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

PhD thesis

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
ABHD6, 12	α,β-hydrolase domain containing proteins 6 and 12
ABPP	activity-based protein profiling
ACE	angiotensin converting enzyme
AEA	anandamide
Aif1	Allograft inflammatory factor 1
AIM2	absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD
ATTACH	Anti-TNF Therapy Against Congestive Heart Failure trial
AVS	infrarenal arteriovenous shunt
BW	body weight
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcomes Study
CARD	caspase recruitment domain
Casp-1	caspase-1
CB1R, CB2R	cannabinoid receptor type 1 and 2
Ccl2	chemokine (C-C motif) ligand 2
CIRT	Cardiovascular Inflammation Reduction Trial
COLCOT	Colchicine Cardiovascular Outcomes Trial
CON	control
Cor	corrected
COX-1, -2	cyclooxygenase -1, -2
Ctgf	connective tissue growth factor
DAGLα β	diacylglycerol lipase α and β
DAMP, PAMP	danger- or pathogen-associated molecular patterns
DCM	non-ischemic (dilated) cardiomyopathy
D-HART	Diastolic Heart Failure Anakinra Response Trial
dsDNA	double stranded DNA
E/e’	ratio of mitral inflow velocity and mitral annular early diastolic velocity
ECS	endocannabinoid system

ELISA	enzyme-linked immunosorbent assay
ESC	endocannabinoid system
FAAH	fatty acid amide hydrolase
FDA	U.S. Food and Drug Administration
FS	fractional shortening
HCM	hypertrophic cardiomyopathy
HF	heart failure
HIN	hematopoietic interferon-inducible nuclear domain
Iba1	ionized calcium binding adaptor molecule 1
ICM	ischemic cardiomyopathy
IL-18	interleukin-18
IL-1R	interleukin-1 receptor
IL-1R1	IL-1 receptor type 1
IL-1RA	IL-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
IL-1β	interleukin-1 beta
Il-23	interleukin 23
IL-6	interleukin 6
IP	immunoprecipitation
IVRT	isovolumetric relaxation time
IκB	inhibitors of κB
JAK	Janus kinase
LAD	left artery descending (postinfarction rat model)
LoDoCo	Low-dose Colchicine Trial
LV	liposome control
LVAW/PW	left ventricular anterior/posterior wall
LVEDV	left ventricular end diastolic volume
LVEF	left ventricular ejection fraction
LVESV	left ventricular end systolic volume
LV-POLY	poly(dA:dT)/liposome complex
Mgl1	macrophage galactose-type lectin 1
MGLL	monoacylglycerol lipase

Mrc2	macrophage mannose receptor 2
MV	mitral valve
NAPE-PLD	N-acyl phosphatidylethanolamine-specific phospholipase D
NF-κB	nuclear factor kappa B
NLRC4	NLR family CARD domain-containing protein 4
NLRP1/NALP1, -3	NLR family, pyrin domain containing 1 and 3
Nppa	natriuretic peptide A
Nppb	natriuretic peptide B
NRL	nucleotide-binding oligomerization domain-like receptors
NSAID	nonsteroidal anti-inflammatory drugs
P2X7, 4	P2X purinoreceptor 7 and 4
Panx1	pannexin-1 channel
PI3K	phosphoinositide 3-kinase
Poly(dA:dT)	poly(deoxyadenylic-deoxythymidylic) acid sodium salt
Prob	probenecid
PRR	pattern recognition receptor
PYD	pyrin domain
PYHIN	pyrin and HIN200 domain–containing proteins
qRT-PCR	quantitative real-time polymerase chain reaction
REDHART	Recently Decompensated Heart Failure Anakinra Response Trial
RENEWAL	Randomized Etanercept Worldwide Evaluation
ROS	reactive oxygen species
RWT	relative wall thickness
STAT	signal transducer and activator of transcription protein
TAC	transverse aortic constriction
TGF-β	transforming growth factor β
TLR4, 9	Toll-like receptors 4, 9
TNFR1, 2	TNF-α receptor 1, 2
TNF-α	tumor necrosis factor alpha
TRAF2	TNF receptor-associated factor 2

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TRPV2	transient receptor potential cation channel subfamily V member 2
TβRI, II	TGF-β receptors I and II
VCU-ART	Virginia Commonwealth University Anakinra Remodeling Trial
Veh	vehicle
WB	Western blot
ZEUS	Ziltivekimab Cardiovascular Outcomes Study

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1. INTRODUCTION

1.1. Definition of heart failure

Chronic heart failure (HF) is defined as a complex clinical syndrome in which there is exertional limitation due to impaired ventricular filling and/or ejection of blood. HF is caused by a loss of a large quantity of functional myocardium after injury to the heart from various causes. The most common etiologies are ischemic heart disease, hypertension or diabetes mellitus, but also important causes of HF are inherited cardiomyopathies, infections, toxins (e.g. cytotoxic drugs, alcohol) or anatomical anomalies (e.g. valvular heart diseases) (1). These critically ill patients suffer from life threatening clinical symptoms including pulmonary edema, chronic hypoperfusion of brain, liver or kidney, thromboembolic events and cardiac cachexia.

The prevalence of diagnosed HF is estimated at 1-2% of adult population in developed countries, and more than 60 million people are living with this condition worldwide (see for review: (2)). The increasing number of patients reflects on one hand the overall aging of the population, while improved survival from myocardial injuries and cardiovascular diseases, and the epidemic of metabolic comorbidities also contributes to the high prevalence of HF. HF inflicts significant morbidity and mortality that consumes a notable part of healthcare resources; HF patients take on average 4-6 HF-related medications, and majority of them is hospitalized one or two times a year (3, 4).

Pathomechanism behind heart failure including the processes of maladaptive hypertrophy and cardiac remodeling has been investigated extensively. These mechanisms are developed as a response to stress signals by the pathological conditions mentioned to adjust to the impaired cardiac function and to maintain cardiac output and tissue perfusion. These compensatory mechanisms become maladaptive over time, and lead to the uncontrollable activation of renin-angiotensin-aldosterone system and autonomic nervous system. Both neurohormonal alternations and increased sympathetic activity may have profound impact on cardiac structure and function by increasing the secretion of angiotensin II, aldosterone and catecholamines, which promote interstitial fibrosis and cellular apoptosis by their downstream signaling and the secondary release of other mediators (5-7). These pathways are the major targets of currently used pharmaceuticals in the management of HF, and angiotensin-converting enzyme inhibitors, angiotensin receptor and beta adrenergic receptor blockers are recommended as first-line treatment

for HF (8). However, the long-term prognosis of HF is still devastating even in case of proper treatment. Therefore, new effective therapeutic strategies that might improve outcome of HF are needed.

1.2. Inflammation and anti-inflammatory therapies in heart failure

Inflammation consists of a complex cascade of molecular and cellular events, which plays an important role in the pathomechanism of cardiovascular diseases such as atherosclerosis, myocardial infarction, stroke or even heart failure (9-13). It has been also shown that the increased number of circulating leukocytes and the level of monocyte- or macrophage-derived pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), transforming growth factor β (TGF-β) or tumor necrosis factor-α (TNF-α) correlates with a more advanced stage and worse outcomes independent of traditional metrics e.g. left ventricular ejection fraction. Furthermore, this strong association between outcome and inflammation is observed in the groups of HF patients both with reduced or preserved ejection fraction (14-18). The increase in the levels of pro-inflammatory cytokines is considered to be a responsive mechanism which generally provides benefits by mediating cardiac repair; nevertheless, sustained elevation of pro-inflammatory cytokines results in a low-grade inflammation that has been identified as a contributor to the development and progression of cardiac injury (19). The safe intervention by anti-inflammatory drugs remains a challenge; however, results from recent preclinical and clinical studies prove that targeting inflammation may offer a novel approach to reduce risk for cardiac diseases and complications. In the following sections, the role of inflammatory pathways and their potential as therapeutic targets are summarized (Fig.1-2).

1.2.1. Inflammatory mediators in the pathomechanism of heart failure

The innate and adaptive inflammatory responses are important for maintaining normal cardiac tissue homeostasis. The immune system responses to cardiac injury by recognizing specific damage or injury associated molecular patterns (DAMPs). DAMPs are various molecules such as double-stranded DNA (dsDNA) from the nucleus or the mitochondria, RNA or organelle components released from damaged cardiomyocytes or other resident cardiac cells (20-22). DAMP-induced inflammatory response is activated by numerous pattern recognition receptors localized on the cell membrane e.g. Toll-like (TLRs), retinoic acid-inducible gene I-like or oligoadenylate synthase-like receptors and

in the intracellular space e.g. nucleotide-binding oligomerization domain-like receptors (NLRs) (23, 24). The DAMPs contribute to mediate initial immune response via cytokines by inducing chemotaxis, adhesion and migration of monocytes and neutrophils to repair cardiac injury. Although pattern recognition receptors are well-characterized in immune cells, their role in cardiac diseases is still under intensive research.

Immune responses by cytokines are generally mediated by activating their specific receptors and converging to regulate transcription factors from which nuclear factor kappa B (NF-κB) is the best characterized (25). NF-κB normally is inhibited by inhibitors of κB (IκBs). Classically, these inhibitors can be degraded by pro-inflammatory signals e.g. cytokines that activate IκB kinases. The phosphorylation and consequent degradation of IκB free NF-κB that in turn translocate to nucleus. In the non-canonical pathway, different signaling is activated which eventually based on the processing of p100 (a precursor of NF-κB) and activation of p52-Re1B dimer. The activation of NF-κB play a crucial role in the pathomechanism of cardiac remodeling and heart failure by fine modulation of cytokine secretion and genes related to hypertrophy and fibrosis (26-28). Nevertheless, it must be emphasized that NF-κB signaling can induce cardioprotective mechanisms beside its well-characterized harmful effects especially when activated in the early phase of cardiac insult (28-30). This presumably contributes to the mixed outcomes of anti-inflammatory therapies in cardiac diseases (see later in details).

In the following section, we summarize the most relevant data on cytokines concentrating on their role in cardiac diseases in particular heart failure and cardiac remodeling.

Tumor necrosis factor-α

TNF-α is one of the key pro-inflammatory cytokines playing a role in the molecular pathomechanism of HF (31-35). The signaling of TNF-α is mediated through NF-κB pathway by two different receptors (TNF-α receptor 1 and 2, TNFR1/2) which have soluble forms as well. It is considered that the two receptors have divergent effects; thus, the relative expression ratio of these can determine the phenotype in a specific tissue. For instance, TNFR1 is associated with death domain that can induce caspase-mediated apoptosis, while TNFR2 lacks it. Moreover, classical NF-κB pathway is activated by TNFR1, while TNFR2 regulates predominantly via non-canonical pathway. However, crosstalk via TNF receptor-associated factor 2 (TRAF2) between the two types of receptors is a known phenomenon (36). Cardiac presence of TNF-α results in depressed

cardiac function by negative inotropic effects, which is believed to be a consequence of crosstalk between TNF-α signaling and sympathetic activity. According to preclinical studies, TNF-α also alters Ca^{2+} homeostasis, sphingolipid mediators and the function of G protein coupled receptor kinases that may lead to beta adrenergic receptor desensitization (37).

