukin 6 (*Il6*) and *Cd206*, macrophage mannose receptor 2 (*Mrc2*), macrophage galactose-type lectin 1 (*Mgl1*) mRNAs showed an M1 to M2 change in macrophage phenotype in TAC hearts while only minor changes were observed in LAD and AVS hearts (*Figure 2C*). Similar to the human tissue, AIM2 showed predominant co-localization with the pan-macrophage marker CD68 in myocardial sections from TAC animals (*Figure 2D*, Supplementary material online, *Figure S2C*).

AIM2 inflammasome activation has been shown to play a significant role in acute ischaemia–reperfusion injury in the liver[36] and early post-infarct HF in diabetic mice,[37] therefore, we aimed to further investigate inflammasome activation in late stage of chronic HF induced by ischaemia–reperfusion injury in a translational pig model as well (*Figure 2E*). We assessed ischaemic left ventricular tissues collected from pigs exposed to ischaemia/reperfusion at three different time points: 3 h (acute), 3 days (subacute), or 2 months (chronic) after ischaemia/reperfusion (*Figure 2E*), representing the acute injury, the early inflammatory and the late remodelling phase, respectively. The detailed characterization of pig model was published previously by our research group.[27,28] Surprisingly, the level of AIM2 protein in heart tissue was not altered at 3 h or 3 days, but it was markedly elevated at 2 months (*Figure 2E*).

3.3 Poly(dA:dT) induces isolated AIM2 inflammasome activation *in vitro*

As our results suggest that AIM2 inflammasome may be a potential player of inflammation in HF, we speculated that inflammasome activation might be a consequence of an interplay between immune cells and cardiac cells. To investigate inflammasome activation *in vitro*, AC16 human cardiac and THP-1 human monocytic cell lines were stimulated with naked or cationic liposome encapsulated (LyoVec™) poly(deoxyadenylic-deoxythymidylic) acid sodium salt [poly(dA:dT)], a specific AIM2 inducer, for 24 h (*Figure 3A*). Naked poly(dA:dT) was unable to induce AIM2 inflammasome activation (*Figure 3B*), however, liposome encapsulated poly(dA:dT) increased the expression of AIM2 in THP-1 cells (*Figure 3C*), suggesting that vesicular uptake of dsDNA is critical in the induction of AIM2 inflammasome activation. Inflammasome activation was confirmed with detection of cleaved caspase-1, IL-18 and IL-1β from the supernatant, and immunofluorescence detection of the inflammasome adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and AIM2 in THP-1 ASC-GFP reporter cell line (*Figure 3C–E*). Interestingly, poly(dA:dT) treatment also led to the induction of AIM2 protein expression in the AC16 cells without significant interleukin release (*Figure 3C and D*).

3.4 Pannexin-1 channel inhibition attenuates AIM2 inflammasome activation in THP1 cells

It has been shown that inflammasome activation by NLRP3 or NALP1 is strongly associated with the activation of purinergic signalling via P2X purinoreceptor 7 (P2X7) and hemichannel PANX1, however, it is unknown whether AIM2 inflammasomes and PANX1 have molecular

