Immune-inflammatory targets in chronic heart failure: inflammasomes and the endocannabinoid system

PhD thesis

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1. INTRODUCTION

1.1. Heart failure

Chronic heart failure (HF) is associated with structural and cellular damage of the heart leading to impaired pump function. Maladaptive activation of the neurohormonal systems to maintain perfusion ultimately induces detrimental effects on cardiac cells leading to cellular damage, fibrosis and cell death. Current therapies (e.g. beta-1 receptor antagonist or ACE inhibitors) for HF aim the interruption of this maladaptive activation, which resulted in significant improvement in the outcome of HF. However, HF still inflicts significant morbidity and mortality that consumes a notable part of healthcare resources. Thus, new effective therapeutic strategies that might improve poor outcome of HF are needed.

1.2. Inflammation in cardiovascular diseases

The role of inflammation and inflammatory mediators (interleukin-1β (IL-1β), interleukin-6, tumor necrosis factor alpha) in HF has been investigated for many years. Increased plasma levels of pro-inflammatory mediators were originally considered to be biomarkers strongly associated with the advanced stage of HF. However, recent studies propose them as active players in the pathomechanism of HF, raising a concept that inflammatory pathways may represent new therapeutic targets.

Preclinical studies have demonstrated the beneficial effects of targeting inflammatory pathways by glucocorticoids, methotrexate, infliximab and many others in various models of myocardial infarction, stroke or HF. Despite the promising preclinical findings, clinical studies have provided conflicting results. Some of the candidate drugs even increased the cardiovascular risks leading to the early termination of related clinical studies. Nevertheless, the recently published results of Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) evaluating the cardiovascular safety and efficacy of canakinumab, a fully human monoclonal antibody against IL-1β, presented promising outcomes for patients with cardiac diseases. Blockade of IL-1β with canakinumab was capable of reducing the incidence of major cardiovascular events such as myocardial infarction, stroke and the hospitalization of HF. The initial optimism about canakinumab faded quickly due to its high cost and possible infectious side effects that finally led to its rejection for the cardiac indications. However, CANTOS and its findings propose that targeting inflammatory mediators e.g. IL-1β may still provide therapeutic promise in cardiac diseases.

IL-1β is secreted 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 regulated by inflammasomes, which are special cytosolic multiprotein complexes. Inflammasome activation is initiated by various sensors recognizing specific pathogen- or danger-associated molecular patterns, leading to cleavage and maturation of caspase-1 enzyme which ultimately cleaves pro-IL-1β to its mature form. Inflammasome assembly is also modulated by numerous related pathways including membrane receptors (e.g. Toll-like receptors) or ion channels (e.g. purinergic and pannexin channels) allowing a fine modulation of immune response.
Recent studies suggest that inflammasome activation might play a role in cardiovascular events. Enhanced inflammasome activity has been shown to be associated with ischemia-reperfusion injury, cardiac remodeling, atherosclerosis and decreased cardiac pump function in preclinical models. Thus, the research on inflammasome inhibitors particularly NACHT, leucine-rich repeat, and pyrin domain-containing protein 3 (NLRP3) selective ones e.g. MCC950 or OLT1177 gains more and more attention. However, increasing number of evidences pointed out that other inflammasome pathways may also contribute to the development of cardiovascular diseases. Furthermore, absent in melanoma 2 (AIM2) and NLR family CARD domain-containing protein 4 (NLRC4) inflammasome activation has been shown in diabetic cardiomyopathy and stroke indicating that caspase-1 activation and IL-1β release might be regulated by multiple inflammasome sensors simultaneously. Therefore, developing new compounds or repurposing already existing drugs as non-selective inflammasome blockers may be innovative strategy for treating cardiovascular diseases in the future.

1.3. Endocannabinoid system in cardiovascular diseases

The endocannabinoid system (ECS) has been proven as a modulator of immune response including the secretion of IL-1β, tumor necrosis factor α or interleukin-6 under certain conditions. The ECS consists of the cannabinoid receptor type 1 and 2 (CB_1R, CB_2R), their endogenous lipid-derived ligands, the endocannabinoids 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the enzymes that play role in their synthesis and rapid degradation including diacylglycerol lipase α and β (DAGL α and β), monoacylglycerol lipase (MGLL), α,β-hydrolase domain containing proteins 6 and 12 (ABHD6, ABHD12), fatty acid amide hydrolase (FAAH) or N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD).

The ECS regulates a broad spectrum of physiological and pathological processes e.g. energy balance, obesity or inflammation. The endocannabinoids have been implicated in the regulation of vascular tone, atherogenesis, fibrosis and cell death in cardiovascular system. These effects of the ECS might be mediated directly via actions on the myocardium, on vascular structures or circulating blood cells as well as indirectly through the nervous system.

