

Multiorgan characterization of inflammasome component expression in a rat model of advanced heart failure

Short title: Multiorgan inflammasome expression in advanced heart failure

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Abstract

Background: Targeting inflammasomes in heart failure (HF) might represent a novel therapeutic option. Nevertheless, previous studies focused only on myocardial inflammasome alterations, and data are scarce regarding their regulation and role in HF-associated multiorgan dysfunction. Therefore, we aimed to determine the myocardial, pulmonary, hepatic and renal expression of various inflammasome components in a rat model of advanced HF.

Methods: Rats underwent transverse aortic constriction (TAC) and were followed-up for 15 weeks. Animals featuring signs of advanced HF were included in the TAC-HF group. TAC rats presenting no HF signs were also investigated (TAC-M group). Sham-operated animals served as controls. The expressions of inflammasome component proteins in left ventricle (LV), right ventricle (RV), lung, liver and kidney tissue were measured with Western blot.

Results: Despite the differences between the clinical state of the TAC-HF and TAC-M groups, severe cardiac dysfunction developed in all TAC animals. Absent in melanoma 2 (AIM2) and NLR family CARD domain-containing protein 4 (NLRC4) inflammasome sensors were upregulated in both the LV and RV of the TAC-HF group compared to Sham. AIM2 and NLR family pyrin domain-containing protein 3 (NLRP3), but not NLRC4 expression were elevated in the lungs of the TAC-HF animals. Inflammasome components were downregulated in the liver and unchanged in kidney tissue in the TAC-HF group. Inflammasome changes were predominantly absent in TAC-M animals.

Conclusions: Inflammasome expression shows distinct patterns in specific organs in advanced HF. Future studies aiming to antagonize inflammation in HF should take these findings into consideration.

Keywords: absent in melanoma 2; advanced heart failure; end-organ damage; multiorgan dysfunction; inflammasome

1. Introduction

Despite the rapidly developing therapeutic options over the past decades, the incidence and mortality of heart failure (HF) still remain high in the general population (1). Advanced HF – a clinical state with refractory symptoms despite optimal medical therapy – has especially poor prognosis (2). Advanced HF can often culminate into multiorgan dysfunction as well (2, 3). The impairment of extracardiac organs can drive the clinical progression independently of cardiac function, thus impacting long-term survival and limiting therapeutic options (3-5). The interplay of the heart and other organs in HF is still under extensive investigations. Pathological hemodynamic conditions lead to the congestion and/or hypoperfusion of end-organs, which initiates several intertwining maladaptive mechanisms (6, 7). One of the best characterized among them is neurohormonal activation, which includes increased sympathetic tone and the stimulation of the renin-angiotensin-aldosterone system (8). Current pharmacological therapy of HF with reduced ejection fraction (HFrEF) aims to mitigate these alterations predominantly.

Besides neurohormonal changes, elevated levels of inflammatory markers are long-known characteristics of HF as well (9-11). All recent evidence points in the direction that systemic inflammation is not only a response to injury in HF, but a causative factor of further decline (12). In preclinical settings, therapeutic targeting of inflammation in HF has been found to successfully improve survival (13, 14). Most clinical trials on larger sample sizes however, failed to replicate these results (15-17). The only promising direction seems to be the antagonization of interleukin-1 beta (IL-1 β), based on the favorable cardiovascular outcomes of the CANTOS trial and its substudies (18-20). On the other hand, IL-1 β blockade also increased the incidence of fatal infections, and all-cause mortality was eventually unchanged in the study population. This suggests the need for a more specific intervention in the complex inflammatory processes. IL-1 β is activated exclusively through the inflammasome pathway (21). Inflammasomes are intracellular pathogen- or danger-sensing multiprotein complexes of the innate immune system, expressed most abundantly by monocytes/macrophages (22). Multiple inflammasome receptor proteins

exist, each of them responds to different types of molecular patterns (22). Besides the diverse sensors, all types of inflammasomes include a common adapter molecule (apoptosis-associated speck-like protein containing a CARD, ASC) and activate caspase-1 finally (22). Gasdermin D (GSDMD), IL-1 β and interleukin-18 (IL-18) are the downstream targets of caspase-1 (22).

Since several upstream inflammasome sensors that respond to different stimuli exist, modulating them would be a feasible alternative therapeutic approach. Numerous experiments in the cardiovascular field – including HF – confirm this notion (23-26). Dapansutrile, a selective oral inhibitor of NLR family pyrin domain-containing protein 3 (NLRP3) has shown promising results in a phase 1B clinical trial on HFrEF patients (27). The role of inflammasomes is well-established in various types of extracardiac organ dysfunction as well (28-30). Cytokines and inflammasomes were also examined in certain organ interactions accompanying HF (31-34). However, the expression of inflammasomes in advanced HF-associated multiorgan failure has not been comprehensively characterized yet.

Our study aimed to characterize the left- and right ventricular (LV and RV, respectively) and extracardiac (lung, kidney, liver) expression of relevant inflammasome elements in a rat model of pressure overload-induced HF. In this regard, we intended to compare the advanced, symptomatic and the asymptomatic HF phenotypes in an otherwise similar population.

2. Methods

2.1. Ethical Approval and Animals

The experimental animals were treated according to the “Principles of Laboratory Animal Care” established by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the US National Institutes of Health (NIH Publication no. 86-23, revised 1996). The investigation conforms to the EU Directive (2010/63/EU). Approval for the study was obtained from the animal ethics committee of Semmelweis University, Budapest, Hungary (PEI/001/2374-4/2015). The results are interpreted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (35). Male Wistar rats (2-3 weeks old, 50-75 g; Toxi-Coop, Dunakeszi, Hungary) were housed in a controlled environment (constant temperature of $22 \pm 2^\circ\text{C}$ with 12:12-h light-dark cycles) and had access to a standard laboratory rat diet and water *ad libitum* throughout the experimental period.

2.2. Transverse Aortic Constriction Model

After a one-week long acclimatization period, rats underwent transverse aortic constriction (TAC) surgery, as described elsewhere (36). In short, animals were placed in a chamber to induce anesthesia by inhalation of 5% isoflurane. The rats were then orotracheally intubated and artificially ventilated with 1.5-2% isoflurane in 100% O₂ for the rest of the procedure. The ventilation parameters were calculated according to the body weight of the animal. Body temperature was maintained at 37°C by using an automatic heating pad. Left anterolateral thoracotomy was performed in the second intercostal space under sterile conditions. The aorta was isolated between the brachiocephalic trunk and the left common carotid artery and constricted to the size of a 21-gauge needle. The thorax was then closed, and the skin layers were sutured together as well. Tramadol (25 mg/kg) was administered subcutaneously for postoperative analgesia. The sham operation was carried out similarly, except the aortic constriction step of the protocol.

2.3. Experimental Groups and Study Design

After TAC operation, a median follow-up period of 15 weeks was utilized. Based on previous experience with the model, we expected an advanced state of HF to develop in a subgroup of animals under this time (36). Since the focus of this study is advanced HF as a clinical state (which is often independent of cardiac function), we applied a simple scoring method to form our study groups from the TAC-operated rats. Cardiac cachexia (defined as 20% less body weight-to-tibial length ratio than the average of the Sham group), visible resting dyspnea/tachypnea and signs of excessive subcutaneous edema were indicative of advanced HF in our animals. Animals presenting at least two of the mentioned clinical signs or symptoms were included in the TAC-HF group ($n=6$, see Supplemental Table S1). The TAC-M group ($n=6$) was formed from rats showing maximum one clinical sign/symptom. The control group (Sham, $n=6$) consisted of sham-operated animals that were followed-up for 15 weeks as well.