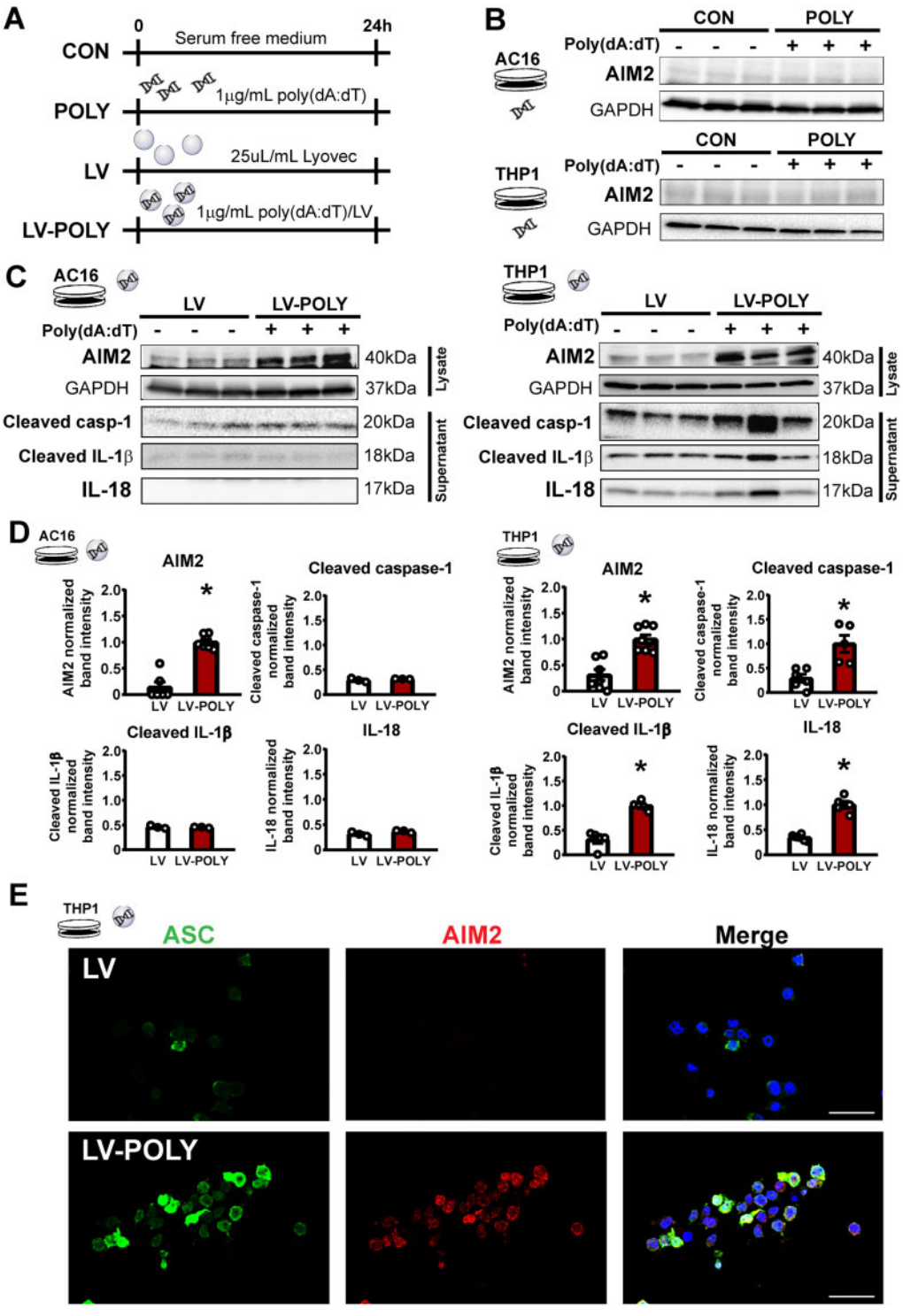

Figure 3 Liposome encapsulated poly(dA:dT) induced the expression of AIM2 and inflammasome activation *in vitro*. (*A*) Experimental protocol for AIM2 induction in human AC16 cardiac and THP1 monocytic cell lines. (*B*) Representative western blot images for naked poly(dA:dT) stimulus on AC16 and THP1 cells. (*C*) Representative western blot images for liposome encapsulated poly(dA:dT) on AC16 and THP1 cell lines. (*D*) Quantification of western blot analysis on poly(dA:dT)-induced AIM2 inflammasome activation in AC16 and THP1 cells. *$P < 0.05$ vs. LV, Student's *t*-test; $n = 4$–6. (*E*) Representative images of immunofluorescence detection of AIM2 (red) and ASC (green) proteins in poly(dA:dT)-stimulated THP1 cells. DAPI (blue) was used for counterstain. Scale bar: 50 μm.