The cannabinoid receptors have been shown to have opposing effects. CB_1R is involved in a series of pathological processes in cardiovascular diseases. Overproduction of endocannabinoids by immune cells can be observed in various conditions including cardiomyopathies and chronic ischemia, and it leads to acute cardiac depression and hypotension via CB_1R signaling. CB_1R activation also promotes fibrosis, inflammation, oxidative stress and apoptosis. CB_1R antagonists improve cardiac function when administered acutely, and attenuate pathological processes in chronic treatment. In contrast, selective agonists of CB_2R are cardioprotective in different models of cardiovascular diseases by reducing inflammation and fibrosis. Interestingly, CB_2R activation has no direct effects on cardiac function.

The pharmacology of ECS has attracted emerging interest as targets for innovative treatment of cancer, chronic pain or anxiety. Besides agonists and antagonists of endocannabinoid receptors, probable modulators of ECS enzymes are intensively investigated as potential new agents as well. Thus, the detailed knowledge of the role of ECS in cardiovascular conditions is essential. Despite the extensive investigation on the endocannabinoids and their receptors, there are only few studies available on the activity of ECS-related biosynthetic or metabolic enzymes in pathological processes of cardiovascular system, and data is lacking on especially human samples.

2. OBJECTIVES

In the present work, we aimed to investigate the activation of inflammasomes and the enzymes of the endocannabinoid system in human chronic HF. To find suitable preclinical models with inflammasome activation that reflects the human condition, we examined failing hearts from various animal models. Additionally, we induced inflammasome activation in human monocytic THP-1 cells as well as in human AC16 cardiac cells *in vitro* to examine the pharmacological inhibition of pannexin-1 with the clinically used uricosuric drug, probenecid. Finally, we intended to study the therapeutic effects of probenecid *in vivo* in a pressure overload-induced chronic HF model.

3. RESULTS

3.1. Inflammasome activation and alterations of the endocannabinoid system in human heart failure

3.1.1. The expression of AIM2 and NLRC4 inflammasome sensors increases in human heart failure

To investigate inflammasome activation in HF, the well-characterized inflammasome sensors (NLRP3, NLRC4, AIM2 and NALP1) were detected by Western blot in heart tissue harvested from healthy donors as well as from HF patients with ischemic (ICM) or non-ischemic (DCM) cardiomyopathy.

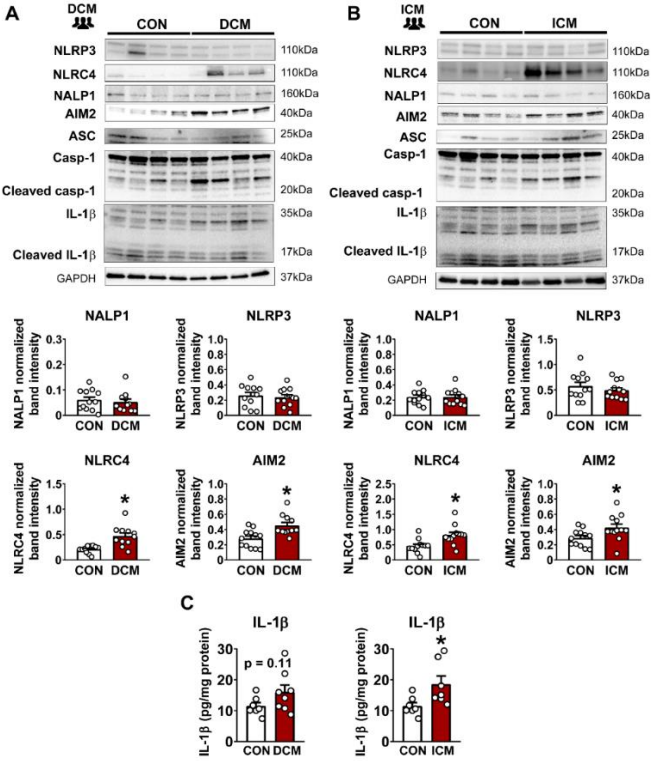

Figure 1 - AIM2 and NLRC4 are the major inflammasome components expressed in human failing hearts Western blot analysis of the inflammasome sensors (NLRP3,

AIM2, NLRC4 and NALP1) and downstream signaling (ASC, caspase-1, IL-1β) in left ventricle of patients with dilated (DCM, A) or ischemic 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.

There was no difference in the expression of NLRP3 as well as NALP1 protein in HF induced by any forms of cardiomyopathies examined (Fig.1A-B). In contrast, the expression of AIM2 increased both in ICM and DCM groups, and we also found a significant increase of NLRC4 protein level in left ventricular tissue of HF patients (Fig.1A-B). Inflammasome activation was further confirmed by detection of the cleaved fragments of caspase-1 and IL-1β and by the detection of elevated IL-1β levels by ELISA in failing hearts (Fig.1A-C).