2.4. Echocardiography

At the end of the follow-up period, echocardiography was carried out by using a 13-MHz linear transducer (GE 12L-RS GE Healthcare, Waukesha, WI, USA) connected to an echocardiographic imaging unit (Vivid I; GE Healthcare), according to our previously described protocol, with minor modifications (37). After induction of anesthesia by inhalation of 5% isoflurane in a chamber, the rats were placed on an automatic heating pad in a supine position. The body temperature of the animals was maintained at 37°C during the whole measurement. Anesthesia was maintained by inhalation of 1.5-2% isoflurane gas in 100% O₂. The thorax of the animals was shaved, and a pre-warmed ultrasound transmission gel was utilized. Two-dimensional and M-mode sonographic recordings in parasternal long- and short-axis (at the midpapillary level) views were acquired. The digital images were analyzed by a blinded investigator using dedicated image analysis software (EchoPAC; GE Healthcare). Three consecutive cardiac cycles were evaluated and averaged to obtain our results. LV end-systolic diameter (LVESD), LV end-diastolic diameter (LVEDD), LV anterior and posterior wall thicknesses in diastole (LVAWTd and LVPWTd) were measured.

Heart rates (HR) were also registered. From the measured parameters, fractional shortening (FS as $[LVEDD-LVESD]/LVEDD$), LV mass (according to the Deveroux formula) and relative wall thickness (RWT as $[LVAWTd+LVPWTd]/LVEDD$) were calculated (38).

2.5. Invasive Hemodynamic Measurement and Tissue Collection

LV pressure-volume (P-V) analysis was performed according to a previously described protocol, with slight modifications (39). In brief, immediately after the echocardiographic measurement was performed, the rats were tracheotomized, intubated, and artificially ventilated with 1% to 1.5% isoflurane gas in 100% oxygen. After median laparotomy, 2 mg/kg rocuronium bromide was administered intraperitoneally for muscle relaxation. A central venous catheter was inserted into the left external jugular vein for fluid supplementation and saline calibration. A 2-Fr microtip pressure-conductance catheter (SPR-838; Millar Instruments, Houston, TX) was inserted into the right carotid artery and subsequently advanced into the ascending aorta. After a period of stabilization, hemodynamic parameters were measured. Ventilation of the animal was stopped during registration for 3-4 seconds to avoid artefacts on the recordings. First, arterial blood pressure readings were taken. The catheter was then carefully moved into the LV under pressure control. After the acquisition of baseline P-V loops, the inferior caval vein was transiently occluded (with a cotton swab carefully pressed against the vessel) to register P-V loops at gradually decreasing preload as well. Using a dedicated P-V analysis program (PVAN; Millar Instruments), the following parameters were calculated: HR, systolic arterial blood pressure (SBP), diastolic arterial blood pressure (DBP), mean arterial pressure (MAP), LVESV, LVEDV, LV end-systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), maximal slope of the systolic pressure increment and the diastolic pressure decrement ($dp/dt_{\max}$ and $dp/dt_{\min}$), EF, stroke volume (SV), cardiac output (CO) and arterial elastance (E_a calculated as $LVESP/SV$). The time constant of the LV pressure decay was calculated according to the Glanz method (τ or Tau). Our load-independent parameters were calculated from the P-V loops registered at transiently decreasing preload. End-systolic P-V relationship (ESPVR; according to the parabolic curvilinear

model) was calculated as a reliable index of LV contractility. To assess ventriculo-arterial coupling (VAC), the ratio of E_a to ESPVR ($VAC=E_a/ESPVR$) was calculated. Furthermore, to characterize LV stiffness, the slope of the end-diastolic P-V relationship (EDPVR) was derived from the P-V analysis. Due to technical issues, we were unable to register the P-V loops of one TAC-HF animal during caval vein occlusion. Parallel conductance and volume calibration of the conductance system were performed as previously described (40). The abdominal aorta was cannulated immediately after the P-V measurements and the animals were euthanized by exsanguination. During this process, arterial blood samples were also collected into serum clot activator or EDTA tubes for serum or plasma samples, respectively. After centrifugation, the supernatant serum and plasma samples were aliquoted and stored at -80°C . Oxygenated Ringer solution (50 mL) was infused retrogradely after the termination of the animal was confirmed based on the ECG recordings. Hearts, lungs, livers and kidneys were harvested and placed immediately into 4°C Ringer solution. After organ weight measurements, 40-50 mg pieces of LV, RV, lung, liver and kidney tissue were sampled and immediately snap-frozen in liquid nitrogen and stored at -80°C for the molecular analyses. A cross section of the hearts (at the level of the ventricles) and 1cm x 1cm pieces of the other organs were placed in 4% buffered paraformaldehyde for the histological analyses as well.

2.6. Histology and Immunohistochemistry

Histology and immunohistochemistry were performed as previously described (41). Formalin-fixed tissue specimens were embedded in paraffin, sliced to $4\text{ }\mu\text{m}$ thick sections and placed on adhesive slides.

Picrosirius red staining was used to evaluate fibrosis. Three subendocardial and three subepicardial representative images were taken from the free LV wall and five representative images from the RV wall of each animal (200x magnification). Representative images of lungs, livers and kidneys were also captured at 20x magnification. After background subtraction, eye-controlled auto-threshold has been set up using ImageJ Software (NIH, Bethesda, MD, US) to detect positive areas. The fractional interstitial myocardial fibrosis area percentage (picrosirius red positive area-to-total area ratio) was determined on

each image, and the mean value of the images represents each animal. Fibrotic area ratios of extracardiac organs were normalized to the means of the respective control groups.

For immunohistochemistry, sections from the lungs and livers were deparaffinized and they were subjected to antigen retrieval (pH=6 citrate buffer, at 95°C for 15 min). Endogenous peroxidase activity was then inhibited (3% H₂O₂ solution in PBS), and the sections were blocked in appropriate sera (2.5% goat, bovine serum or milk in PBS). The primary antibody (CD68 Antibody KP1, cat. no.: sc-20060, Santa Cruz; 1:1000 dilution) was incubated with the slides overnight in diluted blocking solution at 4°C. Sections were then washed thrice in PBS and incubated for an hour with peroxidase polymer-conjugated anti-mouse IgG (cat. no. 7076S, Cell Signaling; 1:5000 dilution). The secondary antibody was washed three times for 10 minutes and diaminobenzidine substrate was added (ImmPACT DAB EqV Peroxidase (HRP) Substrate, Vector Laboratories, Burlingame, CA, US). The specific staining signal was visualized, and images were acquired with Leica DM3000 bright field microscope (Leica Microsystems, Wetzlar, Germany). Representative images of slices were taken on each slide at 20× magnification. Using ImageJ software, color deconvolution was employed to differentiate stains for evaluation. CD68-positive cells were identified by applying a threshold, and touching objects were separated using the watershed option. Subsequently, particles within the range of 70-250 μm^2 were counted. Data was normalized to the mean of control group.

2.7. Western Blot

Western immunoblotting was performed as previously described (42). Frozen samples from LV, RV, lung, liver and kidney tissue were homogenized in 1× radio immunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, US) containing 1× HALT Protease and Phosphatase Inhibitor cocktail (Thermo Scientific, Waltham, MA). Bicinchoninic acid assay (Thermo Scientific, Waltham, MA) was used to assess the protein content of the homogenizates. Homogenizates containing equal amount of protein were mixed with 1/4 volume of Laemmli buffer containing β -mercaptoethanol (Thermo Scientific,

Waltham, MA), then loaded on 4–20% Tris-glycine sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad, Hercules, CA, US). Once separated by electrophoresis, the proteins were transferred onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, US) with Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, US). Membranes were then incubated in 5% bovine serum albumin (Bio-Rad, Hercules, CA, US) dissolved in Tris-buffered saline containing 0.05% Tween-20 (0.05% TBS-T; Sigma, St. Louis, MO, US) for two hours at room temperature. Blot membranes were subsequently incubated with primary antibodies overnight at 4°C (primary antibodies: see Supplemental Table S2 for antibodies, dilution and catalogue number). After washing with TBS-T three times, membranes were incubated with HRP-conjugated secondary antibodies (Cell Signaling, Danvers, MA, US) for 2 hours. Chemiluminescent signal was developed by using enhanced chemiluminescence kit (Bio-Rad, Hercules, CA, US), and detected by Chemidoc XRS+ (Bio-Rad, Hercules, CA, US). Image Lab™ (Bio-Rad, Hercules, CA, US) was used to analyze the acquired images.