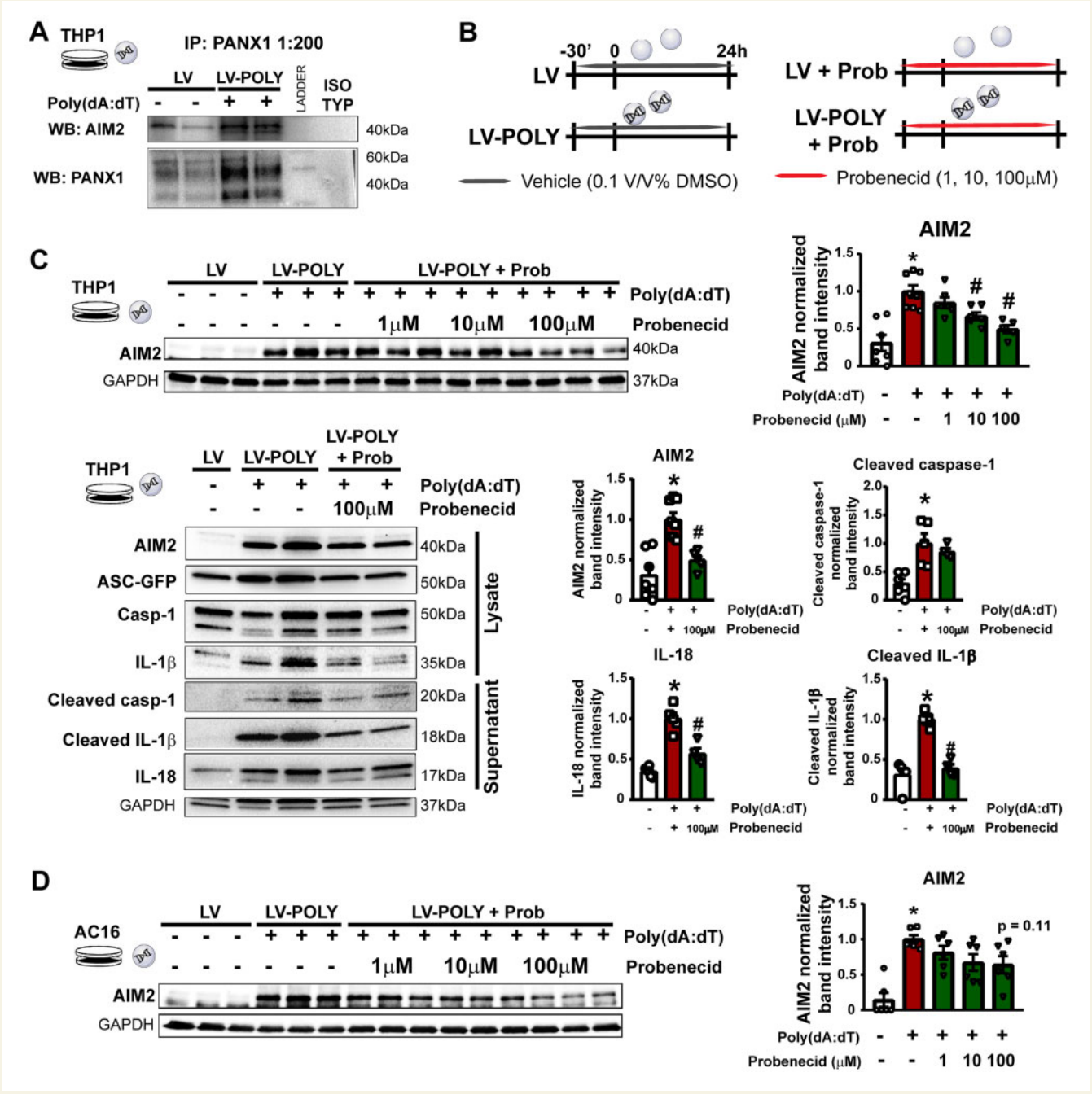

Figure 4 Pannexin-1 channel inhibition attenuates AIM2 inflammasome activation *in vitro*. (*A*) Representative western blot images for co-immunoprecipitation from control and poly(dA:dT)-stimulated THP1 cell lysate. PANX1 is shown as a loading control. Isotype anti-rabbit control was used as negative control. (*B*) Experimental protocol for testing the PANX1 blocker probenecid in cell model for AIM2 inflammasome activation on human AC16 and THP1 cell lines. (*C*) Western blot analysis of AIM2 protein expression on poly(dA:dT)-stimulated THP1 cells in the presence or absence of different concentration of probenecid, and detailed analysis of downstream signalling of AIM2 inflammasome activation in cell lysate and supernatant in the presence of 100 μM probenecid. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. poly(dA:dT) without probenecid; one-way ANOVA; $n = 5–6$. (*D*) Western blot analysis of AIM2 protein expression and cell viability on poly(dA:dT)-stimulated AC16 cells in the presence or absence of different concentration of probenecid. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. poly(dA:dT) without probenecid; one-way ANOVA; $n = 5–6$.