3.1.2. AIM2 inflammasome sensor is expressed in monocytes and macrophages in failing hearts

To assess the presence of macrophages in failing hearts, immunohistochemistry was performed to stain Iba1, a general markers of monocyte-macrophage lineage (Fig.2A). We detected a large population of Iba1 positive monocytes and macrophages in the left ventricle of both control and HF hearts (Fig.2A).

Next, to assess localization of AIM2 inflammasome, immunofluorescence staining was used to confirm the localization of AIM2 inflammasomes by detecting AIM2 in combination with Iba1 (Fig.2B). Immunofluorescence staining showed that AIM2 is localized predominantly in Iba1 positive cells, although AIM2 signals can be detected in other cell types, suggesting that monocytes and macrophages might be key players in the inflammasome activity but their interactions with the other, non-myeloid cells might be also important in the development of the pro-inflammatory milieu in failing hearts (Fig.2B).

Cell death may lead to the release of double-stranded DNA (dsDNA) to the cytosol that can be detected 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 (Fig.2C, red signal) shows tight co-localization with the AIM2 signal (Fig.2C, green signal).

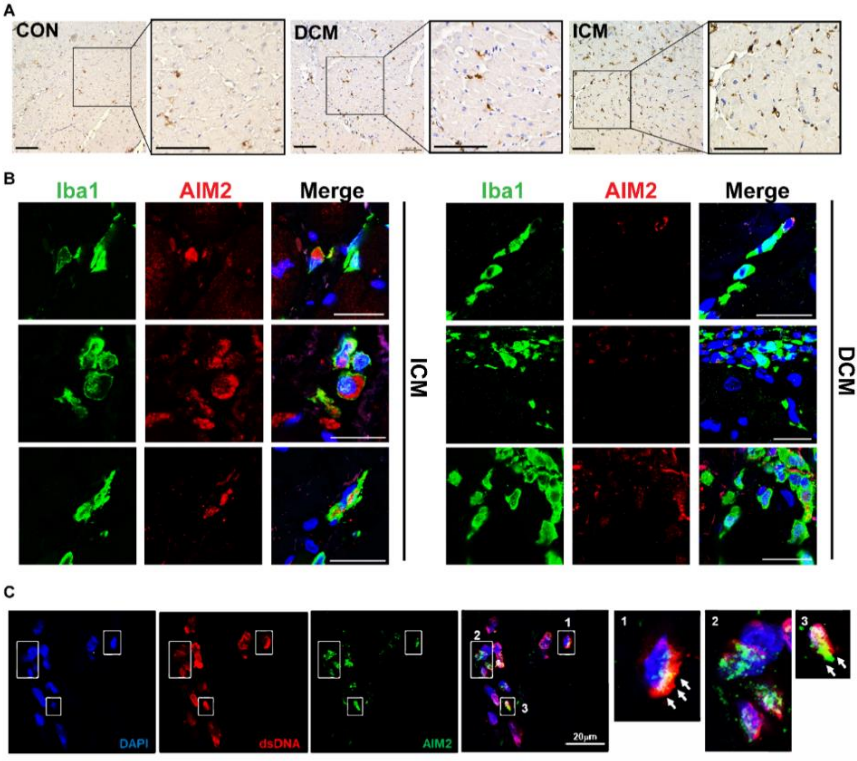

Figure 2 – Double-stranded DNA-sensitive AIM2 inflammasome sensor is expressed in monocytes and macrophages in human failing hearts. (A) Identification of monocytes and macrophages (brown) in human heart tissue by immunohistochemical detection of Iba1. Scale bar: 100μm. (B) Representative images of immunofluorescence detection of AIM2 (red) and Iba1 (green) proteins in failing heart collected from ICM and DCM patients. DAPI (blue) was used for counterstain. Scale bar: 30μm. (C) Representative images of immunofluorescence detection of double-stranded DNA (dsDNA, red) and AIM2 (green) protein in a failing heart collected from a DCM patient. DAPI (blue) was used for counterstain. Scale bar: 20μm.

3.1.3. Ischemic failings hearts show two distinct phenotypes based on the alteration of lipid and hydrolase activity profile

The endocannabinoid system (ECS) has been shown to modulate immune response in both physiological and pathological processes including chronic

inflammation. Despite the growing interest regarding the role of ECS in cardiovascular diseases, enzymes involved in endocannabinoid metabolism during HF have not been investigated before. Thus, to examine the involvement of the ECS in ischemic cardiomyopathy, we used quantitative real-time polymerase chain reaction (qRT-PCR) to measure the expression levels of ECS-related genes in control and ischemic failing hearts (Fig. 3).