Therefore, the role of TNF-α in cardiac physiology and pathophysiology is highly complicated which led to the failure of clinical translation of its inhibitors for cardiac diseases as discussed later in this dissertation.

Transforming growth factor β

TGF-β is a fibrogenic cytokine contributing to cardiac fibrosis, and its expression level is increased in fibrotic myocardium (38). TGF-β is a universal mediator with diverse effects in cardiac fibrosis by binding to TGF-β receptors (TβRI and TβRII) and activating TGF-β/Smad pathway and increasing the transcription of α-smooth muscle actin (39). TGF-β could induce the transformation of fibroblasts and cardiomyocyte hypertrophy, and increase expression of extracellular matrix proteins and secretion of other cytokines (40, 41). TGF-β receptors are expressed on a large population of immune cells such as monocytes, macrophages and mast cells (42). TGF-β can regulate immune function in multiple manner; thus, TGF-β may induce the secretion of profibrotic cytokines as well as suppress the release of these signals in specific microenvironment (43-45). These results suggest that early and moderate activation of TGF-β signaling might be beneficial; however, chronic overactivation is rather detrimental in cardiovascular diseases.

Interleukin-6

IL-6 was shown to be a pleiotropic as well as two-faced mediator in heart failure with wide array of functions through JAK-STAT and phosphoinositide 3-kinase (PI3K) pathways (46, 47). Acute secretion of IL-6 was demonstrated to be cardioprotective in particular during ischemia-reperfusion injury (48), while chronic elevation of IL-6 is a maladaptive response to cardiac insult (47). IL-6 promotes cardiac fibrosis and hypertrophy alone, but it is also critical in angiotensin-induced fibrosis and atherosclerosis (49, 50). In line with that, elevated levels of circulating IL-6 in heart failure patients were observed in clinical studies (10, 31, 51, 52). In contrast, other preclinical studies found that deletion of IL-6 is not sufficient to prevent fibrosis; moreover, lack of IL-6 was associated with cardiac dysfunction (53). Thus, further

preclinical and clinical studies are needed to clarify the role of IL-6 signaling in the progression of heart failure.

Interleukin-1 superfamily

IL-1 superfamily consists of a few cytokines such as IL-1α, IL-1β, IL-18 or IL-33 playing central role in the regulation of immune function. The members of this family share some similar biological features including being secreted as an inactive precursor and requiring further maturation by proteolytic cleavage to become active.

IL-1β is one of the most intensively investigated among the known cytokines that associates with a large number of acute and chronic inflammatory diseases. The downstream signaling of IL-1β and its close relative, interleukin-1α is mediated by IL-1 receptor type 1 (IL-1R1), a cell membrane receptor and its accessory protein (IL-1RAcP) which is recruited by IL-1R1 bound its ligand. The IL-1 receptor type 2 is a decoy receptor without significant downstream signaling; thus, it acts as a suppressor of IL-1 pathway. Another endogenous suppressor is IL-1 receptor antagonist (IL-1RA) that inhibits IL-1R1 and IL-1RAcP transactivation preventing the activation of signaling pathway. Activation of IL-1R1 initiates phosphorylation of several interleukin-1 receptor activated kinases, which eventually leads to the modulation of NF-κB pathway (54).

IL-1β has diverse effects on myocardium leading to impaired cardiac function. IL-1β was shown to decrease responsiveness to sympathetic stimuli, attenuate the expression of important genes of Ca^{2+} homeostasis and inhibit mitochondrial functions (55-57). The impact on Ca^{2+} handling may increase the risk of arrhythmias as well (12). IL-1β also promote cardiac remodeling, atherosclerosis and vascular dysfunction by activating TGF-β signaling and enhancing activity of matrix metalloproteases (58-60). Eventually, activation of IL-1β signaling enhances the secretion of pro-inflammatory cytokines such as IL-6, TNF-α or even IL-1β itself. These findings may facilitate the intensive investigations to translate inhibitors of IL-1 pathway into clinical practice in the management of cardiac diseases.

IL-18 is another member of IL-1 superfamily, and it shares signaling pathways and biological effects similar to IL-1β on cardiac system (61). IL-18 signaling is associated with diet-induced cardiac dysfunction in a mouse model of Western type diet by contributing to systemic inflammation (62). It has been also demonstrated that the deleterious cardiac effects of IL-1β may be partially IL-18-dependent or the cytokines

can act synergistically (63). Treatment with IL-18 neutralizing antibody or recombinant IL-18 binding protein, which is an endogenous inhibitor of IL-18, might be promising as therapeutic strategies; however, there is a general lack of preclinical and clinical studies in relation to cardiac diseases. Additionally, the controversial correlation between cardiac function, the prognosis of HF and circulating level of IL-18 indicate that IL-18 might play a mixed role in the modulation of HF-related inflammation that needs to be clarified in the future (64).

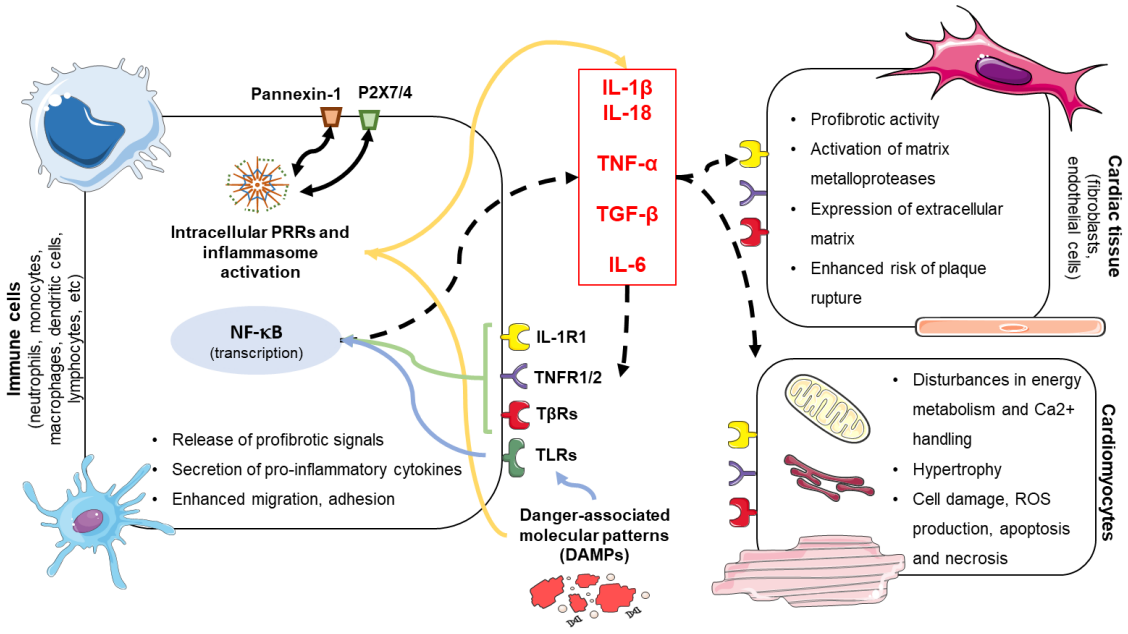

Figure 1 – Summary of cytokine-mediated inflammatory pathways and their effects in cardiac diseases. Danger-associated molecular patterns (DAMPs) as well as pro-inflammatory cytokines released after cardiac insult can modulate the activity of nuclear factor kappa B (NF-κB) and related pathways. NF-κB activity regulates the secretion of profibrotic, pro-hypertrophic and pro-inflammatory cytokines at transcriptional level. The activation of precursor IL-1β and IL-18 is an inflammasome-dependent process, which is induced by sensing intracellular DAMPs with intracellular pattern recognition receptors (PRR). Inflammasome activation may be modulated by cell ion channels including purinergic receptors and pannexin channels. The cytokines act on cardiomyocytes, non-immune cells of cardiac tissue e.g. fibroblasts and endothelial cells and immune cells in paracrine or autocrine manners that results in cardiac remodeling and chronic inflammation. Abbreviations: PRR – pattern recognition receptor, IL-1R1 – interleukin-1 receptor 1, TNFR – tumor necrosis factor receptor, TβR – TGF-β receptor, TLR – Toll-like receptor, P2X4/7 – purinergic receptor 2X 4 and 7, IL-1β/18/6 – interleukin-1β, -18 and -6, TNF-α – tumor necrosis factor-α, TGF-β – transforming growth factor-β, ROS – reactive oxygen species. *(Summary figure was prepared according to references cited in the main text.)*

1.2.2. Inflammasome activation - a key source of active IL-1β

IL-1β is secreted by immune cells as a part of the inflammatory reaction and acts both via autocrine and paracrine manner. IL-1β activity is strictly regulated, as it requires the conversion of the inactive protein, the IL-1β precursor or pro-IL-1β, to the cleaved or active cytokine by proteolysis. A set of endogenous signals including the stimulation of TLRs, TNFRs or even interleukin-1 receptor itself induces the synthesis of the inactive IL-1β precursor via NF-κB and related pathways (30, 65). This process, when precursor IL-1β and inflammasome components are accumulated to create a reserve waiting for further processing, is called “priming”.

The maturation and release of IL-1β is controlled by inflammasomes, which are cytoplasmic multiprotein complexes comprising a sensor protein, inflammatory caspases, and adapter proteins in some cases (Fig.2) (66, 67). Inflammasomes emerged in the last decade to constitute essential processing units contributing to DAMP sensing (24). Inflammasome-related pattern recognition receptors (PRR) are expressed in numerous cell types such as monocytes, macrophages, neutrophils or even in epithelial cells (68). The assembly of inflammasomes is localized in the cytoplasm of immune cells leading to proteolytic activation of caspases and interleukins, which drives the immune responses. Although inflammasome activation has been shown to be vital to host defense, the activation process needs to be strictly regulated to limit collateral damage to the host itself.

A few cytoplasmic PRRs can assemble into the inflammasome complex. These are categorized on the basis of protein domain structures (69). The NOD leucine-rich repeat-containing receptor (NLR) family consists of subfamilies by N-terminal effector domains: the acidic transactivation domain, pyrin domain (PYD), caspase recruitment domain (CARD), and baculoviral inhibitory repeat-like domains (NLRA, NLRP, NLRC and NLRB, respectively). Another class of PRR is represented by PYHIN protein family members, such as absent in melanoma 2 (AIM2) containing hematopoietic interferon-inducible nuclear domain 200 and PYD.

The different inflammasomes converge onto the common signaling pathway, i.e. caspase activation. Inflammasome sensors or so-called scaffold proteins (NLRPs or AIM2) assemble to oligomer complexes, which are sometimes completed with adaptor and effector proteins such as apoptosis-associated speck-like protein containing a CARD

(ASC). The activated caspases (usually caspase-1 in case of canonical inflammasome activation) cleave pro-inflammatory cytokines IL-1β, IL-18 and pore-forming protein gasdermin D (70). The pore-forming activity of gasdermin D is essential for releasing cytokines and mediating a highly inflammatory form of programmed cell death called pyroptosis (71). In case of non-canonical inflammasome activation, caspase-11 in mice or its human orthologues caspase-4/5 can initiate pyroptosis and interleukin cleavage by sensing intracellular pathogens and molecular patterns e.g. lipopolysaccharide via TLR-independent manner (72).