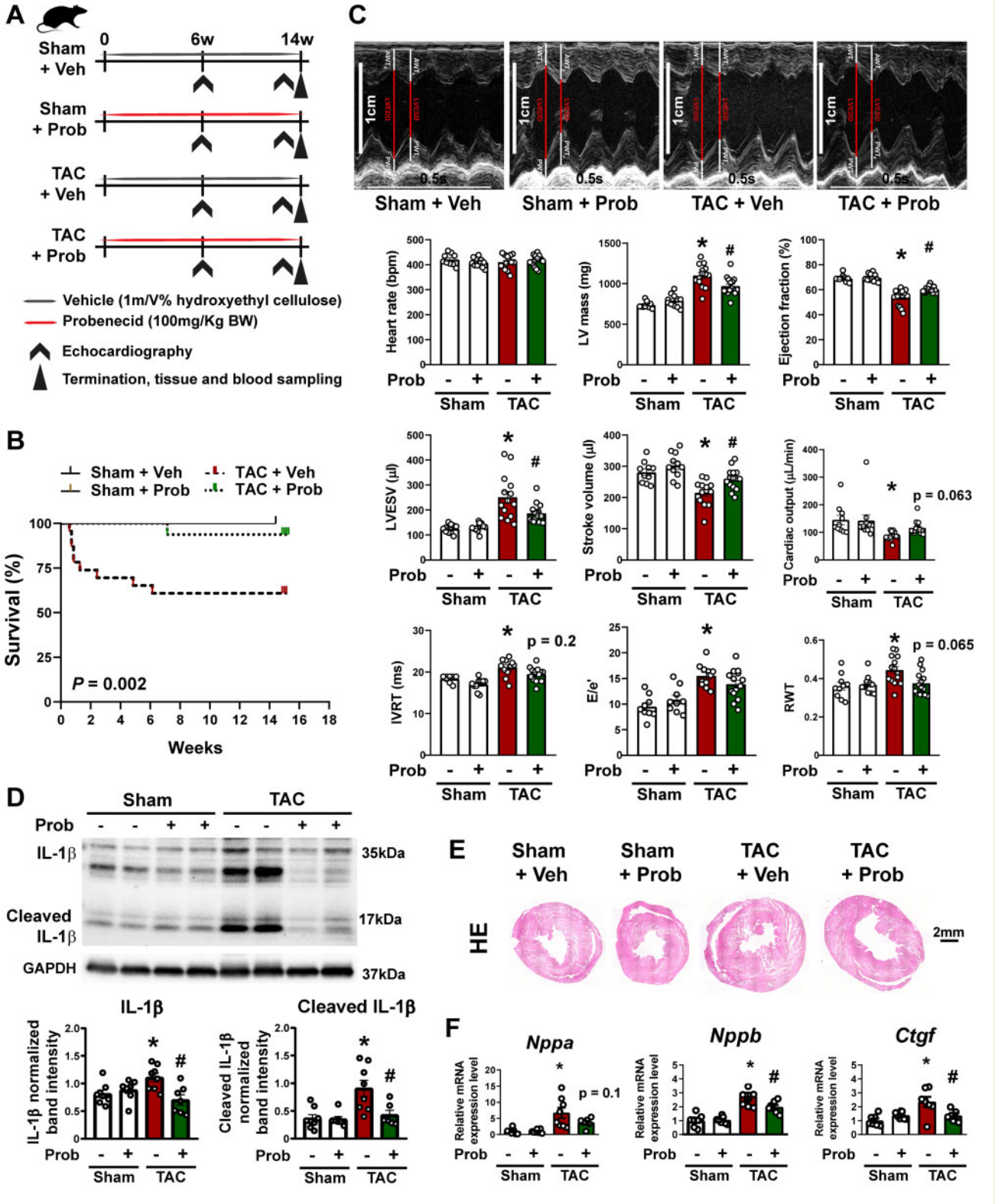

Figure 5 Pannexin-1 channel inhibitor probenecid improves survival and cardiac function *in vivo*. (*A*) Study design for investigating the effects of probenecid (Prob) in a rat model for chronic heart failure (TAC). (*B*) Kaplan–Meier analysis of overall mortality. $P < 0.05$, log-rank (Mantel-Cox) test; $n = 11–23$. (*C*) Representative M-mode echocardiography images and assessment of cardiac function at week 14 after surgery. Scale bar: 1 cm; timestamp: 0.5 s. $^{*}P < 0.05$ vs. Sham + Veh, $^{\#}P < 0.05$ vs. TAC + Veh, two-way ANOVA; $n = 11–17$. (*D*) Western blot analysis and representative images of IL-1β and cleaved IL-1β in left ventricles. $^{*}P < 0.05$ vs. Sham + Veh, $^{\#}P < 0.05$ vs. TAC + Veh; two-way ANOVA; $n = 6–8$. (*E*) Representative histology images (haematoxylin eosin) at week 14. Scale bar: 2 mm. (*F*) Analysis of mRNA expression of hypertrophy and heart failure markers (*Nppa, Nppb*, and *Ctgf*) by qRT-PCR. $^{*}P < 0.05$ vs. Sham + Veh, $^{\#}P < 0.05$ vs. TAC + Veh, one-way ANOVA; $n = 7–8$.

interactions. We performed co-immunoprecipitation on control and poly(dA:dT)-stimulated THP-1 cells, and saw that AIM2 was co-immunoprecipitated with PANX1 in activated cells indicating a potential interaction between the AIM2 inflammasome complex and PANX1 channels (*Figure 4A*). As the opening of PANX1 channels is related to apoptosis and release of 'find me' signals,[38] we tested the effects of probenecid, a potent PANX1 inhibitor, on AIM2 inflammasome activation *in vitro* (*Figure 4B*). Probenecid showed a dose-dependent reduction in the protein expression of AIM2 in both THP-1 and AC16 cells without a significant effect on cell viability (*Figure 4C and D*, Supplementary material online, *Figure S4*). Interestingly, the expression of PANX1 showed no significant differences in PANX1 levels between healthy and failing hearts with high individual variability (Supplementary material online, *Figure S5*).

3.5 Probenecid improves outcomes in pressure overload-induced chronic heart failure in rats