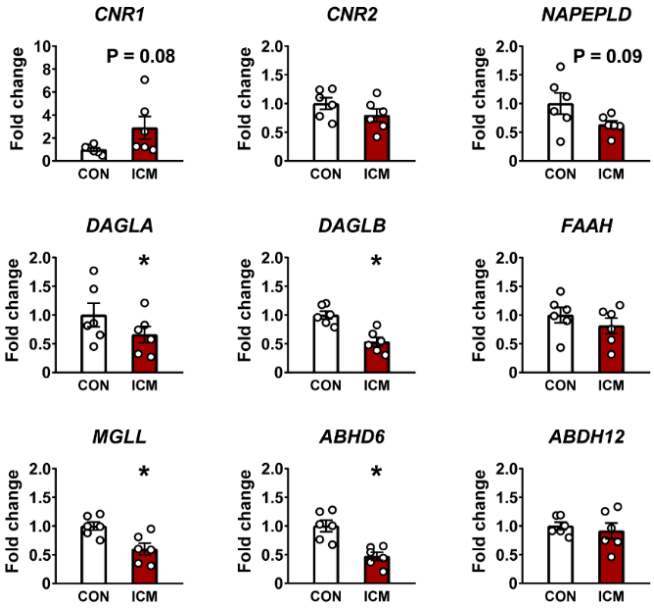

Figure 3 – mRNA expression of type-1 cannabinoid receptors increased and synthetic and hydrolytic enzymes of 2-arachidonoylglycerol decreased in heart samples of ischemic cardiomyopathy patients. Analysis of mRNA expression of endocannabinoid-related genes by qRT-PCR. *p<0.05 vs. control, Student's t-test; n=6.

CNR1 expression remarkably increased in some of the ischemic samples, however the overall increase was not significant (Fig.3). Reduced expression of 2-AG biosynthetic enzyme *DAGLA/B* and the 2-AG hydrolytic enzymes *MGLL* and *ABHD6* was observed in the ischemic tissue (Fig.3). The AEA metabolic enzyme e.g. *FAAH* were not significantly altered, nor was *CNR2* expression (Fig.3).

3.2. Inflammasome activation in animal models for heart failure

3.2.1. AIM2 inflammasome expression is elevated in heart failure induced by pressure-overload and postinfarction but not by volume-overload in rats

To find suitable preclinical animal models to study inflammasome activation, we assessed three pathologically different models of HF i.e. pressure-overload (transverse aortic constriction - TAC), volume-overload (infrarenal arteriovenous shunt - AVS) and the postinfarction HF rat model (LAD), which had been developed and characterized by Mihály Ruppert et al. (Heart and Vascular Center, Semmelweis University, Hungary).

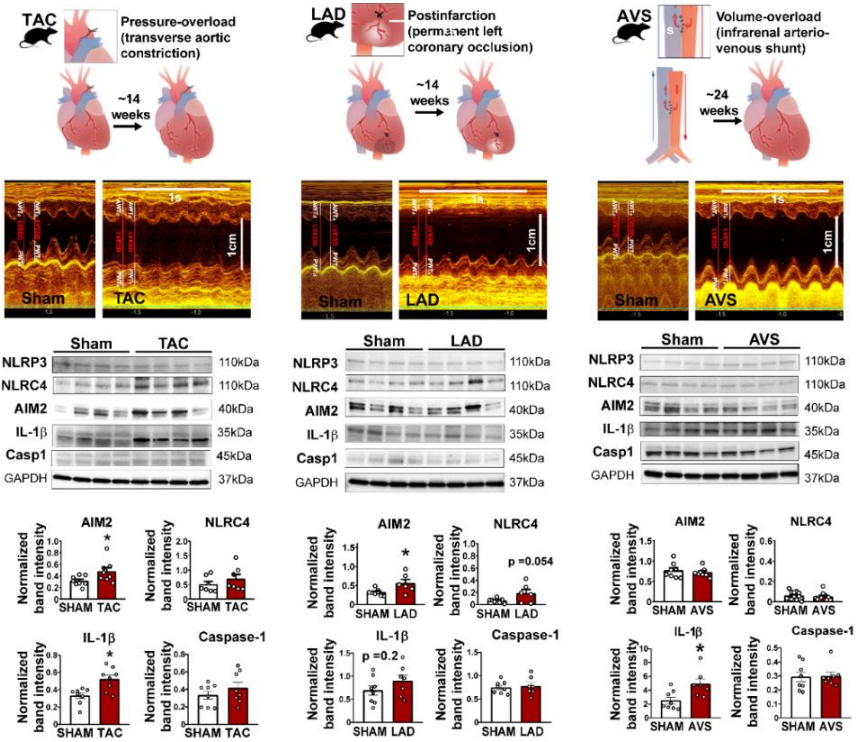

Figure 4 - AIM2 inflammasome expression increased in the late phase of chronic heart failure in rats. Pressure-overload, postinfarction and volume-overload-induced