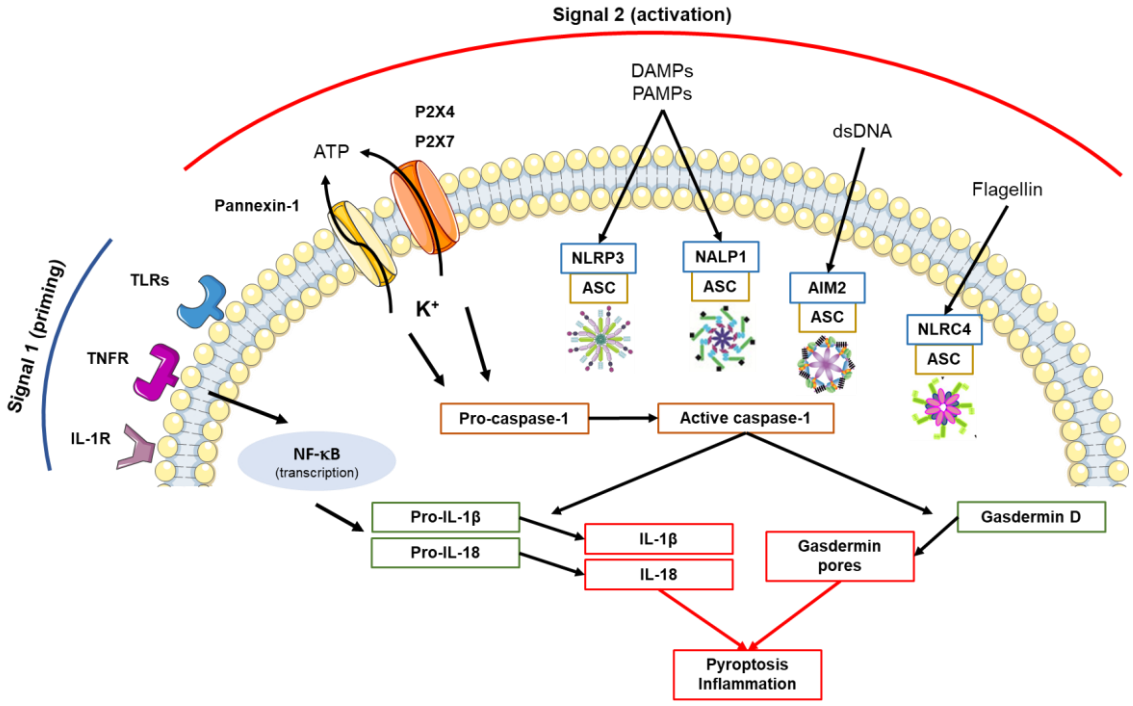

Figure 2 – Inflammasome signaling – priming and activation. The transcription of precursor interleukins ("priming") is modulated by different receptors via nuclear factor kappa B (NF-κB) and related pathways. Specific signals such as danger- of pathogen-associated molecular patterns (DAMP, PAMP) induce the assembly of inflammasome complex leading to caspase activation and cleavage of precursor interleukins and/or gasdermin pore-forming ("activation"). Abbreviations: IL-1R – interleukin-1 receptor, TNFR – tumor necrosis factor receptor, TLR – Toll-like receptor, P2X4/7 – purinergic receptor 2X 4 and 7, dsDNA – double stranded DNA, IL-1β/18 – interleukin-1β and -18. *(Summary figure was prepared according to references cited in the main text.)*

In the past decade, researchers have focused on the role of inflammasomes, in particular NLRP3, in large number of diseases including some common cardiovascular conditions (73, 74). According to preclinical studies, NLRP3 inflammasome activation is an important player in early fibrosis, cardiac remodeling and ventricular dysfunction after

ischemic injury which can be attenuated by NLRP3 inhibition (75-78). Additionally, the NLRP3 inflammasome also contributes to the pathogenesis and progression of cardiac dysfunction induced by early pressure-overload, infection, angiotensin II-related hypertrophy, obesity or aging (79-83). NLRP3 inflammasome activation has been shown to be localized in immune cells; nevertheless, there are controversial reports showing that cardiomyocytes and fibroblasts may be capable of expressing inflammasome components and even to undergo pyroptotic cell death (75, 76, 79).

In addition to the best characterized NLRP3 inflammasome activation, other inflammasome pathways have been identified playing a role in cardiovascular diseases as well. According to recent reports, NLRP1 and NLRC4 are associated with cardiac diseases such as stroke or cardiomyopathies (11, 84)**.** Non-NLR type inflammasome sensor AIM2, which senses cytoplasmic dsDNA, has been proven to participate in inflammatory responses after cardiac events as well. The AIM2 inflammasome has shown to display enhanced activity in response to acute myocardial infarction, diabetic cardiomyopathy, stroke and atherosclerosis in various animal models (11, 20, 21, 85-87). In summary, these promising data from basic and preclinical research facilitate the translation of various anti-inflammatory strategies into clinical practice in the management of chronic cardiovascular conditions.

1.2.3. Anti-inflammatory therapies in cardiac diseases

A large set of studies were published studying anti-inflammatory therapies in HF. Preclinical studies have demonstrated the beneficial effects of targeting inflammatory pathways in various models for atherosclerosis, myocardial infarction, stroke or HF. Despite the promising preclinical findings, clinical studies have provided disappointing and conflicting results so far (Table 1). The literature on using anti-inflammatory drugs in cardiovascular diseases are revised in the following section.

Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs), including diclofenac, naproxen, ibuprofen and others inhibit prostaglandin synthesis by blocking cyclooxygenase-1 and -2 (COX-1, -2) enzymes leading to reduction of inflammation, fever and pain. Both non-selective and COX-2-selective NSAIDs aside from low-dose aspirin, which is widely used as secondary prevention of acute coronary syndromes and stroke (88, 89), are

associated with increased cardiovascular risk particularly with major cardiac events such as myocardial infarction (90). The use of NSAIDs is not recommended after acute coronary syndromes for at least 3-6 months (91). NSAIDs may increase the risk for developing heart failure and its complications even without the history of previous cardiac events (90, 92). Interestingly, ibuprofen and some other NSAIDs can still be used in the management of pericarditis (93).

Glucocorticoids

Glucocorticoids are released from the adrenal cortex cyclically and in response to stress signals. These hormones act on intracellular glucocorticoid receptors which are expressed nearly on all tissues in the human body. Thus, the effects of glucocorticoids are highly variable depending on the cell and tissue context. In the cardiovascular system, glucocorticoids have significant impact on the development and maturation of cardiac myocytes (94, 95). In pathological conditions, glucocorticoids may increase contractility and cardiomyocyte survival (96). However, chronic glucocorticoid administration with persistently high levels may lead to the increased risk of various systemic side effects such as diabetes mellitus, hyperlipidemia or hypertension which are the major risk factors of cardiovascular events. Moreover, high dose glucocorticoids can act on mineralocorticoid receptors as well; the stimulation of which promotes fibrosis and cardiac remodeling (97). Therefore, clinical translation of anti-inflammatory therapy by glucocorticoids is limited due to diverse side effects which exceed the potential benefits.

TNF-α inhibitors

TNF-α inhibitors, a group of biologic agents in the treatment of inflammatory diseases, arose as innovative therapeutic agents in HF after observations that TNF-α may play a role in impaired cardiac pump function and cardiac remodeling (98-100). Despite the numerous promising preclinical results (32, 33, 101), Randomized Etanercept Worldwide Evaluation (RENEWAL) study revealed that etanercept did not improve outcomes at primary endpoints; moreover, it suggested that etanercept tended to increase HF hospitalization resulting in early termination of similar trials (35). Anti-TNF Therapy Against Congestive Heart Failure trial (ATTACH) confirmed these results (102). In accordance with the findings of RENEWAL, not only that TNF-α inhibitors were unable to improve outcomes but high dose infliximab (10mg/body weight kg) significantly increased all-cause mortality and HF-related hospitalization. This double-edged effect of

TNF-α may be explained by its complex role in cardiovascular diseases; TNF-α acts via NF-κB pathway which also participate in cardioprotection by reducing mitochondrial dysfunction, cell damage and reactive oxygen species activation (103).

Methotrexate

Methotrexate is an immunosuppressant drug, which is widely used to treat proliferative, inflammatory and autoimmune diseases. As an antimetabolite it can inhibit tetrahydrofolate synthesis competitively by blocking dihydrofolate reductase enzyme. Additionally, it was also reported that methotrexate can inhibit IL-1β and IL-6 signaling (104, 105). Previous observations on patients with rheumatoid arthritis and other inflammatory diseases revealed that methotrexate treatment reduces cardiovascular events compared to other therapies (106, 107). Therefore, a large prospective randomized study (Cardiovascular Inflammation Reduction Trial, CIRT) was conducted to evaluate the efficacy of low-dose methotrexate in the prevention of cardiovascular events among patients with history of atherosclerosis (108). CIRT showed no benefits of methotrexate in the prevention of cardiovascular events or HF hospitalization. Interestingly, methotrexate was not capable of reducing the serum levels of IL-1β and IL-6, indicating a distinct mechanism behind the original observation among rheumatologic patients.

IL-1 inhibitors

As described in the previous section, the role of IL-1β in cardiac diseases has been demonstrated before. Thus, targeting IL-1β and its signaling pathway by different biologic agents such as anakinra (a recombinant human analogue of IL-1RA) or canakinumab (a fully human monoclonal antibody against IL-1β) in the prevention and management of cardiovascular disease gained interest for many years. Previous clinical observations on small numbers of rheumatologic patients provided promising evidences on the efficacy of blocking IL-1β as therapeutic strategy for cardiac conditions (109).

Pilot studies with anakinra (Virginia Commonwealth University Anakinra Remodeling Trial, VCU-ART and VCU-ART2) suggested mixed effects of anakinra; anakinra did not prevent new cardiac events but tended to decrease the risk of novel onset HF (110). Other clinical trials with anakinra e.g. Diastolic Heart Failure Anakinra Response Trials (D-HART and D-HART2) and Recently Decompensated Heart Failure Anakinra Response Trial (REDHART) had similar results; there were slight improvements in some of the primary endpoints (111, 112). Furthermore, anakinra was well-tolerated in these studies.

The phase II study on the efficacy of canakinumab (Canakinumab. Anti-Inflammatory Thrombosis Outcome Study, CANTOS) included 10,061 patients with history of myocardial infarction and serum C-reactive protein level ≥ 2mg/L (113). Blockade of IL-1β with canakinumab (single dose of 150mg in every 3 months) was capable of reducing the incidence of major cardiovascular events such as myocardial infarction, stroke and cardiovascular related death as well as the hospitalization for HF exacerbation. However, the initial optimism about canakinumab faded quickly. Canakinumab increased the risk of severe infections; thus, all-cause mortality was not reduced presumably due to increased fatality caused by infections. Ultimately, U.S. Food and Drug Administration (FDA) rejected canakinumab in cardiac indications.