2.8. Serum and Plasma Measurements

Circulating markers of liver function (albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; total bilirubin) were measured in the clinical routine diagnostic laboratory of Semmelweis University Heart and Vascular Center (Budapest, Hungary) from frozen serum and plasma samples. Plasma N-terminal prohormone of brain natriuretic peptide (NT-proBNP) concentration was assessed using Rat N-terminal pro-brain natriuretic peptide, NT-proBNP ELISA KIT (#CSB-E08752r; Cusabio Technology, Houston, TX, US) according to the manufacturer's protocol.

2.9. Statistical Analysis

All values are expressed as mean $\pm$ standard error of the mean. The distribution of the datasets was tested by the Shapiro–Wilk normality test. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test were carried out in case of normal distribution, Kruskal–Wallis test followed by Dunn's *post hoc* test were used in case of non-normal distribution to compare the three study groups. A P value of

239 <0.05 was considered statistically significant. Furthermore, two additional categories ($P < 0.01$ and $P <$
240 0.001) were introduced to indicate the strength of the observed statistical difference. Statistical analysis
241 was performed with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, US).

3. Results

3.1. Characterization of the TAC model

According to the measured cardiac functional parameters, both TAC-M and TAC-HF groups featured severe systolic and diastolic LV dysfunction. The average of the EF calculated from the parameters obtained during the P-V measurements decreased below 40% in both TAC groups (Figure 1C). NT pro-BNP, widely used in clinical settings to monitor disease severity in HF patients, was also found to be elevated in all TAC animals (Figure 1D). Arterial and LV systolic pressures (SBP and LVESP, see Table 1) increased in the aortic constricted rats, indicating profoundly elevated afterload. LV contractility failed to compensate the increment in afterload, which was demonstrated by the impairment of VAC ratio (E_a /ESPVR, see Figure 1E-F). Moreover, the changes in the maximal velocity and the time constant of LV pressure decline ($dPdt_{min}$ and τ , respectively) in the TAC-M and TAC-HF animals reveal the disturbance of LV active relaxation in the diastole (Figure 1G, Table 1). Passive filling of the LV was also impaired in these groups, as evidenced by the elevation of the LVEDP and the slope of EDPVR (Figure 1E and 1G).

Besides the functional alterations, the structural remodeling of the heart also signaled the advanced state of HF pathology. The approximately two-fold growth of the echocardiography-derived LV mass and heart weight-to-tibial length ratio of TAC-M and TAC-HF groups compared to the Sham animals confirmed the development of myocardial hypertrophy (Figure 1B, Figure 2D). Wall thickness parameters (LVAWTd and LVPWTd, Table 1.) and relative wall thickness (RWT, Table 1.) showed greater increase in the TAC-M groups, suggesting a rather concentric geometry of hypertrophy. In the TAC-HF group however, ventricular dilation was predominant, as demonstrated by the alterations of ventricular diameters and volumes (LVESD, LVEDD, LVESV and LVEDV, Table 1.). Histological analysis detected significant interstitial myocardial fibrosis in the LV of both TAC groups. (Figure 2F and 2H). Excessive fibrosis was found in the RV of the TAC-HF group as well, while the TAC-M group showed only a tendency towards RV fibrosis

(Figure 2G-H). The isolated RV weight-to-tibial length ratio was also found to be elevated in the TAC-M and TAC-HF groups compared to Sham (Figure 2E).

3.2. Myocardial Expression of Inflammasome Components

In the LV, expression of inflammasome components showed an overall increasing tendency in the TAC-HF group (see Figure 2A and 2C). Among the inflammasome sensors, NLR family CARD domain-containing protein 4 (NLRC4) and absent in melanoma 2 (AIM2) were upregulated compared to the controls. Pro-IL-1 β expression also featured a more than two-fold elevation in the TAC-HF animals. The most abundant pro-caspase-1 isoform (with 40 kDa molecular weight) was found to be statistically significantly upregulated as well. None of the changes described above were present in the LV of the TAC-M group, only the expression of GSDMD increased in this group. The inflammasome components in the RV of the TAC-HF rats showed a similar pattern of upregulation (Figure 2B-C). Interestingly, the fold changes were even higher than those of the TAC-HF LV, while the TAC-M group showed no statistically significant changes (except for the change in GSDMD expression) in the RV, either.

3.3. Lung Tissue Changes and Inflammasome Component Expression

Wet lung weight normalized to tibial length increased in all of the TAC animals, indicating pulmonary congestion (Figure 3E). Histological analyses detected CD68+ leukocyte infiltration in the lung tissue of the TAC-HF rats (and a strong tendency in the TAC-M group as well), while fibrosis was not found to be significantly different in any of the groups (Figure 3B-C and 3F-G). The expression of certain inflammasome-related proteins was elevated in the lungs of TAC-HF animals as well (Figure 3A and D). In contrast to the myocardial findings, NLRP3 expression showed a marked growth along with AIM2, while NLRC4 remained unchanged compared to the controls. In addition, pro-caspase-1 and pro-IL-1 β were significantly upregulated in the TAC-HF group as well. Inflammasome component expressions were practically similar to the controls in the TAC-M group, only AIM2 and pro-IL-1 β showed a tendency towards elevation.

3.4. Liver Tissue Changes and Inflammasome Component Expression

The mean liver weight-to-tibial length ratio was unaltered in the TAC animals (Figure 4E). The TAC-HF group showed a tendency towards liver fibrosis and CD68+ infiltration, although not statistically significantly (Figure 4B-C and 4F-G). Plasma albumin was found to be decreased and total bilirubin concentrations elevated in the TAC-HF group, while transaminases displayed no significant changes (Table 2). These liver function tests indicate preserved liver parenchyma, and the dominance of congestion (instead of hypoperfusion) in the clinical picture. Interestingly, inflammasomal components were repressed in the liver of TAC-HF animals, while the TAC-M group did not present any meaningful alterations (Figure 4A and 4D).

3.5. Kidney Tissue Changes and Inflammasome Component Expression

Both TAC groups featured renal atrophy with decreased kidney weights and considerable fibrosis (Figure 5B and 5D-E). The fibrosis in the TAC-HF group differed statistically significantly from both the Sham and the TAC-M groups as well. In spite of the described kidney changes, inflammasome component expressions were unchanged in the TAC animals (Figure 5A and 5C).

4. Discussion

In the present study, we established a small animal model showing a spectrum of clinical progressions that ranged from asymptomatic (TAC-M group) to symptomatic, advanced HF (TAC-HF group). We intended to characterize the cardiac and extracardiac organ expression of inflammasome components with or without the presence of HF signs. We observed several changes in the TAC-HF animals, such as the priming of inflammasomes in the myocardium, where NLRC4 and AIM2 were the prominently upregulated sensor proteins. The RV was associated with a more abundant inflammasome expression, even though the LV was primarily affected in the utilized model. Instead of NLRC4, the expression of NLRP3 was elevated along with AIM2 in the lung. Inflammasome components were downregulated in the liver and unaltered in the kidney. The majority of these inflammasome changes were not detectable when the same animal model developed a milder, asymptomatic phenotype of HF (TAC-M group).