rat models of chronic heart failure with representative M-mode echocardiographic images, Western blot analysis of the inflammasome sensors and downstream signaling. Scale bar (echocardiography): 1cm, timestamp: 1s; *p<0.05 vs. corresponding Sham, Student's t-test; n=6-8. *(Representative echocardiographic images were taken by Mihály Ruppert, Heart and Vascular Center, Semmelweis University, Hungary.*)

Pressure-overload induced excessive myocardial hypertrophy and fibrosis in TAC animals, while volume-overload and ischemic conditions developed severe dilation (Fig.4).

The expression of NLRP3 did not increase in any of the HF rat groups compared to corresponding shams. The protein expression of AIM2 increased significantly in TAC and LAD, but not in AVS rats (Fig.4). A tendency towards elevation in the protein level of NLRC4 was observed in TAC and LAD animals (Fig.4). In accordance with these results, the tissue level of IL- 1β increased significantly in TAC and in AVS animals, and tendentiously in LAD rats (Fig.4).

Similar to human study, we found enhanced presence of monocytes and macrophages in rat failing hearts by assessing *Aif1* and *Cd68* mRNA expression with qRT-PCR analysis (Fig.5A). In line with the Western blot results, AIM2 showed co-localization with the monocyte-macrophage marker CD68 in myocardial sections from TAC animals (Fig.5B).

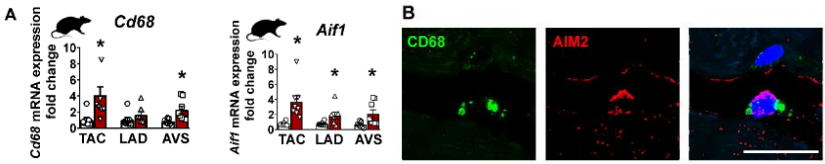

Figure 5 – Detection of macrophages in rat failing hearts. (A) Analysis of mRNA expression of macrophage marker *Cd68* and *Aif1* by qRT-PCR. *p<0.05 vs. corresponding Sham, Student's t-test; n=6-8. (B) Representative images of immunofluorescence detection of AIM2 (red) and CD68 (green) proteins in a failing heart harvested from a TAC animal. DAPI (blue) was used for counterstain. Scale bar: 20μm.

3.3. Targeting AIM2 inflammasome activation by pannexin-1 inhibitor probenecid

3.3.1. Liposome encapsulated poly(dA:dT) induces AIM2 inflammasome activation in monocytic and cardiomyocyte cell lines *in vitro*

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(dA:dT), a specific AIM2 inducer, for 24 hours (Fig.6A).

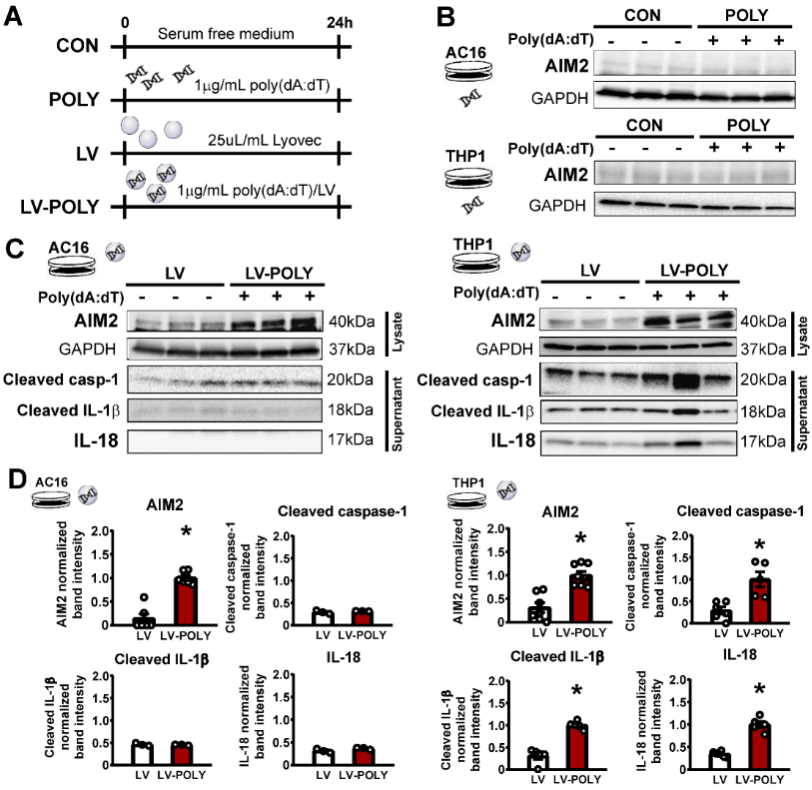

Figure 6 - Liposome encapsulated poly(dA:dT) induced the expression of AIM2 and inflammasome activation in vitro (A) Experimental protocol for AIM2 induction in human AC16 cardiac and THP1 monocytic cell lines. (B) Representative Western blot images for naked poly(dA:dT) stimulus on AC16 and THP1 cells. (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.