Of note, the use of rilonacept, a chimeric fusion protein of IL-1R1 and IL-1RAcP acting as a decoy receptor for the members of IL-1 superfamily, and gevokizumab, another monoclonal antibody against IL-1β, was associated with unfavorable U-shaped dose-response curve and discouraging real-life experiences leading to the premature termination of their testing in cardiovascular indications (114).

In addition to direct blockers of IL-1β, the inhibitors of IL-1β secretion and activation may represent a new innovative way of anti-inflammatory therapy. NLRP3 inflammasome inhibitors such as inzomelid (developed by Roche) or dapansutrile (OLT1177) are intensively investigated for the use of inflammatory indications. Dapansutrile has been tested for acute myocardial infarction and heart failure in phase 1 (77). However, these results are not reported yet (115).

Colchicine

Colchicine is an anti-inflammatory drug which is extensively used in the management of acute attacks of gout. It can act through various mechanisms of action. It inhibits tubulin polymerization and microtubule generation presumably resulting in decreased migration and adhesion of immune cells, but also has significant effect on cytokine production and downstream signaling (116, 117).

There are now clinical data available, that suggests that low dose (0.5mg) colchicine may reduce cardiovascular risk after ischemic events according to low-dose colchicine trials (LoDoCo and LoDoCo2) (118, 119) and Colchicine Cardiovascular Outcomes Trial (COLCOT) (120). Of note, non-cardiovascular mortality increased tendentiously in colchicine-treated groups in LoDoCo2 which phenomenon was not observed in

COLCOT. Still, the mixed tolerability, controversial outcomes and long-term effects of colchicine in trials require further investigations.

IL-6 inhibitors

IL-6 inhibitors e.g. tocilizumab or sarilumab are essential biological agents in the management of inflammatory diseases. As it was mentioned before, elevated serum IL-6 has been observed in high-risk cardiovascular patients. The beneficial results of CANTOS study in prevention of atherosclerotic complications were associated with not only the reduced level of IL-1β but IL-6; furthermore, the residual inflammatory risk has shown a stronger relation to serum IL-6 than serum IL-18 (121).

Population studies suggest that anti-IL-6 therapies such as tocilizumab are safe in high-risk cardiovascular patients with rheumatic diseases, and these can decrease the levels of circulating inflammatory markers; however, tocilizumab has been shown to induce disturbances in lipid homeostasis, which may be disadvantageous in the prevention of cardiac diseases (122, 123). Nevertheless, innovative new IL-6 inhibitor ziltivekimab has been shown to be effective for reducing inflammatory markers in patients with high serum level of high-sensitivity C-reactive protein and chronic kidney disease in phase II trial RESCUE without significant adverse events (50). Therefore, new clinical trial called Ziltivekimab Cardiovascular Outcomes Study (ZEUS) has been scheduled to 2021 in order to investigate the efficacy of IL-6 inhibition in the prevention of cardiovascular events (124).

Table 1 – Summary of anti-inflammatory therapies in cardiovascular diseases.

Drug	Clinical trials	Outcome, results	References
Nonsteroidal anti-inflammatory drugs	metanalyses on safety	cardiovascular risk↑	(90)
Glucocorticoids	QUEST-RA	controversial; strongly depending on dose and patient population	(107)
Tumor necrosis factor-α inhibitors	RENEWAL ATTACH	cardiovascular risk ~ or ↑	(35, 102)
Methotrexate	CIRT	no improvement at end points	(108)
Interleukin-1β inhibitors	VCU-ART D-HART REDHART CANTOS	anakinra: no significant improvement at primary end points canakinumab: cardiovascular mortality and risk↓, but overall mortality ~	(110-113)

Colchicine	LoDoCo COLCOT	cardiovascular risk↓ non-cardiovascular mortality ↑ in LoDoCo2	(119, 120)
Interleukin-6 inhibitors	RESCUE ZEUS (ongoing in 2021)	tocilizumab, sarilumab: cardiovascular events ~, inflammatory markers↓, but lipid profile disturbances ziltivekimab: inflammatory and thrombotic biomarkers in patients with chronic kidney disease↓	(50, 124)

1.3. The endocannabinoid system and its role in cardiovascular diseases

The endocannabinoid system (ECS) has been proven as a modulator of the cardiovascular system and inflammation under certain conditions in particular in obese and atherosclerotic patients (125). The cannabinoid receptors and their lipid-derived ligands, the endocannabinoids, receive attraction for being promising therapeutic targets for numerous diseases; however, the clinical translation of newly developed ECS modulators has encompassed challenges mostly due to serious adverse effects. For instance, cannabinoid receptor antagonist rimonabant, approved in 2006 for the management of obesity and its complications, was withdrawn two years later due to serious psychiatric side effects despite its clear effectiveness in its indication (126). In 2016, phase 1 trial on the promising anxiolytic and analgesic medication BIA 10-2474, which is an inhibitor of endocannabinoid degrading fatty acid amide hydrolase (FAAH), resulted in fatal outcome: one volunteer died, and others suffered permanent brain damage (127, 128). These unexpected adverse events and fails have pointed out the urgent need for broadening the knowledge on ECS in physiological and pathological processes.

1.3.1. The endocannabinoid system: endocannabinoids, receptors and enzymes

The ECS consists of cannabinoid receptors, the endocannabinoids, their synthetic or catabolic enzymes and transport proteins. The most significant and characterized ECS components, i.e. the two main types of cannabinoid receptors, endocannabinoids and related enzymes are reviewed in the following section.

Cannabinoid receptors

The cannabinoid receptor type 1 and 2 (CB_1R and CB_2R) are the products of two different genes (*CNR1* and *CNR2*, respectively). These G protein-coupled receptors are expressed

in various types of organs and tissues, and they are involved in several physiological and pathological processes (129).

CB_1R is abundant in the central and the peripheral nervous systems, and it is responsible for the psychotropic effects of cannabis and its constituents as well as for the effects on appetite and energy balance. CB_1R is also expressed in other organs and tissues in low-to-moderate level. CB_1R in gastrointestinal tract and enteral nerves regulates motility, enteroendocrine and barrier functions (130). On the other hand, CB_1R can be upregulated in pathological conditions e.g. in fibrosis, ischemia or even insulin resistance which are mainly associated with elevated CB_1R level in the liver or the heart (131, 132).

In contrast to CB_1R, CB_2R shows a higher expression on immune cells and peripheral organs, while much lower level can be detected in the central nervous system mostly on resident immune cells (133). By the high abundance on immune cells, CB_2R has a wide variety of immunomodulatory function. A series of papers reported both anti- and pro-inflammatory effects of CB_2R induced by endocannabinoids (134). Interestingly, selective exogenous agonists of CB_2R show predominantly anti-inflammatory than pro-inflammatory effects. The activation of CB_2R attenuates leukocyte migration and the secretion of pro-inflammatory cytokines such as TNF-α, IL-6 or IL-12, but increases the level of anti-inflammatory cytokine IL-10. In line with the aforementioned results, despite the low expression level in nervous system CB_2R still plays an important role in neurological disorders particularly if inflammation is present (135).

Thus, modulating CB_1R- or CB_2R-mediated signaling selectively are believed promising approaches in the treatment of different diseases; however, the issue of adverse events still needs to be explored in the future.

Endocannabinoids and their metabolism

The most investigated players of ECS are the phospholipid-derived endocannabinoid 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (also known as anandamide; AEA) which are widely expressed throughout the human body. The properties of the two endocannabinoids to cannabinoid receptors are highly diverse; AEA is a high-affinity partial agonist of CB_1R, but nearly inactive on CB_2R, while 2-AG can act as full agonist on both receptors with moderate affinity. Additionally, both ligands can modulate other receptors e.g. subtypes of transient receptor potential channels and G protein coupled receptors.

As the endocannabinoids are secreted on demand, the tissue level of endocannabinoids is strongly dependent on the balance between biosynthesis and degradation by specific enzymes (Fig.3). The 2-AG is produced from diacylglycerol (DAG), by the activity of diacylglycerol lipase α and β (DAGLα/β) enzymes. The AEA with other N-acyl ethanolamines is released from N-acyl-phosphatidylethanolamine (NAPE) by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) enzyme. The degradation of 2-AG and AEA are mediated by hydrolases; AEA is catabolized through the activity of FAAH, while 2-AG is hydrolyzed by several enzymes including monoacylglycerol lipase (MGLL), α,β-hydrolase domain containing proteins 6 and 12 (ABHD6, ABHD12). Both endocannabinoids are hydrolyzed to arachidonic acid (AA) and serve as substrates of AA-metabolizing enzymes such as COX-2 or lipoxygenases (136, 137).

1.3.2. Endocannabinoid system in cardiac physiology and pathology

Endocannabinoids have been implicated in the regulation of vascular tone, atherogenesis, oxidative stress, inflammation, fibrosis and cell death in cardiovascular system (Fig.3). These effects can be mediated mainly by cannabinoid receptors directly through the myocardium, vascular structure or circulating blood cells as well as indirectly through the nervous system (138-141).

CB_1R has been shown to be 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 (142, 143). In accordance with these observations, CB_1R antagonists may improve cardiac function when administered acutely, and may also attenuate pathological processes upon chronic treatment (144). CB_1R activation promotes atherosclerosis and inflammation independently from the lipid profile, and inhibition of CB_1R is suggested to be antiatherogenic. Furthermore, elevated expression of CB_1R was detected in patients with ischemic heart disease (145, 146). CB_1R may also play a role in remodeling, fibrosis and tissue damage as receptor inhibitor rimonabant mitigated these effects (144, 147, 148).

In opposition to the main effects of CB_1R, the selective agonists of CB_2R might be cardioprotective by reducing inflammatory signaling and fibrosis. Endothelial CB_2R agonist attenuates the release of TNF-α and chemokines that leads to decreased leukocyte migration (149, 150). In addition, there are evidences on the role of CB_2R in the

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prevention of atherosclerosis and related inflammation (151, 152). Interestingly, CB_2R activation has no direct effects on cardiac function (153). Despite the accumulating evidences of the beneficial effects of CB_2R activation in cardiovascular system, the role of this receptor is still under investigation.