Advanced HF is associated with frequent hospitalizations and high mortality rates, significantly burdening both the healthcare system and the individual patients (43, 44). The emergence of extracardiac organ dysfunction further limits therapeutic options and the prognosis of the disease becomes particularly unfavorable (3, 45). Multiple pathomechanisms contribute to end-organ damage in HF, the process is still not uncovered entirely (46). Hemodynamic disturbances result in various kinds of tissue damage, which can trigger sterile inflammation by presenting endogenous danger signals to the immune system (47). In HF, the inflammatory response manifests both systemically and locally in the impacted organs, including the heart itself (48-51). Inflammatory mediators exert a detrimental effect on myocardial contractility and other extracardiac organs, resulting in a positive feedback loop that can undermine the clinical status further (52-54). Several efforts have already been made to utilize anti-inflammatory therapies in HF. IL-1 β inhibition seems to offer certain cardiovascular benefits, but the improvement of all-cause mortality in an advanced HF population is still to be achieved (18-20). Since the so far tested pharmacotherapies act systemically, the unwanted side effects such as infections or cancer can outweigh the cardiovascular gains.

Further attempts should focus specifically on the active inflammatory pathways in HF, not overlooking the end-organs, either.

We intended to gain a better understanding of multiorgan inflammasome activation patterns in advanced HF. We applied TAC-operated rats, a well-characterized surgical HF model in our experiment. In line with previous results, the animals featured considerable systolic and diastolic dysfunction, the structural remodeling of the LV and elevated plasma NT-proBNP levels as well (55). Moreover, by the end of the fifteen-week-long follow-up period, clinical signs of severe HF (edema, resting dyspnea and tachypnea, cardiac cachexia, reduced physical activity and appetite) were observable in a fraction of the animals. Since the whole clinical picture, rather than mere cardiac function, was the focus of our investigation, we formed our TAC-HF group based on the presence of these clinical signs. Another subpopulation of our animals showed no resting symptoms and developed a milder HF phenotype under the same monitoring period. This variable response to similar pressure overload in experiments is known in the literature, and we decided to use these animals (TAC-M group) as a model of a benign clinical progression (56). We aimed to measure the expression levels of inflammasome pathway components in the LV, RV, lung, liver and kidney tissues of our models. Given the diversity of signals that activate them, inflammasome receptor proteins are ideal candidates of targeted anti-inflammatory therapy (57). Clinical studies are already in progress to verify the potential of inflammasome inhibitors (27, 58).

According to previous literature, the LV myocardium seems to express multiple inflammasome sensor proteins in HF (41, 59, 60). The role of NLRP3 is well-established in the pathophysiology of HF (59). Recent studies confirmed the myocardial activation of other receptors as well. Durga Devi *et al.* described impaired mitophagy and mitochondrial DNA release in the peri-infarct region of a diabetic murine model of myocardial infarction, which also resulted in AIM2 and NLRC4 activation (60). In addition, Wang *et al.* managed to alleviate cardiac dysfunction and remodeling in a rat model of diabetic cardiomyopathy by AIM2 gene silencing (61). Moreover, our previous work also verified the pathophysiological role of the

351 AIM2 inflammasome pathway (and partially NLRC4 as well) in various translational HF models and human
352 samples (41). Similarly to these prior results, we detected the upregulation of the AIM2 and NLRC4
353 proteins in the LV tissue of animals with advanced HF (Figure 2A).

354 AIM2 is known as a cytosolic DNA receptor, capable of sensing pathogen-derived or self-DNA released
355 upon tissue damage (22, 62). Besides a possible local self-perpetuating cycle eliciting tissue damage
356 through AIM2, exogenous pathogens also seem a viable trigger for inflammasome activation in the
357 myocardium. This assumption can be based upon the fact that NLRC4 is activated mainly by bacterial
358 antigens according to current knowledge (63, 64). It has been suggested long ago that gastrointestinal
359 changes caused by HF can induce an adverse feedback effect (65). This includes bacterial translocation
360 through gut barriers impaired by congestion, which would serve as a source of extracardiac inflammation
361 (66). NLRC4 was not upregulated in the hearts of our clinically compensated models (Figure 2A-B),
362 suggesting the possibility that such interactions with the intestinal system are only a feature of advanced
363 HF. Gastrointestinal disturbances in the TAC-HF group can also explain the presence of cardiac cachexia,
364 which served as a basis of group selection in current experimental setting (Table 1). Interestingly, none of
365 the inflammasome components were found to be upregulated in the liver tissue of our TAC animals, even
366 though the liver is the first organ to meet bacterial antigens exiting the gut circulation.

367 LV failure results in the eventual impairment of RV through post-capillary pulmonary hypertension (PH)
368 (67, 68). Although the hemodynamic examination of the pulmonary circulation and the RV was not
369 possible in the current experiment, the detected alterations in both TAC-HF and TAC-M groups (elevated
370 LVEDP, pulmonary congestion, structural RV remodeling, see Figures 1-3) were consistent with these
371 mechanisms. Recent studies postulate that inflammation might further damage the pulmonary
372 vasculature and the RV independently of the hemodynamic conditions. Al Qazazi *et al.* studied multiple
373 preclinical models of PH and concluded that the activation of NLRP3 inflammasomes contributed to the
374 decompensation of RV function in PH (34). Moreover, they discovered opposing polarization of

macrophages in the lung and RV tissue, suggesting the presence of different pathomechanisms in the hemodynamically connected organs. Two other contemporary works employed external stressors to induce inflammation in PH and LV HF animal models, revealing significant RV deterioration as a result (33, 69). In line with these results, we found increased number of CD68+ monocytes/macrophages in the lungs of TAC-HF and TAC-M animals (see Figure 3G). However, the priming of the inflammasome pathway was only present in the lung and RV tissue of the clinically progressed TAC-HF group (see Figures 2-3). In addition, the expression patterns of the inflammasome receptors were different in the lung and the heart – NLRC4 activation was specific to the myocardium, while NLRP3 was upregulated only in the lung. It is also interesting to note that inflammasome expressions were comparable in the two ventricles, even trending towards higher levels in the RV. According to our findings, the lung is a possible additional source of inflammatory response in advanced HF, contributing to further RV decline. It is also important to note that current trends in pharmaceutical innovation are favoring NLRP3 inhibitors such as dapansutride, which has already shown promising results in systolic HF patients in a phase 1B trial (27). Based on our results however, the expression of NLRP3 seems less specific to the myocardium in HF. The success of previously utilized NLRP3 inhibitor therapies in HF might have depended on the involvement of pulmonary damage in the clinical picture, while the selective modulation of NLRC4 or AIM2 seems a more promising approach for HF.

The pathological alterations of liver in HF are mainly determined by the presence of hypoperfusion and systemic congestion (70). The elevation of circulating transaminase levels indicates hypoperfusion, while a cholestatic profile of liver enzymes and hypoalbuminaemia demonstrate congestion (71, 72). Liver function tests in our TAC-HF group suggested the dominance of congestion, which can be the result of the postulated RV dysfunction of these animals. The unchanged liver function of the TAC-M rats is in good agreement with the absence of clinical HF signs. In this context, downregulation of inflammasomes in the TAC-HF group is puzzling, especially alongside with the elevated CD68+ cell count (Figure 4D and 4G).

None of the measured liver parameters indicate parenchymal cell loss or other disturbances that can account for a relative decrease of protein expression in the TAC-HF group. The histochemical marker CD68 marks either resident Kupffer cells in the liver tissue or infiltrating monocytes/macrophages (73, 74). Both these cell types are known to participate in liver changes associated with HF (75, 76). Their ability to express inflammasomes has also been confirmed (74, 77). According to literature data, the upregulation of inflammasomes is expected throughout the spectrum of liver diseases, even in advanced cirrhosis (78, 79). Further research is needed to uncover the underlying factors behind these findings.