Naked poly(dA:dT) was unable to induce AIM2 inflammasome activation (Fig.6B), however, liposome encapsulated poly(dA:dT) increased the expression of AIM2 in THP-1 cells (Fig.6C), suggesting that vesicular uptake of dsDNA is critical in the induction of AIM2 inflammasome activation. Inflammasome activation was confirmed with the detection of cleaved caspase-1, IL-18 and IL-1β from the supernatant in THP-1 cell line (Fig.6C-E). Interestingly, poly(dA:dT) treatment also led to the induction of AIM2 protein expression in the AC16 cardiac cells without significant interleukin release (Fig.6C-D).

3.3.2. Pannexin-1 channel inhibitor probenecid reduces AIM2 inflammasome activation in monocytes/macrophages and cardiomyocytes *in vitro*

We performed co-immunoprecipitation on control and poly(dA:dT)-stimulated THP-1 cells, and saw that AIM2 was co-immunoprecipitated with pannexin-1 channels (PANX1) in activated cells indicating a potential interaction between the AIM2 inflammasome complex and PANX1 channels (Fig.7). As the opening of PANX1 channels is related to apoptosis and release of "find me" signals, we tested the effects of probenecid, a potent PANX1 inhibitor, on AIM2 inflammasome activation *in vitro* (Fig.8). Probenecid showed a dose-dependent reduction in the protein expression of AIM2 in both THP-1 and AC16 cells (Fig.8A-B).

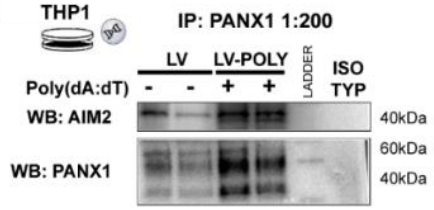

Figure 7 - Pannexin-1 channel inhibition attenuates AIM2 inflammasome activation in vitro Representative Western blot images for co-immunoprecipitation from control and poly(dA:dT)-stimulated THP1 cell lysate. PANX1 is shown as a loading control. Isotype anti-rabbit control was used as negative control.

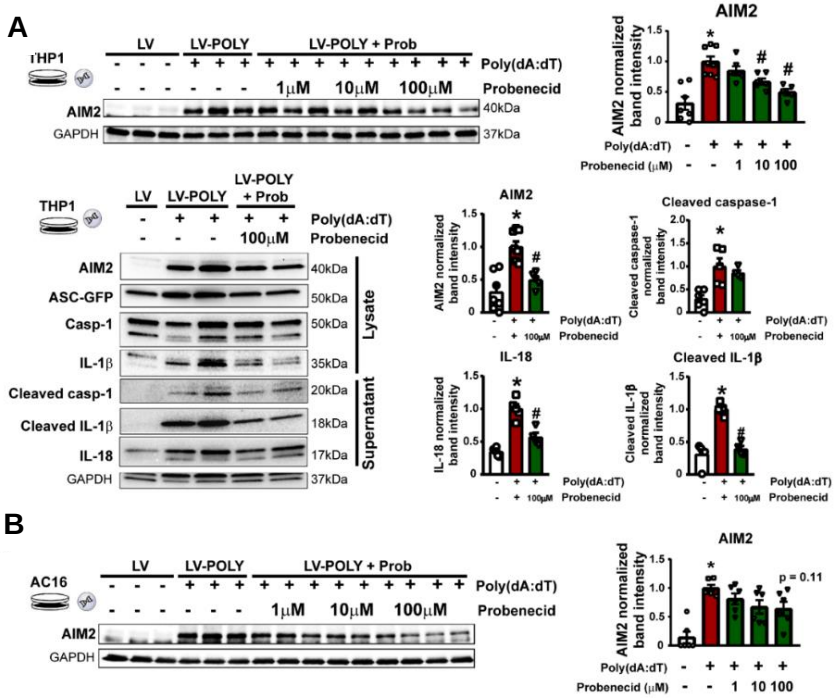

Figure 8 - Pannexin-1 channel inhibition attenuates AIM2 inflammasome activation in vitro (A) Western blot analysis of AIM2 protein expression on poly(dA:dT)-stimulated THP1 cells in the presence or absence of different concentration of probenecid, and detailed analysis of downstream signaling of AIM2 inflammasome activation in cell lysate and supernatant in the presence of 100μM probenecid. *p<0.05 vs control; #p<0.05 vs poly(dA:dT) without probenecid; one-way ANOVA; n=5-6. (B) 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.