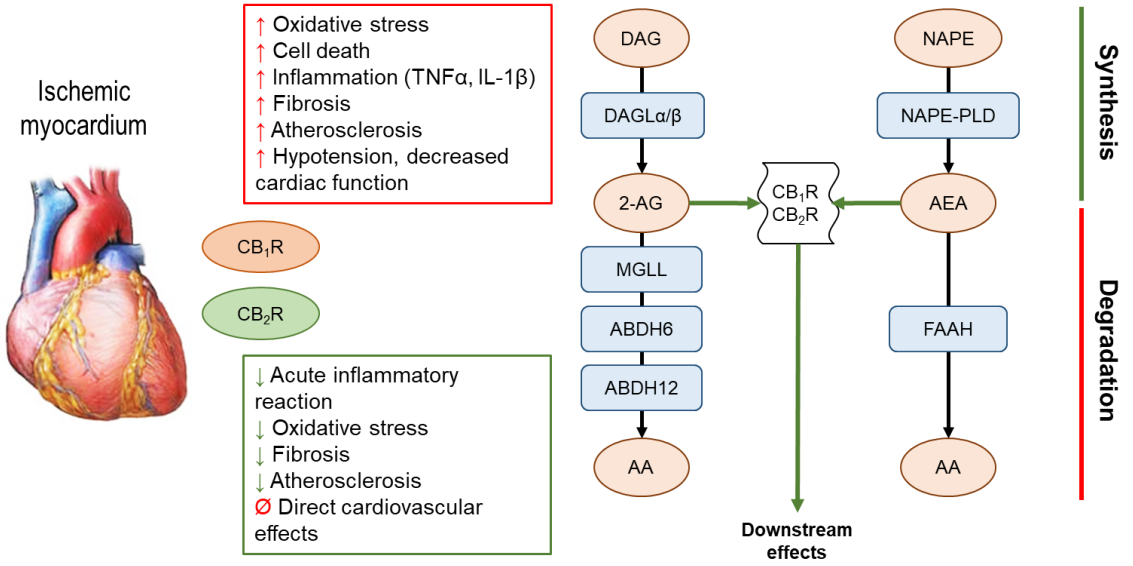

Figure 3 – The endocannabinoids and their receptors in ischemia-related cardiac diseases. The endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide (AEA) act through cannabinoid receptor type 1 and 2 (CB1R, CB2R) with opposing effects; CB1R facilitates inflammation, damage, atherosclerosis and fibrosis, while CB2R attenuates these harmful effects. The endocannabinoids are secreted on demand and degraded rapidly mediated by synthetic enzymes and hydrolases (see section 1.3.1. for details). *(Summary figure was prepared according to references cited in the main text.)*

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2. OBJECTIVES

The number of preclinical studies is increasing quickly on the role of inflammation and inflammatory pathways in cardiovascular diseases; however, the clinical translation of anti-inflammatory agents and therapies is still controversial. The clinical ineffectiveness or unexpected adverse effects have pointed out the importance of detailed knowledge on the complexity and connections between signaling pathways and disease stages as well as the critical differences between animal models and real-life human observations. The enthusiastic efforts for developing new drugs with unique mechanisms of action e.g. NLRP3-selective or endocannabinoid degrading-enzyme inhibitors arises the urgent need for investigating the role of these pathways in cardiovascular diseases and in human samples.

Therefore, the major objectives of this work were:

1. First, to investigate the inflammasome activation and endocannabinoid-related signaling pathways in human failing hearts.

2. To find a suitable translational animal models for examining relevant inflammasome activation in heart failure.

3. To test the potential anti-inflammatory effects of pannexin-1 (Panx1) channel inhibitor probenecid *in vitro* and *in vivo*.

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3. RESULTS

3.1. Inflammation and endocannabinoid system in human heart failure

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

To investigate inflammasome activation in the late-stage of HF, expression of well-characterized inflammasome sensors (NLRP3, NLRC4, AIM2 and NALP1) were detected by Western blot in left ventricular tissue harvested from healthy donor patients (CON, n=12) as well as from HF patients with ischemic (ICM, n=12) or non-ischemic (DCM, n=11) cardiomyopathy. Patient characteristics are summarized in Table 2.

Table 2 - Patient characteristics. Data are expressed as mean and range. *p<0.05 vs CON; #p<0.05 vs DCM, †p<0.05 vs ICM. Abbreviations: CON – control, ICM – ischemic cardiomyopathy, DCM – dilated cardiomyopathy, HCM – hypertrophic cardiomyopathy, ACE – angiotensin converting enzyme. (154)

Parameter	CON	ICM	DCM	HCM
Gender (male/female)	6/6	11/1	10/1	2/3
Age (years)	32.6 (17-52)	57.8 (38-67)*#	39.1 (23-53)	49.6 (33-66)
Body mass index (kg/m^2)	24.0 (18-31)	26.9 (20-32)	24.9 (20-31)	22.3 (13-30)
Left ventricular end-diastolic dimension (mm)		72.3 (64-82)	75.4 (57-92)	57.0 (38-70)
Left ventricular end-systolic dimension (mm)		65.4 (58-75)	67.3 (51-85)	43.5 (43-44)
Ejection fraction (%)	> 60	20.7 (10-33)	17.0 (10-25)	34.4 (13-70)
Posterior wall (mm)		9.0 (6-11)	10 (8-12)	9.5 (7-12)
Septal thickness (mm)		8.6 (6-13)	9.9 (7-12)	13.2 (8-16)
Pulmonary artery diameter (mm)		27.0 (22-34)	29.5 (22-34)	20.3 (19-22)
Right atrial pressure (mmHg)		9.9 (1-21)	15.9 (5-31)†	9.4 (4-19)
Mean pulmonary wedge pressure (mmHg)		25 (7-45)	21 (12-31)	15 (7-22)
Pulmonary vascular resistance (wood units)		1.7 (0.6-2.2)	2.4 (0.7-6.6)	2.0 (0.6-4.1)
Cardiac index (L/min/m^2)		2.2 (1.3-3.9)	2.1 (1.4-3.0)	2.2 (1.4-3.0)
Heart rate (/min)	103 (70-125)	79 (70-95)	104 (99-110)	60 (50-70)
Systolic arterial blood pressure (mmHg)	123 (90-150)	104 (76-135)	99 (80-115)	103 (80-141)
Diastolic arterial blood pressure (mmHg)	74 (50-90)	56 (15-72)	62 (31-77)	54 (31-70)
N-terminal pro-BNP (pg/mL)		2798 (338-7699)	7796 (1050-25539)	12163 (4159-26284)
Medications				
ACE inhibitors	0	10	8	4

Beta receptor blockers	0	12	11	5
Digoxin	0	3	3	1
Ivabradine	0	1	3	0
Diuretics	0	12	11	5
Mineralocorticoid receptor inhibitor	0	12	11	4
Statins	0	12	3	2
Antiplatelets	0	8	1	1

As it can be seen in Figure 4, there was no difference in NLRP3 protein expression in the HF groups compared to control. In contrast, the expression of AIM2 remarkably 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.4A-B).

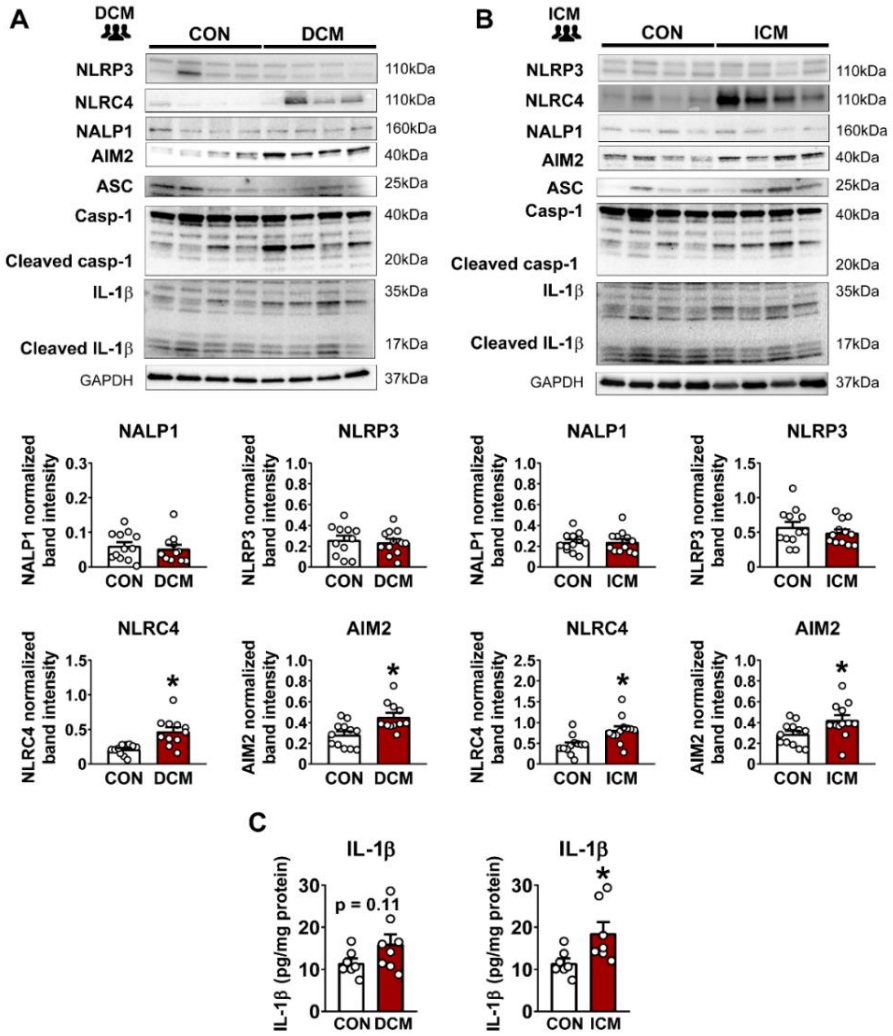

Figure 4 - AIM2 and NLRC4 are the major inflammasome components expressed in human failing hearts. Western blot analysis of the inflammasome sensors (NLRP3, AIM2, NLRC4 and NALP1) and downstream signaling (ASC, caspase-1, IL-1β) in left ventricle of patients with dilated (DCM, A) or

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. (154)

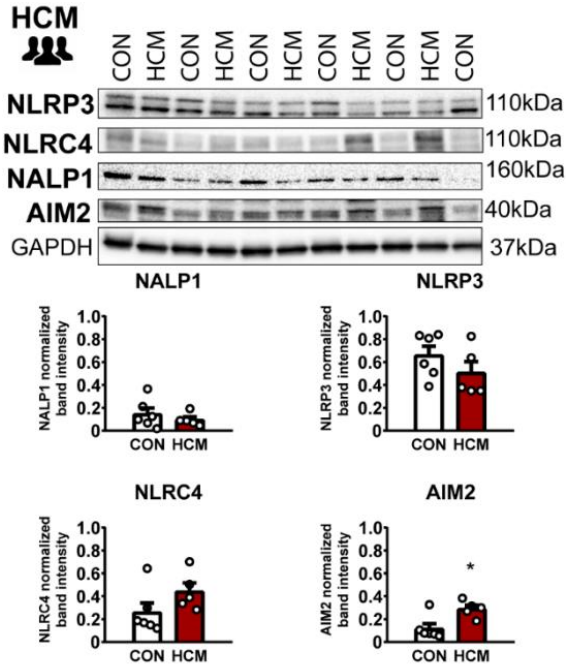

Figure 5 - AIM2 is upregulated in human hearts from patients with hypertrophic cardiomyopathy. Western blot analysis and representative images of the main inflammasome sensors (NLRP3, AIM2, NLRC4 and NALP1). GAPDH is shown as loading control. Results are expressed as mean ± SEM; *p<0.05 vs. CON, Student's t-test, n=5-6. (154)

The 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 (Fig.5). The expression of NALP1 protein did not change in HF induced by any forms of cardiomyopathies examined (Fig.4A-B, Fig.5). Inflammasome activation was further confirmed by detection of downstream signaling components such as the cleaved fragments of caspase-1 and IL-1β and by the detection of elevated IL-1β levels by ELISA in failing hearts (Fig.4A-C).