To test if probenecid improves cardiac function *in vivo*, we investigated probenecid in the rat HF model induced by TAC (*Figure 5*, Supplementary material online, *Tables S5* and *S6*). In these rats, cardiac function was assessed at 6 weeks and, 14 weeks after TAC, while the rats were orally treated with probenecid (100 mg/kg body weight/day) or vehicle (hydroxyethyl cellulose) control. We evaluated mortality throughout the whole study. The group treated with vehicle and having TAC surgery showed a reduced survival rate compared with vehicle-treated sham operated rats (*Figure 5B*). On the other hand, the group treated with a 100 mg/kg dose of probenecid showed significant amelioration of mortality compared with vehicle-treated TAC rats in Kaplan–Meier analyses (*Figure 5B*). As published previously[39] and shown above (*Figure 2A*) TAC surgery resulted in HF development. 14 weeks after TAC, left ventricular ejection fraction (LVEF) was reduced compared with baseline from 69.2 ± 1.8% to 54.0 ± 2.0% and from 69.7 ± 0.9% to 60.2 ± 0.6% in rats allocated to vehicle or probenecid treatment groups, respectively (*Figure 5C*, Supplementary material online, *Tables S5* and *S6*). Thus, compared with vehicle, oral probenecid treatment of rats with TAC significantly prevented deterioration of LVEF. In accordance, at 14 weeks after TAC, left ventricular end-systolic volumes increased more in the vehicle group compared with the probenecid treated group (*Figure 5C*, Supplementary material online, *Tables S5* and *S6*). In accordance with our previous observation above (*Figure 2*), the protein levels of IL-1β and its mature form increased 14 weeks after TAC surgery, which was reduced by probenecid treatment (*Figure 5D*). In addition, treatment with probenecid prevented development of left ventricular hypertrophy (*Figure* 5C, E, and *F*). Fourteen weeks after TAC, in vehicle-treated TAC operated rats the left ventricular mass significantly increased (compared to sham) with a significant reduction after probenecid treatment (*Figure 5C and E*). This was further confirmed by analysis of pro-hypertrophic genes (*Nppa* and *Nppb*) and the pro-fibrotic factor connective tissue growth factor (*Ctgf*) (*Figure 5F*). All these transcripts were significantly induced by TAC surgery, and their up-regulation was prevented by probenecid (*Figure 5F*).