3.4. Pannexin-1 channel inhibitor probenecid improves outcome in pressure-overload heart failure model *in vivo*

To test if PANX1 channel inhibition improves cardiac function *in vivo*, we investigated probenecid in the rat HF model induced by pressure-overload

(Fig.9). The rats were orally treated with probenecid (100 mg/kg BW/day) or vehicle (hydroxyethyl cellulose) control. Cardiac function of rats was assessed at 14 weeks after TAC.

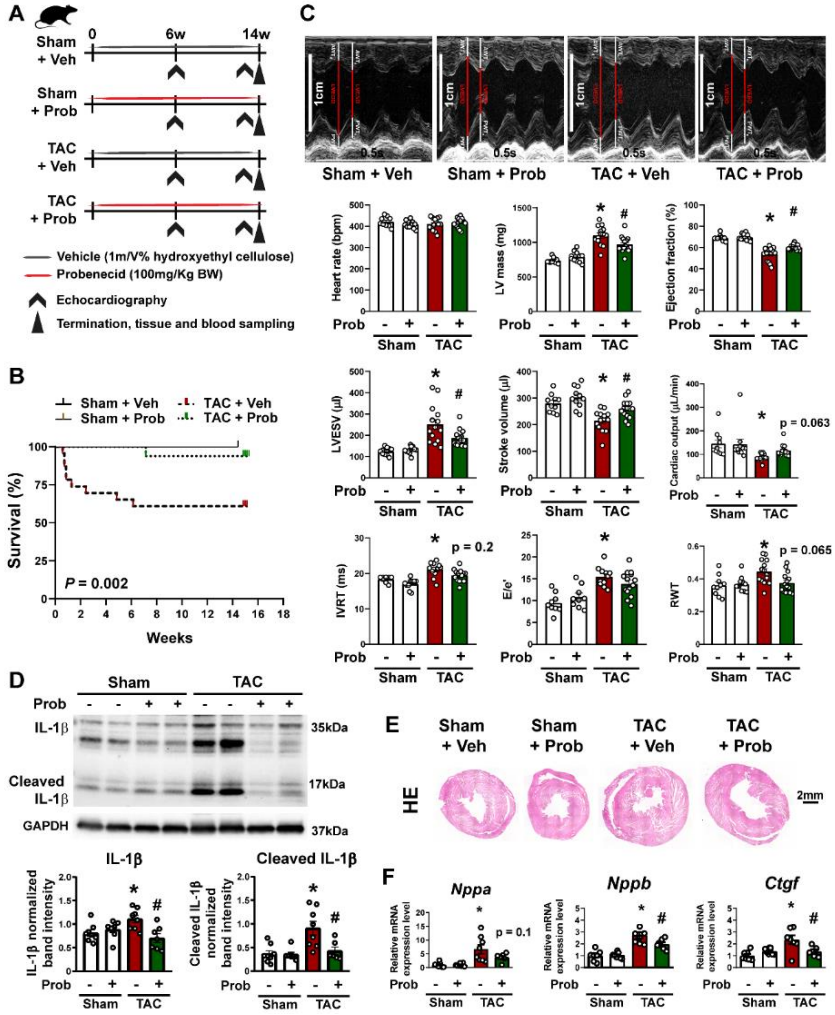

Figure 9 - Pannexin-1 channel inhibitor probenecid improves survival and cardiac function in vivo (A) Study design for investigating the effects of probenecid (Prob) in

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: 1cm; timestamp: 0.5sec. *$p<0.05$ 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 ventricle of heart. *$p<0.05$ vs. Sham + Veh, #$p<0.05$ vs. TAC + Veh; two-way ANOVA; n=6-8. (E) Representative histology images (hematoxylin eosin) at week 14. Scale bar: 2mm. (F) Analysis of mRNA expression of hypertrophy and 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. *(Echocardiography and data analysis were performed by Alex Ali Sayour, Heart and Vascular Center, Semmelweis University, Hungary.*)