3.1.2. AIM2 inflammasome sensor is expressed in monocytes and macrophages in heart

Inflammasomes are predominantly expressed in the innate immune cells e.g. in monocytes, macrophages or granulocytes. To assess the presence of monocytes and macrophages in failing hearts, immunohistochemistry was performed to stain Iba1 and CD68, markers of monocyte-macrophage lineage (Fig.6-7). The number of cells were

counted as well. We observed a tendency of increase in the total number of monocytes and macrophages in the left ventricle (Fig.7).

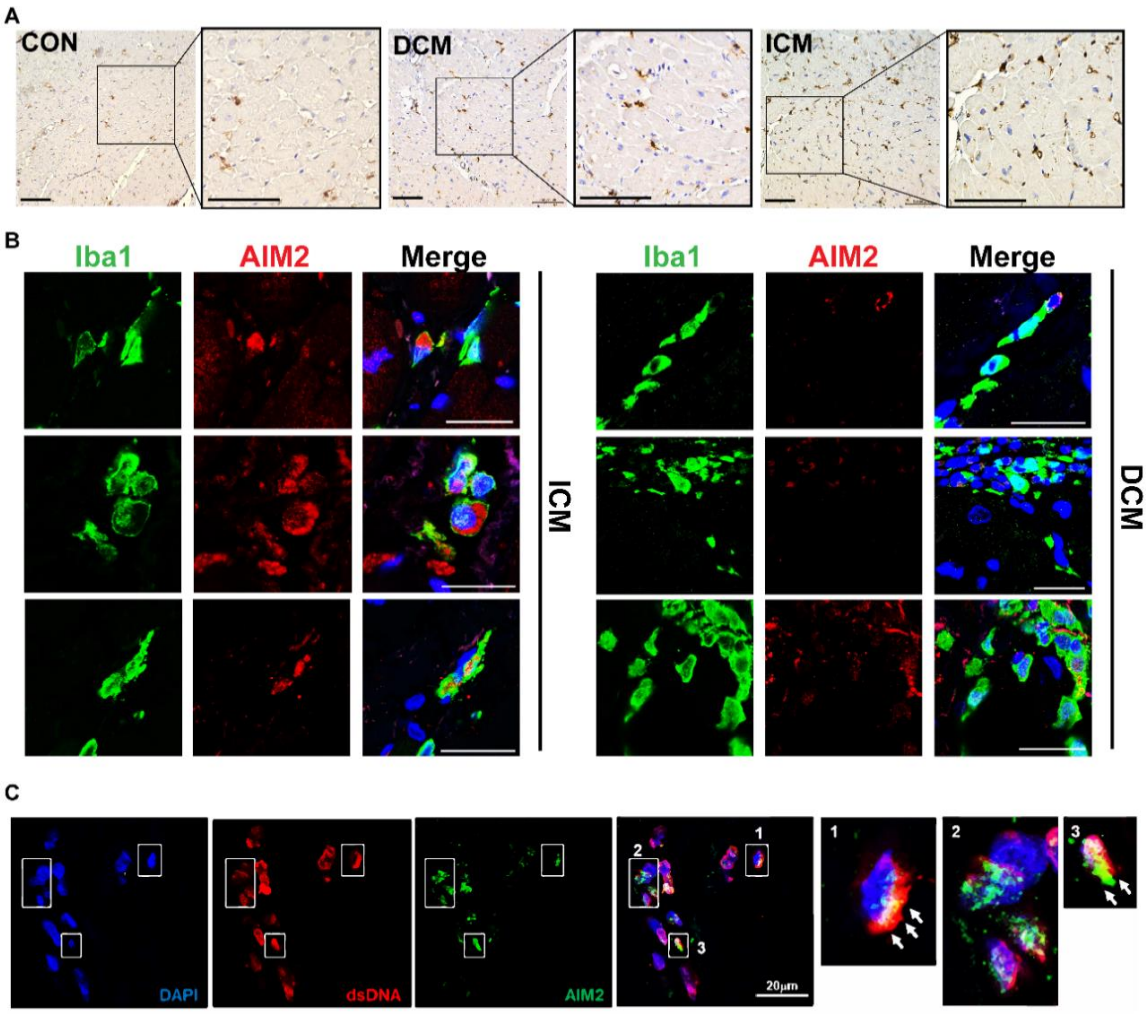

Figure 6 – Double-stranded DNA-sensitive AIM2 inflammasome sensor is expressed in monocytes and macrophages in human failing hearts. (A) Identification of monocytes and macrophages in human heart tissue by immunohistochemical detection of Iba1. Scale bar: 100μm. (B) Representative images of immunofluorescence 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. (154)

Despite the number of investigations on the inflammasome activation in cardiac diseases, there is a general lack of reliable evidence in which cell types of human hearts the inflammasomes are expressed. Immunofluorescence staining was performed to confirm the localization of AIM2 inflammasomes by detecting AIM2 in combination with monocyte/macrophage marker Iba1 (Fig.6B). Immunofluorescence staining showed that

AIM2 is localized mainly in Iba1 positive monocytes and macrophages, although AIM2 signals can be detected in other cell types, suggesting that primarily monocytes and macrophages might contribute to the enhanced inflammasome activity. However, their interactions with the surrounding cells might be also substantial for developing a pro-inflammatory milieu in failing hearts (Fig.6B). Interestingly, immunofluorescence assay revealed that a subpopulation of Iba1 positive cells shows low or undetectable AIM2 expression indicating the presence of a heterogeneous macrophage population in the myocardium of HF patients (Fig.6B).

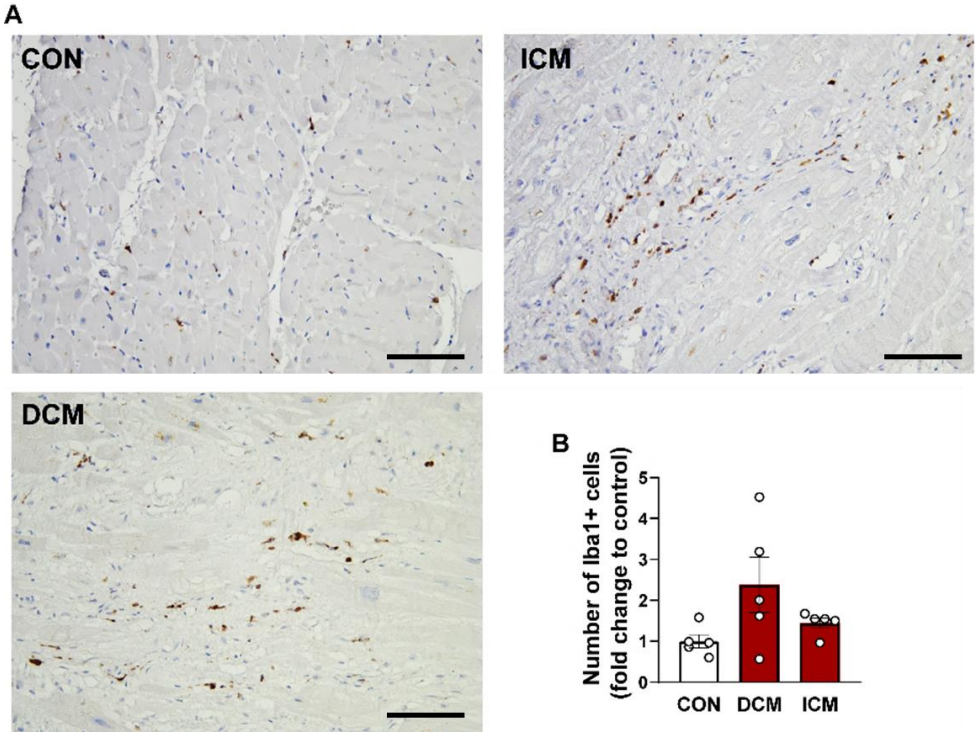

Figure 7 – The population of macrophages in left ventricle of failing hearts showed a slight tendency to expand. (A) Identification of monocytes/macrophages in human heart tissue by immunohistochemical detection of CD68. Scale bar: 100µm. (B) Quantification of macrophages based on Iba1+ cells in human failing hearts. $p>0.05$ vs. CON, one-way ANOVA, n=5. (154)

Extensive cell death may lead to the release of nuclear or mitochondrial dsDNA to the cytosol that can be recognized by the AIM2 inflammasome initiating the release of IL-1β and IL-18. We performed co-staining of dsDNA and AIM2 in sections from failing human hearts, and found that extranuclear dsDNA (Fig.6C, red signal) shows tight co-localization with the AIM2 signal (Fig.6C, green signal) further confirming the presence of activated AIM2 inflammasomes.

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

Despite the growing interest in the role of endocannabinoid signaling in cardiac diseases associated with atherosclerosis and dyslipidemia, the ECS-related enzymes in HF has not been investigated before. To examine the involvement of the ECS in HF with ischemic origin, we used quantitative real-time polymerase chain reaction (qRT-PCR) to measure the expression levels of ECS-related genes in control (n=6) and ischemic (n=6) failing hearts (Fig.8).

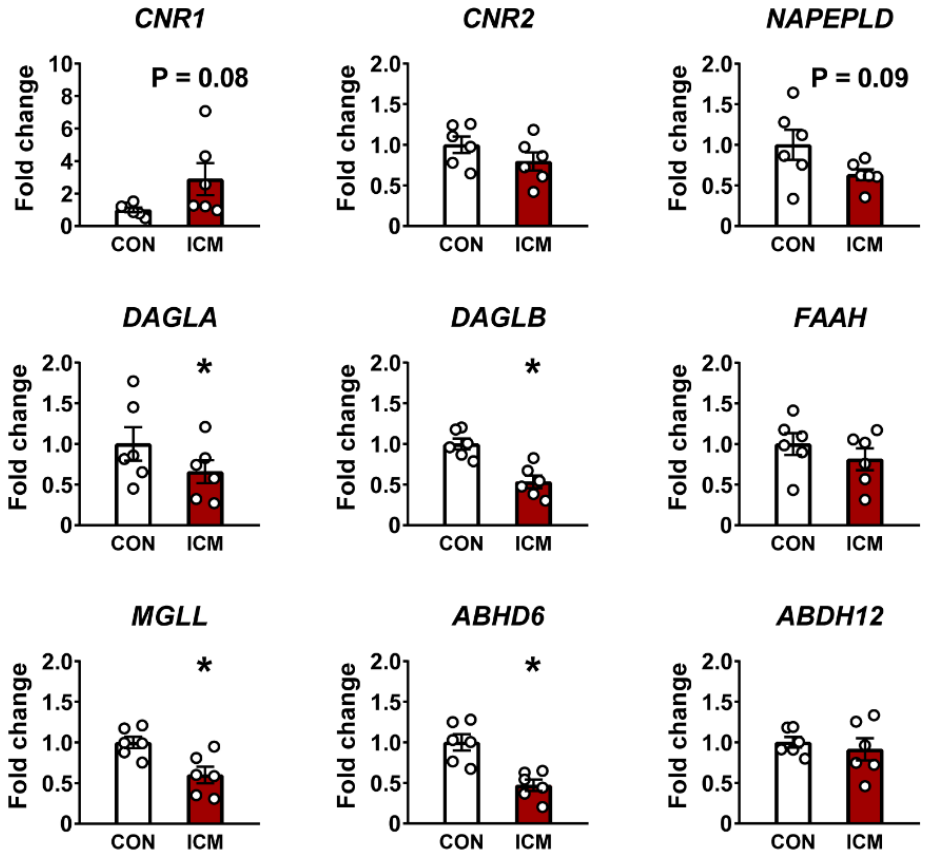

Figure 8 – mRNA expression of enzymes of endocannabinoid-related enzymes decreased. Analysis of mRNA expression of endocannabinoid-related genes by qRT-PCR. *Rplp0* was used as reference. Results are normalized to control, and expressed as mean ± SEM. *p<0.05 vs. control, Student's t-test; n=6. (155)

CNR1 mRNA expression was increased in some of the ischemic samples, however the overall increase was not significant (Fig.8). Reduced mRNA expression of 2-AG biosynthetic enzyme DAGLβ and the 2-AG hydrolytic enzymes MGLL and ABHD6 was detected in the ischemic hearts. The AEA metabolic enzymes did not change significantly, nor did *CNR2* expression.