4. Discussion

We detected enhanced AIM2 inflammasome expression in failing hearts harvested from human patients as well as from different small and large animal models of chronic HF, highlighting the importance of chronic inflammatory reactions in these conditions. In addition, increased NLRC4 expression was observed in human failing hearts as well. We assessed inflammasome activation in cardiac cells and macrophages*in vitro*, and showed that dsDNA is capable of inducing the AIM2 inflammasome in both cell types, suggesting that necrotic DNA might be the major trigger of the AIM2 inflammasome *in vivo*. In addition, we showed that the AIM2 inflammasome associated PANX1 channels may play a role in inflammasome activation, since the PANX1 inhibitor probenecid significantly reduced IL-1β secretion and maturation. Furthermore, chronic treatment with probenecid improved outcomes of pressure overload-induced HF.[40] These anti-inflammatory properties of probenecid could facilitate potential repurposing and use of this uricosuric drug to in chronic HF.[40,41]

The role of inflammatory mediators (such as interleukins and other cytokines) in cardiovascular diseases has been extensively studied over the last decades, nevertheless, clinical translation of these results was rather mixed and controversial.[8,42] Results of the CANTOS trial, however, pointed out that just by neutralizing IL-1β, with canakinumab, marked reductions can be achieved in incidence of major cardiovascular adverse events of post-infarction patients, highlighting the central role of IL-1β in these disease states.[9,43] However, there are major limitations of the use of canakinumab (e.g. price, infectious adverse reactions), ruling it out from the routine tools of current cardiovascular therapy. In light of these data, it is obvious that modulating new targets of IL-1β-related pathways might be of high therapeutic importance.

Our present human and translational animal data provides evidence for AIM2 and NLRC4 inflammasome activation in HF. We also show that co-activation of multiple types of inflammasomes is a possible phenomenon, suggesting that single inflammasome targeting may not be an optimal strategy in case of cardiovascular diseases including atherosclerosis[22] and chronic HF.

Bacterial and viral particles were considered as the primary triggers of inflammasome activation, however, it became evident that during sterile inflammatory conditions, DAMPs may also promote inflammasome activity. Among these, the AIM2 inflammasome is known to be activated by dsDNA.[44] It is reasonable to hypothesize that dsDNA was a major contributor to AIM2 inflammasome activation in our study as well, since the chronic remodelling process associates with a low degree of apoptotic/necroptotic cell death resulting in a concomitant monocyte/macrophage infiltration and inflammasome activation.[45] A similar activation pattern has been described in case of chronic renal failure,[46] as well as in animal models of atherosclerosis.[20] The background of myocardial NLRC4 activation in HF is even more surprising. Currently, the most characterized trigger of NLRC4 is flagellin of Gram negative bacteria.[47,48] It is presumable that HF-induced hypoperfusion of the intestines leads to dysbiosis, and increased gut permeability,[19] promoting a low grade systemic inflammatory state. This is supported by studies showing gut microbiome modulation as a relevant target to alleviate the systemic inflammatory state during the course of human HF.[49] This hypothesis might provide an explanation for increased NLRC4 expression in human failing hearts; nevertheless, it is unknown whether significant gut hypoperfusion could have developed in our animal models. On the other hand, in animal models of stroke a similar co-activation pattern of AIM2 and NLRC4 has been described previously,[19,37] suggesting that the activation of these two inflammasomes might be linked.

The complex pathways converging to inflammasome activation and signalling involve triggers that may influence inflammasome activity and assembly by mechanisms that associate with lysosomal membrane rupture,[50] as well as autoregulatory signalling by the products IL-1β and IL-18. However, the