The vehicle-treated TAC rats showed an increase in mortality compared to sham. The mortality was reduced among probenecid treated TAC animals compared with vehicle-treated ones in Kaplan–Meier analyses (Fig.9B). At week 14, left ventricular ejection fraction (LVEF) was reduced compared to baseline from $69.2 \pm 1.8\%$ to $54.0 \pm 2.0\%$ and from $69.7 \pm 0.9\%$ to $60.2 \pm 0.6\%$ in rats allocated to vehicle or probenecid treatment groups, respectively (Fig.9C). Probenecid treatment significantly prevented reduction of LVEF in TAC group. In accordance, at 14 weeks after TAC, left ventricular end-systolic volumes increased more in the vehicle group than in the probenecid treated group (Fig.9C). The protein levels of IL-1β and its mature form increased 14 weeks after TAC surgery, which was reduced significantly by probenecid treatment (Fig.9D). In addition, treatment with probenecid prevented development of left ventricular hypertrophy (Fig.9C, E-F). At 14 weeks, the left ventricular mass of vehicle-treated TAC animals was increased but it was reduced by probenecid treatment (Fig.9C, E). This was further confirmed by analysis of pro-hypertrophic genes (*Nppa* and *Nppb*) and the pro-fibrotic factor *Ctgf* that were significantly induced by TAC surgery, and their upregulation was prevented by probenecid (Fig.9F).

4. CONCLUSIONS

Here we have demonstrated with a series of experiments on human failing heart tissues that AIM2 and NLRC4 inflammasome activation play a role in the later stage of chronic HF. It was also shown that monocytes and macrophages are the main scene of AIM2 inflammasome activation. In addition, the investigation on the role of endocannabinoid system in human ischemic cardiomyopathy identified a subgroup within the ischemic specimens that showed increased mRNA expression of the CB1 receptor, and displayed

reduced expression and activity of many hydrolases responsible for the degradation or biosynthesis of endocannabinoids.

Human findings related to changes in the inflammasome expression profile were further confirmed in preclinical animal models such as pressure-overload and postinfarction heart failure rat models; however, lack of AIM2 inflammasome activation in volume-overload model points out the possible disease and stage specificity of the inflammasome expression and activation pattern. Our results highlight the importance of specific inflammation patterns and the involvement of multiple pathways. Thus, we propose development of 'board spectrum' inflammasome inhibitors instead of inflammasome-specific ones.

In this study, AIM2 inflammasome has been found to be associated with pannexin-1 channels by co-immunoprecipitation. We have shown that probenecid, a pannexin-1 channel inhibitor drug, is able to reduce AIM2 inflammasome activation by reducing the expression of AIM2 inflammasome sensor, its downstream signaling and cleavage of effector caspase-1 or IL-1β in both human monocytic and cardiomyocyte lines *in vitro*. In addition, probenecid improves outcomes of heart failure in pressure overload rat model by reducing mortality, improving cardiac function and reversing cardiac remodeling. The recently described anti-inflammatory properties as well as previously published beneficial (e.g. non-injurious positive inotropic) effects on cardiac function may speed up the repurposing of probenecid for the treatment of heart failure.

5. LIST OF OWN PUBLICATIONS

Publications related to the candidate's Ph.D. dissertation

1. **Onódi, Z***, Ruppert, M*, Kucsera, D, Sayour, AA, Tóth, VE, Koncsos, G, Novák, J, Brenner, GB, Makkos, A, Baranyai, T, Giricz, Z, Görbe, A, Leszek, P, Gyöngyösi, M, Horváth, IG, Schulz R, Merkely, B, Ferdinandy, P, Radovits, T and Varga, ZV (2021). "AIM2-driven inflammasome activation in heart failure." Cardiovasc Res. *IF: 10.787 *equal contribution to this study*

2. van Esbroeck, ACM, Varga, ZV, Di, X, van Rooden, EJ, Tóth, VE, **Onódi, Z**, Kuśmierczyk, M, Leszek, P, Ferdinandy, P, Hankemeier, T, van der Stelt, M and Pacher, P (2020). "Activity-based protein profiling of the human failing ischemic heart reveals alterations in hydrolase activities involving the endocannabinoid system." Pharmacol Res 151: 104578. *IF: 7.658*

Publications independent of the candidate's Ph.D. dissertation

3. **Onódi, Z**, Pelyhe, C, Nagy, CT, Brenner, GB, Almási, L, Kittel, Á, Manček-Keber, M, Ferdinandy, P, Buzás, EI and Giricz, Z (2018). "Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography From Blood Plasma." Front Physiol 9: 1479. *IF: 3.201*

4. Baranyai, T, Herczeg, K, **Onódi, Z**, Voszka, I, Módos, K, Marton, N, Nagy, G, Mäger, I, Wood, MJ, El Andaloussi, S, Pálinkás, Z, Kumar, V, Nagy, P, Kittel, Á, Buzás, EI, Ferdinandy, P and Giricz, Z (2015). "Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods." PLoS One 10(12): e0145686. *IF: 3.057*

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