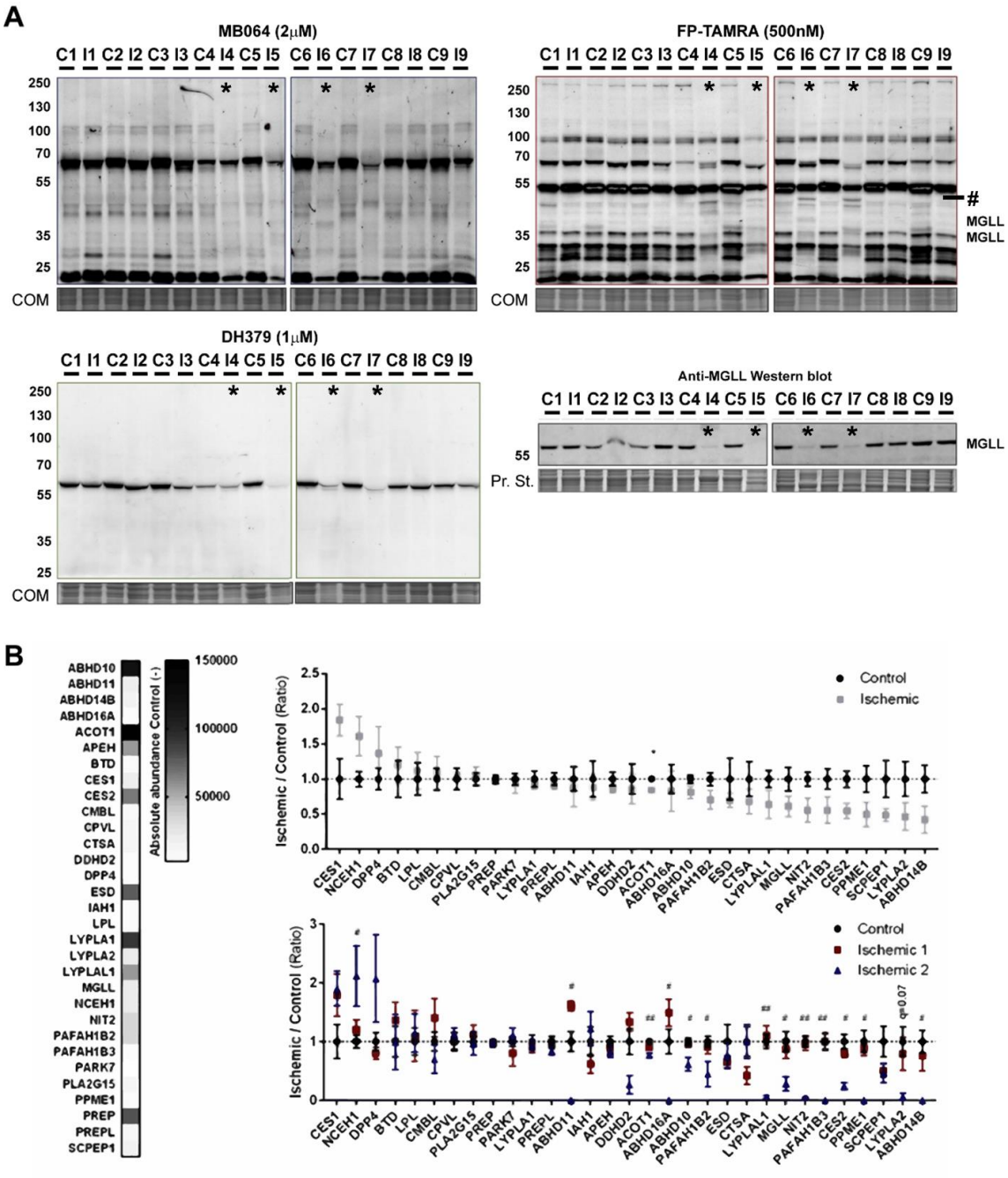

Figure 9 – Overall hydrolase activity was reduced in a subgroup of ischemic failing hearts. (A) Gel-based activity-based protein profiling of ischemic and control hearts. Coomassie blue (COM) was shown as loading control. Asterisk (*) was used to indicate a subgroup of ischemic samples with distinct lipid profile. (B) Activity-based proteomics on ischemic and control hearts. Heat map summary of mean abundance of hydrolases and hydrolase activity relative to mean control. Ischemic samples were categorized in subgroups (below). Results are expressed as mean ± SEM, #p<0.05, ##p<0.01 vs control; Student's t-test, n=9. (*Experiments and data analysis were performed by van Esbroeck et al, Leiden Institute of Chemistry, Leiden University, the Netherlands*.) (155)

The activity of the ECS-related metabolic enzymes was investigated by activity-based protein profiling (ABPP, Fig.9), which was performed exclusively in the laboratory of Prof. Mario van der Stelt by Annelot C.M. van Esbroeck (Leiden Institute of Chemistry, Leiden University, the Netherlands). The tissue samples from control and failing hearts were lysed and labeled with fluorescent activity-based probes to visualize the targets in gels. The tailored lipase probe MB064 preferentially reacts with the DAGLα, DAGLβ, ABHD6, and ABHD12. FP-TAMRA, a broad spectrum serine hydrolase probe, labels ABHD6, MGLL and FAAH. Probe DH379 selectively labels DAGL and ABHD6. The overall hydrolase activity was reduced in a subgroup of ischemic samples (Fig.9A, indicated with *) separated previously based on a significantly distinct lipid profile (data not shown). Additionally, MGLL activity and expression was reduced in these samples as well, while an additional band was observed in the activity profile of ischemic subgroup (Fig. 9A, indicated with #). The remaining ischemic samples only showed mild deviations compared to controls (Fig.9A). There were no significant differences in the overall protein staining. Of note, the other ECS metabolic hydrolases, including DAGLα (~120 kDa), DAGLβ (~70 kDa), ABHD6 (~35 kDa), and FAAH (~60 kDa) were not detected in human heart tissue (Fig.9A).

The biotinylated counterparts of FP-TAMRA and MB064, FP-biotin and MB108 respectively, were then used for target identification by mass spectrometry-based chemical proteomics (Fig. 9B). 31 hydrolases were identified, which included MGLL as the only ECS-related hydrolase (Fig. 9B). A slight, but not significant downregulation of several hydrolases, including MGLL was observed (Fig. 9B). Separation of the ischemic samples into two subgroups (based on lipid profile, data not shown) revealed that 13 hydrolase activities, including MGLL, were drastically reduced in the subgroup with an altered lipid profile (Fig.9B). Hydrolase activities from the first subgroup, however, were not significantly altered (Fig.9B).

3.2. Inflammation 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

It was previously demonstrated that NLRP3 inflammasome activation might play a significant role in initiating inflammatory reactions in animal models of early-stage HF. However, there is no data on the activation of other inflammasome types, especially in a

later stage of HF. To find suitable translational animal models to study inflammasome activation, we assessed three pathologically different models of HF. The pressure-overload (transverse aortic constriction - TAC), volume-overload (infrarenal arterio-venous shunt - AVS) and the postinfarction HF rat model (LAD) were developed and characterized by Mihály Ruppert et al. (Heart and Vascular Center, Semmelweis University, Hungary) (156). The functional characterization of each model with transthoracic echocardiography is shown in Table 3. In line with the fact that the three models are pathologically diverse, marked differences were found in morphology and function. Pressure-overload induced excessive cardiac fibrosis with hypertrophy in TAC animals, while volume-overload and ischemia promoted severe dilation as shown by the left ventricular dimensions and relative wall thicknesses (Tabl.3, Fig.10).

Table 3 – Echocardiography data on chronic heart failure rat models. Abbreviations: LV – left ventricle; FS – fractional shortening; RWT – relative wall thickness; LVEDV – left ventricular end-diastolic volume; LVESV - left ventricular end-systolic volume; LVEF – ejection fraction; TAC – transverse aortic constriction; LAD – left anterior coronary artery occlusion; AVS – arteriovenous shunt. *(Echocardiography and data analysis were performed by Mihály Ruppert, Heart and Vascular Center, Semmelweis University, Hungary.*) (154)

Group	**Short axis transection (mm)**						**LV mass (g)**	**FS (%)**	**RWT**	**LVEDV (uL)**	**LVESV (uL)**	**LVEF (%)**
	LV anterior wall		**LV internal**		**LV posterior wall**							
	Diastole	Systole	Diastole	Systole	Diastole	Systole						
Sham	1.89±0.04	3.10±0.08	7.59±0.33	4.21±0.25	2.17±0.09	3.34±0.09	1.19±0.07	45±1	0.54±0.03	594±43	207±18	65±1
TAC	2.82±0.11	3.45±0.14	9.07±0.31	6.95±0.34	3.23±0.20	3.73±0.21	2.83±0.16	24±2	0.68±0.04	849±47	528±52	39±4
LAD	1.17±0.07	1.26±0.05	10.23±0.40	8.62±0.49	1.77±0.10	2.65±0.11	1.26±0.09	16±2	0.29±0.02	1045±96	711±100	34±4
P (sham vs TAC)	**<0.001**	**0.051**	**0.005**	**<0.001**	**<0.001**	**0.108**	**<0.001**	**<0.001**	**0.024**	**0.001**	**<0.001**	**<0.001**
P (sham vs LAD)	**<0.001**	**<0.001**	**0.001**	**<0.001**	**0.011**	**<0.001**	**0.506**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Sham	1.88±0.08	2.95±0.16	8.25±0.34	4.53±0.33	2.05±0.12	3.31±0.20	1.29±0.05	45±3	0.49±0.04	709±36	215±25	70±2
AVS	2.05±0.08	3.02±0.12	13.30±0.37	8.77±0.21	2.51±0.07	4.06±0.32	3.51±0.23	34±2	0.35±0.01	2574±170	1054±57	59±1
P (sham vs AVS)	**0.148**	**0.729**	**<0.001**	**<0.001**	**0.005**	**0.064**	**<0.001**	**0.005**	**0.002**	**<0.001**	**<0.001**	**<0.001**

Protein expression of NLRP3 did not increase in any of the HF groups as compared to corresponding sham groups. As it was observed in human experiments, the expression of AIM2 protein increased significantly in TAC and LAD, but not in AVS rats (Fig.10). A tendency towards elevation in the level of NLRC4 was detected in TAC and LAD groups (Fig.10). In accordance with the elevation in the expression levels of inflammasome

sensors, the tissue level of IL-1β increased in TAC animals and, interestingly, in AVS animals (Fig.10).