Both of our aortic constricted groups developed kidney atrophy and fibrosis without any changes of the inflammasome components (Figure 5C-E). Chronic kidney disease and renal failure patients are known to have elevated levels of circulating inflammatory cytokines, the role of IL-1 β in diseased or fibrotic kidney tissue has also been proven (80-82). Yet, there is little data about the direct function of inflammasomal proteins in cardiorenal interactions (83, 84). A functionally intact kidney atrophy without inflammation has already been described in a murine TAC model by Richards *et al.* (85). The atrophy of kidneys was also discovered in a transgenic mouse model of dilatative cardiomyopathy, where renal fibrosis and elevated inflammatory cytokine levels were identified (86). However, the expression of IL-1 β was unchanged in this experiment. Taken together, the pathomechanism of kidney tissue damage associated with HF is likely to unfold without the involvement of the inflammasome pathway according to our results.

5. Conclusions

In conclusion, we provided the first comprehensive characterization of inflammasome component protein expressions in a small animal model of advanced heart failure induced by pressure overload in multiple key organs affected by the disease. In line with previous findings, we found the priming of the AIM2 and NLRC4 inflammasome sensors in the LV myocardium. The RV featured a comparable degree of inflammasome upregulation, despite the pathological stimulus of our model affecting primarily the LV. We discovered a different pattern of inflammasome expression in the lung, as the AIM2 and NLRP3, but not the NLRC4 inflammasome sensors were upregulated. Surprisingly, downregulated inflammasomes in the liver and unchanged expression profile were found in the kidney, despite the histological evidence of typical HF-associated pathological alterations. In a group of similar HF model animals displaying no clinical signs of decompensation, milder cardiac dysfunction and moderate, yet significant organ deviations were measured. The inflammasome changes of the advanced HF group were absent in these compensated animals.

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8. Disclosures

P.F. is the founder and CEO of Pharmahungary Group, a group of R&D companies; the other authors have nothing to declare.

9. Author contributions

The following authors contributed equally: D.N. and Zs.O.; Z.V.V. and M.R.

D.N, Zs.O., B.M., P.F., T.R., Z.V.V. and M.R. conceived and designed research; D.N., Zs.O., M.K., A.T., T.B. and M.R. performed experiments; D.N., Zs.O., M.K., A.T., T.B., A.O., A.A.S., B.A.B., Z.V.V. and M.R. analyzed data; D.N., Zs.O., T.R., Z.V.V. and M.R. interpreted results of experiments; D.N., Zs.O., A.O. and

454 R.M. prepared figures; D.N., Zs.O., T.R., Z.V.V., and M.R. drafted manuscript; D.N., Zs.O., T.B., A.O., A.A.S.,
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749 **Table 1.** Characterization of the TAC model.

	Sham	TAC-M	TAC-HF
<i>n</i>	6	6	6
Body weight, g	531.7 ± 16.2	473.7 ± 21.9	439.3 ± 25.9*
<i>Echocardiographic data</i>			
HR, beats/min	383.9 ± 9.66	330.0 ± 7.05**	300.7 ± 9.02***
LVAWTd, mm	1.91 ± 0.05	2.76 ± 0.16**	2.33 ± 0.20
LVPWTd, mm	2.00 ± 0.09	3.28 ± 0.24**	3.29 ± 0.29**
LVEDS, mm	4.32 ± 0.26	5.95 ± 0.27**	7.20 ± 0.42***#
LVEDD, mm	7.55 ± 0.41	8.57 ± 0.21	9.60 ± 0.34**
FS, %	42.9 ± 0.95	30.6 ± 2.71	25.0 ± 3.44**
RWT	0.53 ± 0.04	0.71 ± 0.04*	0.59 ± 0.06
<i>Hemodynamic data</i>			
HR, beats/min	337.6 ± 12.1	301.9 ± 6.53*	290.2 ± 8.83**
SBP, mmHg	128.1 ± 8.25	253.8 ± 8.57***	224.2 ± 13.2***
DBP, mmHg	96.7 ± 7.26	95.15 ± 6.67	85.6 ± 5.48
MAP, mmHg	107.2 ± 7.56	148.0 ± 6.38**	131.8 ± 7.55
LVESV, µl	125.8 ± 10.7	208.4 ± 21.9*	291.4 ± 26.7***#
LVEDV, µl	291.8 ± 21.7	319.5 ± 34.9	369.1 ± 20.6
LVESP, mmHg	124.3 ± 8.01	246.7 ± 8.73***	206.0 ± 11.3***#
dPdt _{max} , mmHg/sec	8,652 ± 710.4	8,818 ± 547.2	6,874 ± 469.0
dPdt _{min} , mmHg/sec	-10,564 ± 1,020	-8,547 ± 312.6	-5,919 ± 277.7***#

SV, μ l	166.1 ± 12.9	111.2 ± 18.3	$77.72 \pm 13.1^{**}$
CO, ml/min	55.7 ± 3.67	$33.3 \pm 5.54^{**}$	$22.2 \pm 3.28^{***}$

750

751 Values are means $\pm$ SE; Normal distribution of the data was assessed with Shapiro-Wilk test. One-
752 way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Kruskal–Wallis test
753 followed by Dunn's *post hoc* test were carried out depending on the distribution of the datasets.

754 * P < 0.05 vs. Sham; ** P < 0.01 vs. Sham; *** P < 0.001 vs. Sham; # P < 0.05 vs. TAC-M.

755 *n*, number of rats; HR, heart rate; LVAWTd and LVPWTd, left ventricular anterior and posterior
756 wall thickness in diastole; LVESD and LVEDD, left ventricular end-systolic and end-diastolic
757 diameter; FS, fractional shortening; RWT, relative wall thickness; SBP and DBP, systolic and
758 diastolic blood pressure; MAP, mean arterial pressure; LVESV and LVEDV, left ventricular end-
759 systolic and end-diastolic volume; LVESP, left ventricular end-systolic pressure; dP/dt_{max} and
760 dP/dt_{min} , maximal slope of the systolic pressure increment and the diastolic pressure decrement;
761 SV, stroke volume; CO, cardiac output

762 **Table 2.** Circulating biochemical markers of liver function.

	Sham	TAC-M	TAC-HF
<i>n</i>	6	6	6
albumin, g/l	26.0 ± 0.37	24.58 ± 0.97	22.7 ± 0.68*
AST, IU/l	106.8 ± 5.85	112.8 ± 16.9	133.8 ± 9.50 ¹
ALT, IU/l	55.3 ± 3.20	43.8 ± 4.24	41.8 ± 5.17
total bilirubin, µmol/l	1.20 ± 0.14	1.08 ± 0.19	2.50 ± 0.26**###

763
764 Values are means ± SE; Normal distribution of the data was assessed with Shapiro-Wilk test. One-
765 way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Kruskal–Wallis test
766 followed by Dunn's *post hoc* test were carried out depending on the distribution of the datasets.