best characterized triggers are the classic mediators promoting inflammasome priming [triggered by e.g. Toll-like receptors 4, 9 (TLR4, TLR9), and TNFα receptors], and inflammasome oligomerization (influenced by the purinergic receptors and the associated pannexin-1 channels), PANX1 channels have so far been described as critical modulators of NALP1, NLRP3 as well as of non-canonical inflammasome activities via ATP release.[38,51,52] Nonetheless, whether PANX1 is involved in the activation of AIM2 inflammasomes has not been studied yet. By co-immunoprecipitation experiments, we have shown here first in the literature that PANX1 channels associate to the AIM2 inflammasome as well, and showed a prominent anti-inflammatory effect of the PANX1 channel inhibitor probenecid *in vitro*. The anti-inflammatory effect of probenecid was mediated by decreasing Il-1β level in a rabbit sepsis model.[53] We have seen a reduction in the expression of AIM2 and its downstream signalling *in vitro* in both dsDNA stimulated monocytes/macrophages and cardiac cells. In addition to AIM2 inflammasome inhibition, PANX1 channels may play a role in leukocyte migration and in modulation of the NFκB pathway.[54,55] A recent study has also confirmed that probenecid improves cardiac function at early phase of post-infarction HF via inhibiting endothelial PANX1 channels and consequential leukocyte infiltration.[56] Therefore, we propose that probenecid might be a 'broad-spectrum' inflammasome inhibitor besides its well-characterized uricosuric properties. Probenecid has been previously demonstrated to improve outcome in an animal model of ischaemic HF with a shorter 4-week follow-up period by exerting positive inotropic effects via transient receptor potential vanilloid type-2 (TRPV2), and the positive inotropic effect was confirmed in a small number of patients with HFrEF.[40,57] We now show that probenecid is able to prevent adverse cardiac remodelling upon a more prolonged period of pressure overload *in vivo*; however, the interplay between anti-inflammatory effects of probenecid and its action on TRPV2 as well as on myocardial contractility was not investigated within the frame of this study which should be acknowledged as a limitation. Nevertheless, these already published beneficial effects (action on TRPV2 and contractility) and the novel anti-inflammatory effects might explain the recently observed clinical benefits of probenecid use in patients suffering from HF, as well as the epidemiological observation, that patients receiving probenecid therapy for gouty arthritis have better cardiovascular outcomes.[40,41] Thus, we believe that probenecid fulfils many of the characteristics desirable for a repurposed drug for the treatment of chronic HF.

4.1 Limitation

We have shown that probenecid has a significant inhibitory effect on AIM2 inflammasome *in vitro* and it improves survival and cardiac function in a rat model for HF *in vivo*. However, to identify precisely the contribution of PANX1-mediated AIM2 inflammasome inhibition besides the other well-known effects of probenecid, further *in vivo* studies using genetically modified mice might be necessary.

5. Conclusion

We have shown with a series of experiments on human failing heart tissues as well as in various translational *in vivo* animal models (pressure or volume overload-induced rat HF models, and post-infarction rat and pig HF models) and in *in vitro* cell culture experiments, that inflammasome activation is primarily characterized by the activation of the AIM2 and NLRC4 inflammasome during chronic HF. We believe that our results highlight the importance of disease-, and disease-stage specific differences of inflammasome activation patterns. IL-1β and the upstream inflammasome inhibition has been shown as an intriguing therapeutic target in the CANTOS trial, therefore inhibition of AIM2 by probenecid may reveal a promising new therapeutic concept promoting drug repurposing efforts in the treatment of chronic HF.

Data availability

The datasets used and/or analysed are available from the corresponding author upon request.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

Z.O. participated in study design and performed *in vitro* experiments, analysed data, and drafted the manuscript. M.R., D.K., A.A.S., and T.R. designed and performed *in vivo* rat experiments, analysed data and wrote the manuscript. P.L. collected human heart samples and provided clinical data. Z.G., G.B.B., A.M., I.H., and M.G. designed and performed *in vivo* pig experiments and evaluated results. G.K. and V.E.T. performed *in vitro* experiments and evaluated results. P.F., R.S., A.G., T.R., and B.M. revised the manuscript, the intellectual content and provided professional advice. Z.V.V. designed experiments, wrote manuscript, revised the intellectual content, and provided professional advice. All authors read and approved the final manuscript.

Conflict of interest: P.F. is the founder and CEO of Pharmahungary Group, a group of R&D companies. R.S. received honoraria for lecturing from Sanofi that is not related to the present study. The remaining authors declare no conflict of interest.

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Translational perspective

Targeting interleukin-1β and its release by the inhibition of inflammasomes may be a potential therapeutic approach in cardiovascular diseases including heart failure. Absent in melanoma 2 inflammasome activation was identified in human heart failure samples which was confirmed in various translational small and large animal models of chronic heart failure as well. Our findings suggest that NLRP3-independent inflammasome inhibitors (e.g. probenecid) might be novel agents in the treatment of chronic heart failure.