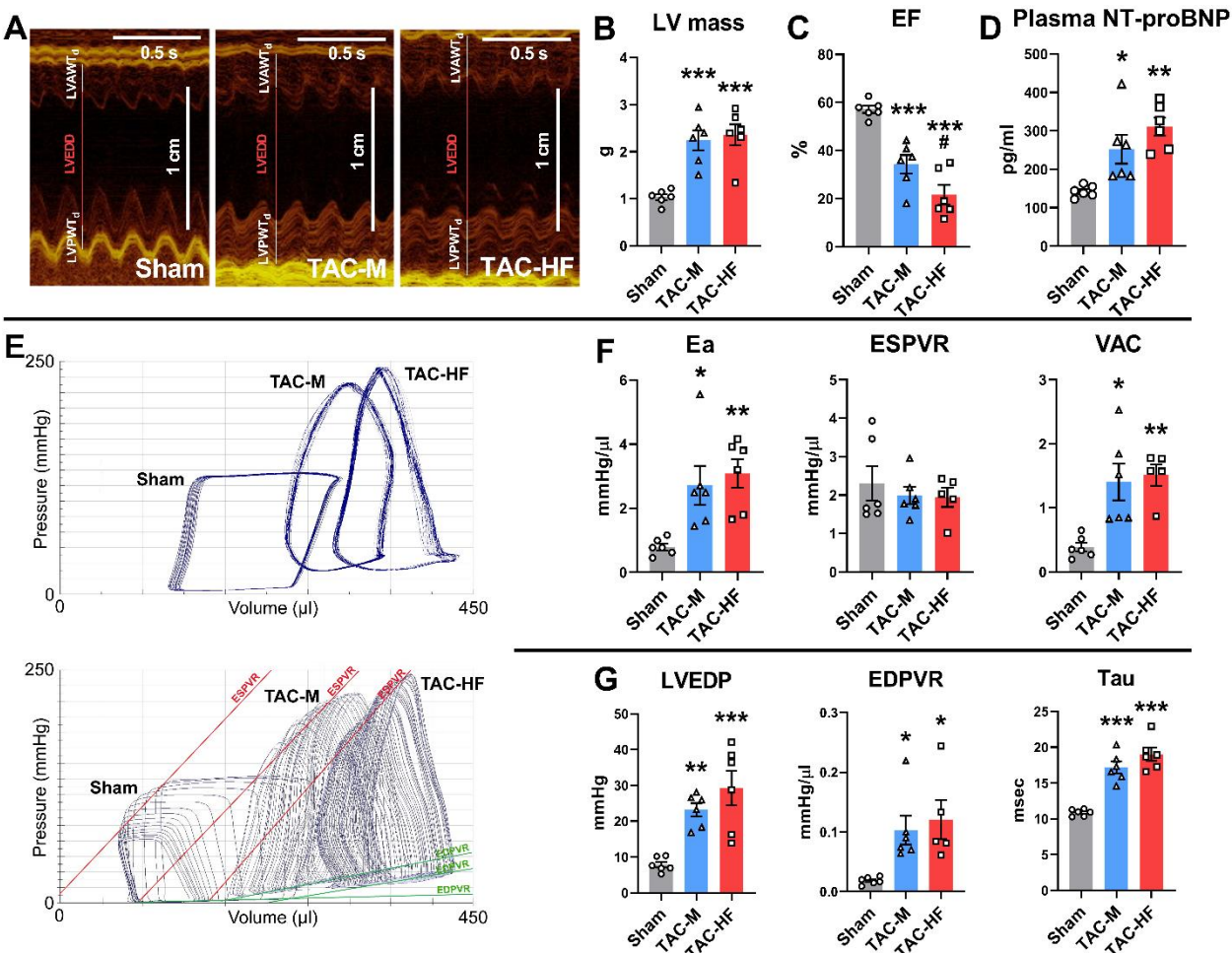
767 * P < 0.05 vs. Sham; ** P < 0.01 vs. Sham; ### P < 0.001 vs. TAC-M.

768 *n*, number of rats; AST, aspartate aminotransferase; ALT, alanine aminotransferase

769 ¹: *n*=4 due to technical reasons

12. Figures with Figure Legends

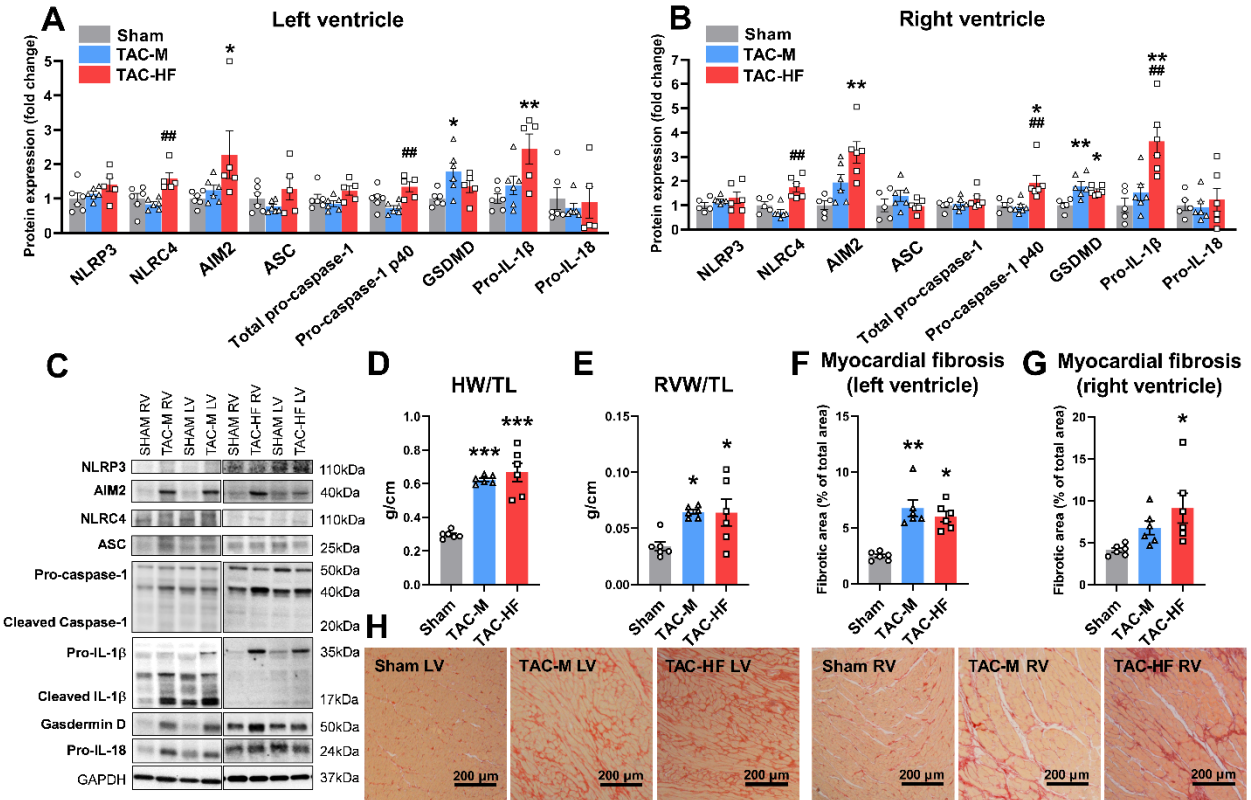
Figure 1.



Characterization of the TAC model. Upper panel: morphology and clinical parameters of heart failure. A: Representative M-mode echocardiographic images at the midpapillary muscle level. B: Left ventricular mass (LV mass) calculated from echocardiographic data. C: ejection fraction (EF) calculated from invasive P-V measurement data. D: plasma N-terminal prohormone of brain natriuretic peptide (NT-proBNP) levels measured with ELISA. Lower panel: left ventricular hemodynamic parameters from invasive P-V measurements. E: Representative original steady-state recordings (top) and loops obtained at different preloads during transient vena cava occlusions (bottom). F: systolic parameters – arterial elastance (E_a), slope of end-systolic pressure-

781 volume relationship (ESPVR) and ventriculo-arterial coupling (VAC). G: diastolic parameters –
782 left ventricular end-diastolic pressure (LVEDP), slope of end-diastolic pressure-volume
783 relationship (EDPVR) and time constant of left ventricular pressure decay (Tau).
784 Values are means $\pm$ SE; Normal distribution of the data was assessed with Shapiro-Wilk test. One-
785 way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Kruskal–Wallis test
786 followed by Dunn's *post hoc* test were carried out depending on the distribution of the datasets.
787 $n=6$ (except for the ESPVR, VAC and EDPVR parameters in the TAC-HF group, where $n=5$ due
788 to technical reasons). * $P < 0.05$ vs. Sham; ** $P < 0.01$ vs. Sham; *** $P < 0.001$ vs. Sham; # $P <$
789 0.05 vs. TAC-M.
790 TAC, *transverse aortic constriction*; HF, *heart failure*; ELISA, *enzyme-linked immunosorbent*
791 *assay*; LVPWT_d, *left ventricular posterior wall thickness in diastole*; LVEDD, *left ventricular end-*
792 *diastolic diameter*; LVAWT_d, *left ventricular anterior wall thickness in diastole*.

Figure 2.

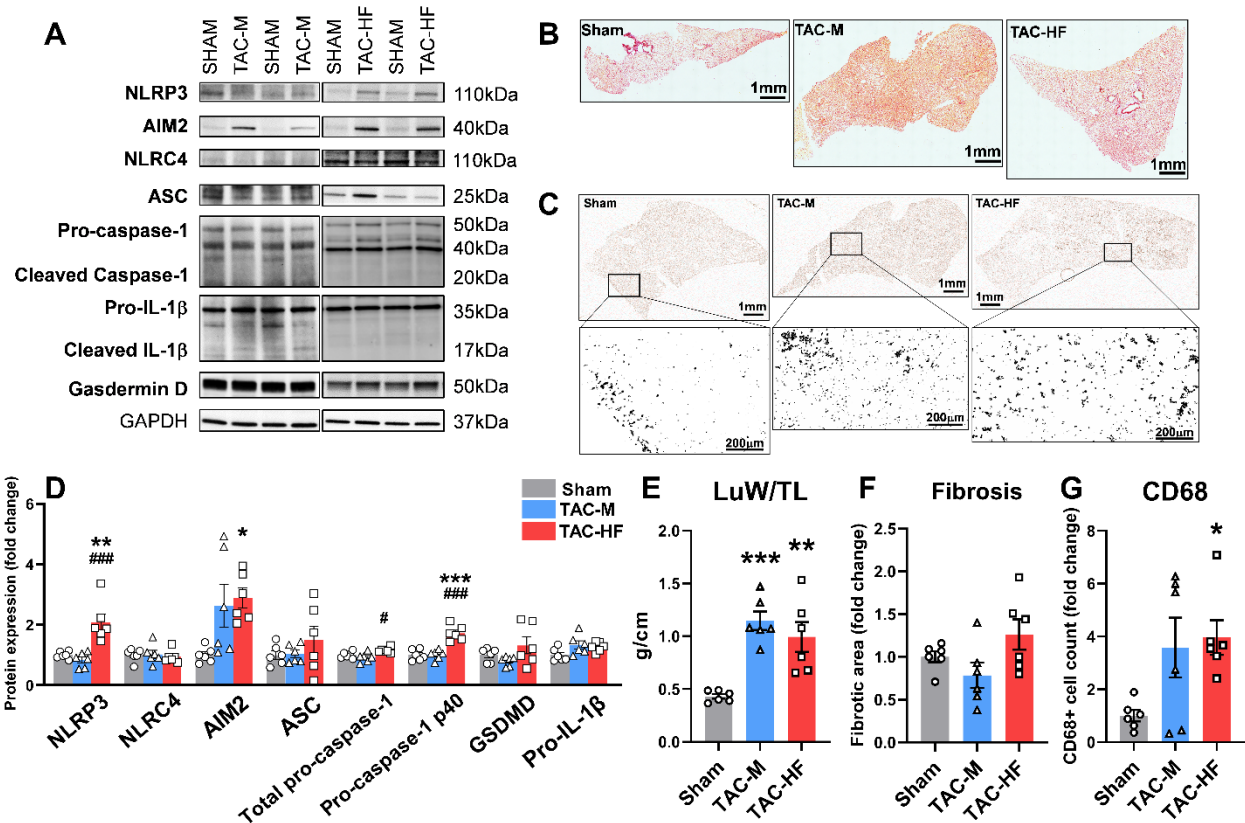


Myocardial tissue changes. A and B: Western blot analysis of left and right ventricular major inflammasome component expression levels normalized to GAPDH. C: Representative images of blots. Heart weight (D) and right ventricle weight (E) normalized to tibial length. F and G: Left and right ventricular fractional area of interstitial fibrosis. H: Representative photomicrographs with picrosirius red staining of left and right ventricular myocardium (red color indicates fibrotic area). 50x magnification, scale bar 200 μ m.

Values are means $\pm$ SE; Normal distribution of the data was assessed with Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Kruskal-Wallis test followed by Dunn's *post hoc* test were carried out depending on the distribution of the datasets. $n=6$ (except for the Western blot measurements in the LV of the TAC-HF group and the RV of the

805 Sham group, where $n=5$ due to technical reasons). * $P < 0.05$ vs. Sham; ** $P < 0.01$ vs. Sham; ***
806 $P < 0.001$ vs. Sham; ## $P < 0.01$ vs. TAC-M.
807 *TAC, transverse aortic constriction; HF, heart failure; NLRP3, NLR family pyrin domain-*
808 *containing protein 3; NLRC4, NLR family CARD domain-containing protein 4; AIM2, absent in*
809 *melanoma 2; ASC, apoptosis-associated speck-like protein containing a CARD; GSDMD,*
810 *gasdermin D; Pro-IL-1 β , Pro-interleukin-1 beta; Pro-IL-18, Pro-interleukin-18; GAPDH,*
811 *glyceraldehyde-3-phosphate dehydrogenase; LV, left ventricle; RV, right ventricle; HW, heart*
812 *weight; TL, tibial length; RVW, right ventricle weight.*

Figure 3.

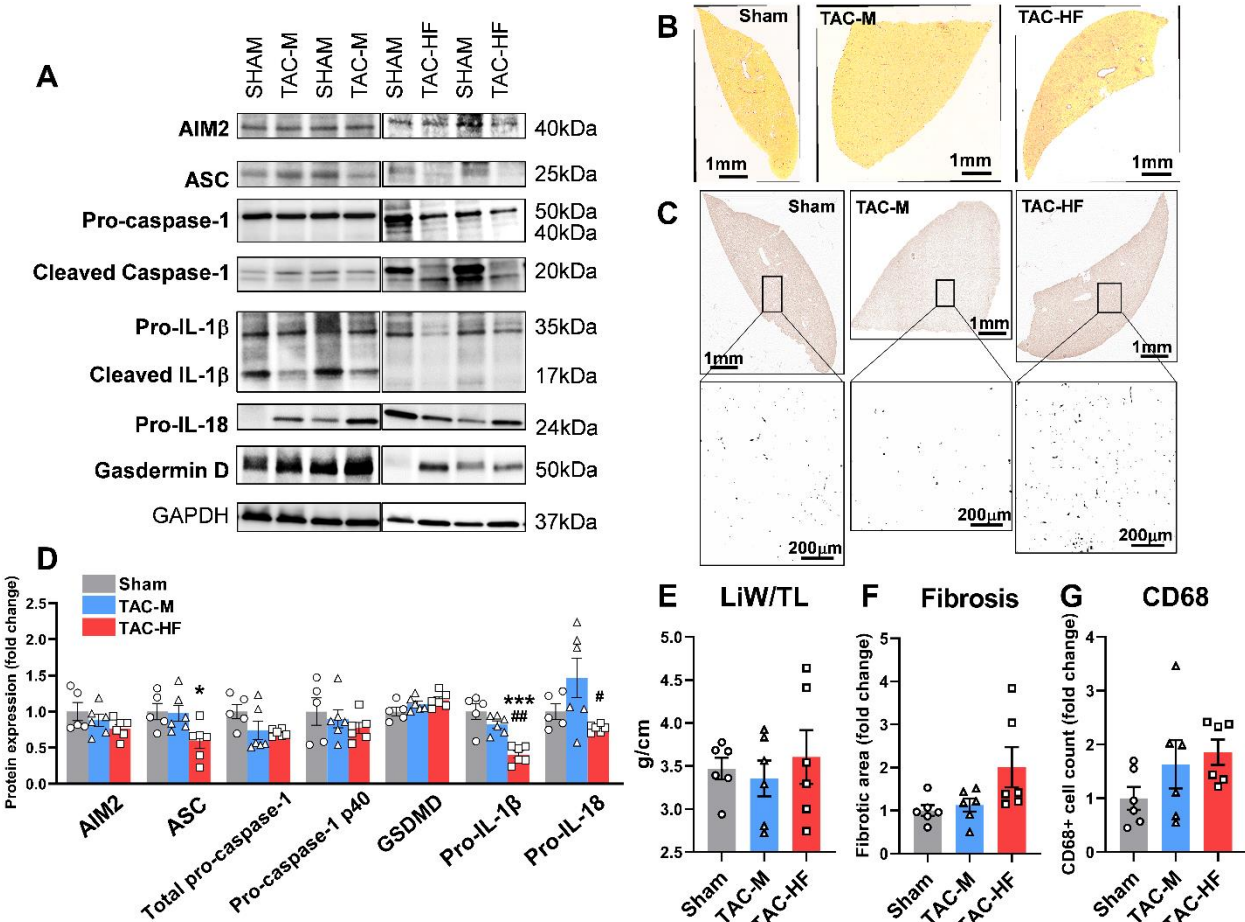


Lung tissue changes. Western blot analysis of major inflammasome component expression levels normalized to GAPDH (D) and representative images of blots (A). B and F: Histological evaluation of relative fibrosis area in lung tissue on picrosirius red-stained sections. Scale bar 1 mm. C and G: Identification of macrophages in lung tissue by immunohistochemical detection of CD68. Scale bar 1 mm on top and middle images, 200 μm on bottom images. E: Lung weight normalized to tibial length.

Values are means ± SE; Normal distribution of the data was assessed with Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Kruskal–Wallis test followed by Dunn's *post hoc* test were carried out depending on the distribution of the datasets. *n*=6 in all groups. * *P* < 0.05 vs. Sham; ** *P* < 0.01 vs. Sham; *** *P* < 0.001 vs. Sham; ### *P* < 0.001 vs. TAC-M.

826 *TAC, transverse aortic constriction; HF, heart failure; NLRP3, NLR family pyrin domain-*
827 *containing protein 3; AIM2, absent in melanoma 2; NLRC4, NLR family CARD domain-containing*
828 *protein 4; ASC, apoptosis-associated speck-like protein containing a CARD; Pro-IL-1 β , Pro-*
829 *interleukin-1 beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSDMD, gasdermin D;*
830 *LuW, lung weight; TL, tibial length.*

Figure 4.



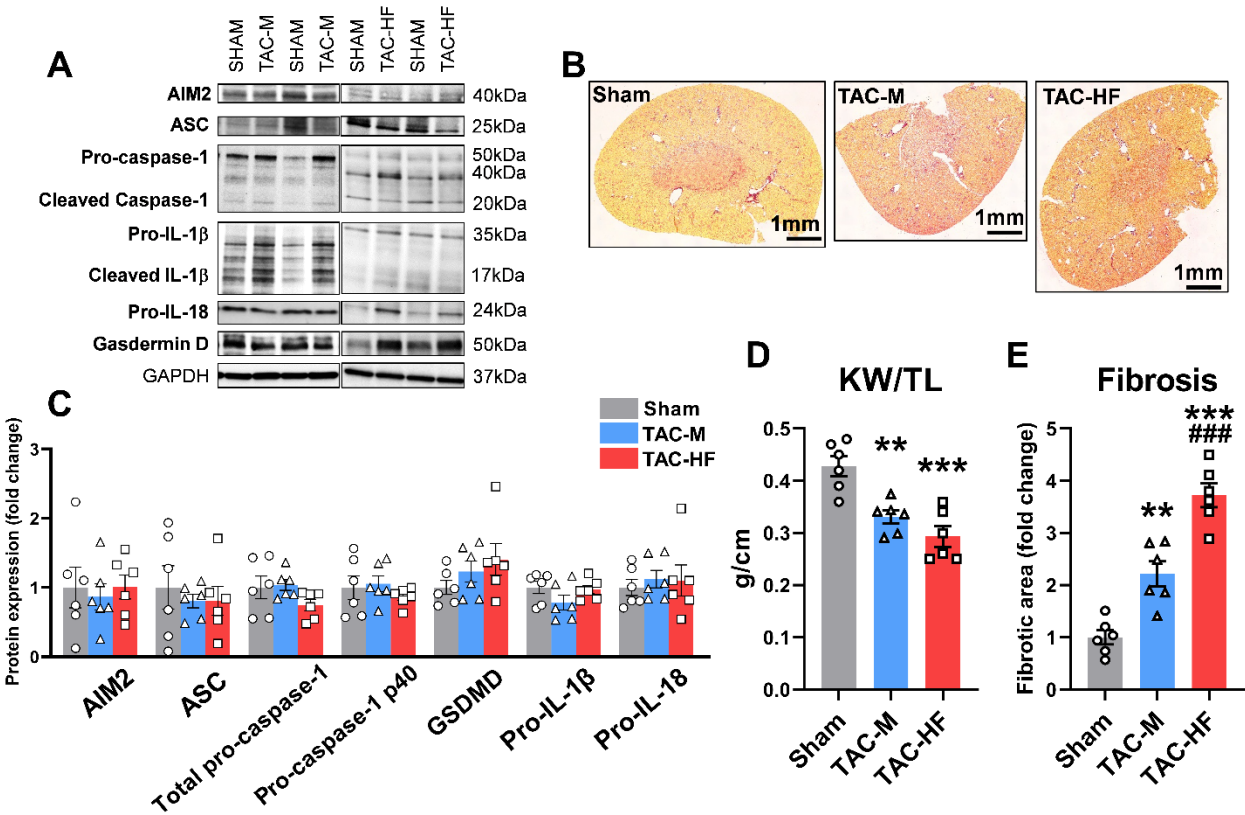
Liver tissue changes. Western blot analysis of major inflammasome component expression levels normalized to GAPDH (D) and representative images of blots (A). B and F: Histological evaluation of relative fibrosis area in liver tissue on picrosirius red-stained sections. Scale bar 1 mm. C and G: Identification of macrophages in liver tissue by immunohistochemical detection of CD68. Scale bar 1 mm on top and middle images, 200 μ m on bottom images. E: Liver weight normalized to tibial length.

Values are means $\pm$ SE; Normal distribution of the data was assessed with Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Kruskal–Wallis test followed by Dunn's *post hoc* test were carried out depending on the distribution of the datasets.

842 $n=6$ (except for the Western blot measurements in the Sham group where $n=5$ due to technical
843 reasons). * $P < 0.05$ vs. Sham; *** $P < 0.001$ vs. Sham; # $P < 0.05$ vs. TAC-M; ## $P < 0.01$ vs.
844 TAC-M.

845 *TAC, transverse aortic constriction; HF, heart failure; AIM2, absent in melanoma 2; ASC,*
846 *apoptosis-associated speck-like protein containing a CARD; Pro-IL-1 β , Pro-interleukin-1 beta;*
847 *Pro-IL-18, Pro-interleukin-18; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSDMD,*
848 *gasdermin D; LiW, liver weight; TL, tibial length.*

Figure 5.



Kidney tissue changes. Western blot analysis of major inflammasome component expression levels normalized to GAPDH (C) and representative images of blots (A). B and E: Histological evaluation of relative fibrosis area in kidney tissue on picosirius red-stained sections. Scale bar 1 mm. E: Kidney weight normalized to tibial length.

Values are means $\pm$ SE; Normal distribution of the data was assessed with Shapiro-Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Kruskal–Wallis test followed by Dunn's *post hoc* test were carried out depending on the distribution of the datasets. $n=6$ in all groups. ** $P < 0.01$ vs. Sham; *** $P < 0.001$ vs. Sham; ### $P < 0.001$ vs. TAC-M.

TAC, transverse aortic constriction; HF, heart failure; AIM2, absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing a CARD; Pro-IL-1 β , Pro-interleukin-1 beta;

861 *Pro-IL-18, Pro-interleukin-18; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSDMD,*
862 *gasdermin D; KW, kidney weight; TL, tibial length.*