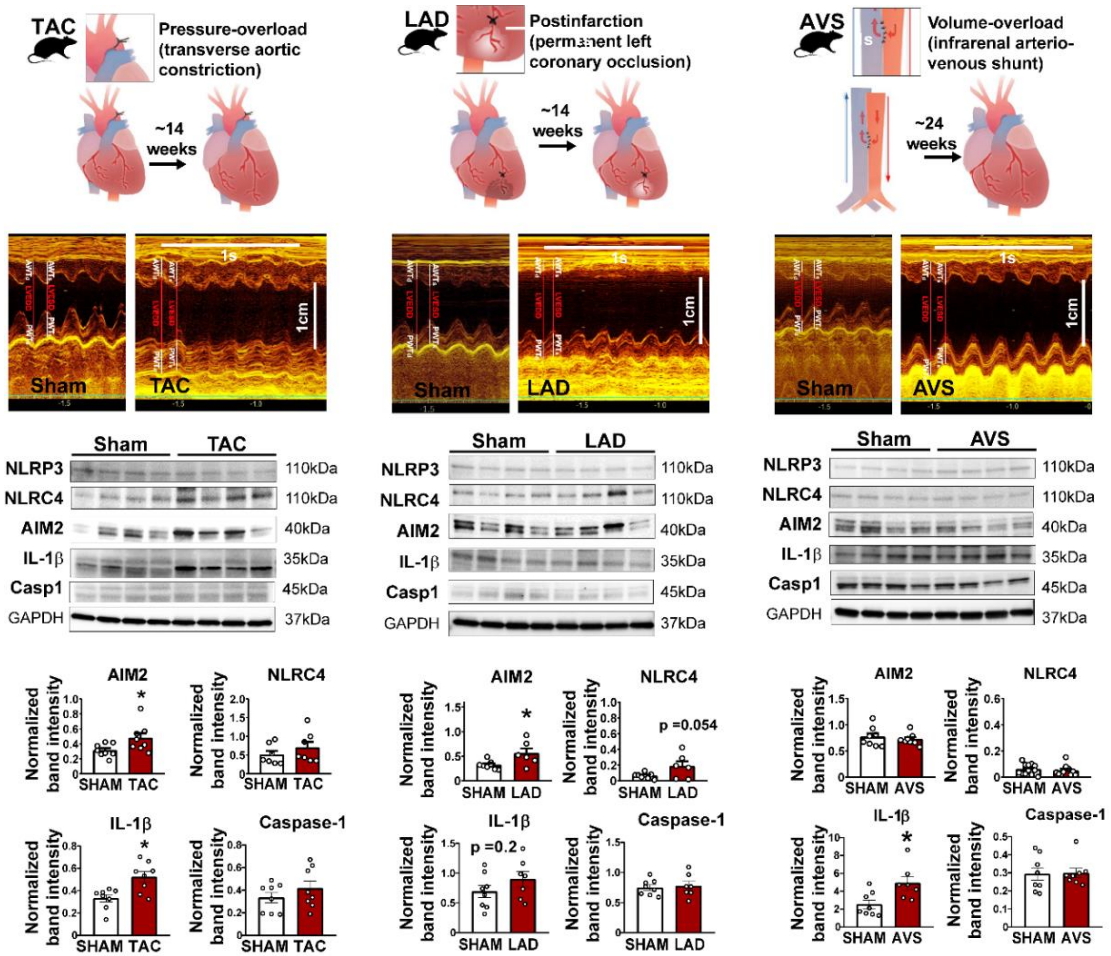

Figure 10 - AIM2 inflammasome expression increased in the late phase of chronic heart failure in rats. Pressure-overload, post-infarction and volume-overload-induced rat models of chronic heart failure with representative M-mode echocardiographic images, Western blot analysis of the inflammasome sensors 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.)* (154)

We found increased mRNA expression of the macrophage markers *Aif1* (gene of Iba1 protein) and *Cd68* with qRT-PCR analysis in rat failing hearts indicating enhanced monocyte and macrophage presence (Fig.11A). By detecting AIM2 and monocytes/macrophages with immunofluorescence, AIM2 showed predominant co-localization with the pan-macrophage marker CD68 in myocardial sections from TAC animals which is in line with our human results (Fig.11B). Surprisingly, detection of *Ccl2*, *Il23*, *Il6* and *Cd206*, *Mrc2*, *Mgl1* mRNAs showed an M1 to M2 change in macrophage

phenotype in TAC hearts while only slight changes were found in LAD and AVS hearts (Fig.11C).

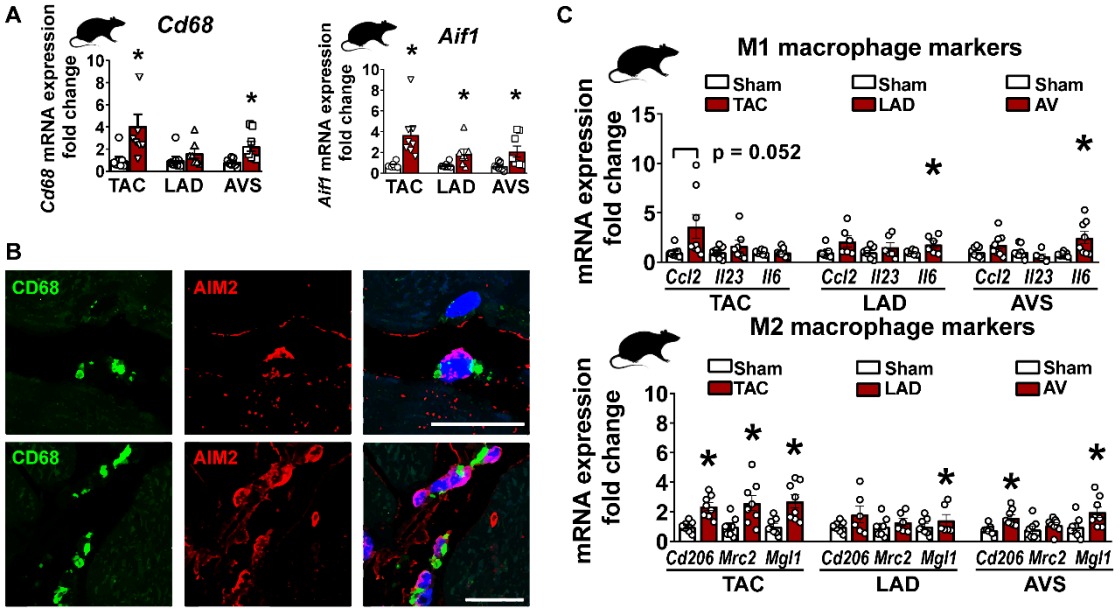

Figure 11 – Expansion of macrophage population is observed in rat failing hearts. (A) Analysis of mRNA expression of macrophage marker *Cd68* and *Aif1* by qRT-PCR. *Rplp0* was used as reference. Results are normalized to the control and expressed as mean ± SEM. *p<0.05 vs. corresponding Sham, Student's 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. (C) Analysis of mRNA expression of the M1 and M2 macrophage markers (*Ccl2, Il23, Il6* and *Cd206, Mrc2, Mgl1*, respectively) by qRT-PCR. *Rplp0* was used as reference. Results are normalized to the control and expressed as mean ± SEM. *p<0.05 vs. corresponding Sham, Student's t-test; n=6-8. (154)

3.2.2. AIM2 expression increased in the chronic stage of heart failure in porcine model for myocardial ischemia-reperfusion

We aimed to further investigate inflammasome activation in late stage of chronic heart failure induced by ischemia-reperfusion injury in a translational pig model as well (Fig.12). We assessed ischemic left ventricular samples harvested from pigs exposed to ischemia and reperfusion at three different time points: 3 hours (acute), 3 days (subacute) or 2 months (chronic) after ischemia-reperfusion (Fig.12), representing the acute injury, the early inflammatory and the late remodeling phase, respectively. The characterization of pig model was published previously by our research group (157, 158). Surprisingly, the level of AIM2 protein in heart tissue was not altered at 3 hours or 3 days, but it was markedly elevated at 2 months (Fig.12).

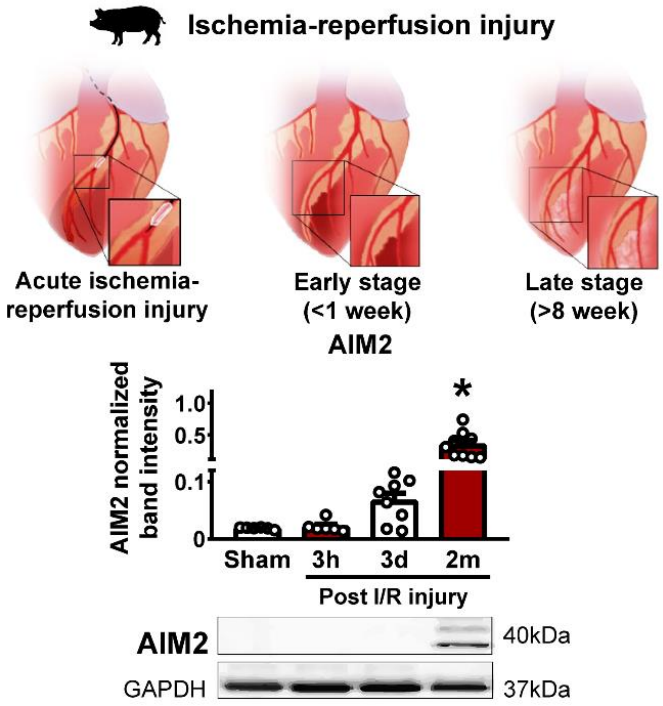

Figure 12 – AIM2 inflammasome sensor is expressed in the late stage of postinfarction heart failure in pigs. Chronic ischemia/reperfusion-induced pig heart failure model with Western blot analysis of time-dependent AIM2 protein expression. *p<0.05 vs. Sham, one-way ANOVA; n=6-8. (154)

3.3. *In vitro* platform of AIM2 inflammasome activation and anti-inflammatory effect of probenecid

3.3.1. Setting up a cell model for AIM2 inflammasome activation

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.13A). Naked poly(dA:dT) was unable to induce AIM2 inflammasome activation (Fig.13B), however, liposome encapsulated poly(dA:dT) increased the expression of AIM2 in THP-1 cells (Fig.13C), suggesting that vesicular uptake of dsDNA is essential in the induction of AIM2 inflammasome activation. Inflammasome activation was confirmed with detection of downstream signaling as well; we detected significantly increased level of cleaved caspase-1, IL-18 and IL-1β from the supernatant of THP-1 cells (Fig.13C-D). To visualize inflammasome activation, we performed immunofluorescence detection of the inflammasome adaptor protein ASC and AIM2 in THP-1 cell line (Fig.13E). Interestingly, poly(dA:dT) treatment led to the induction of AIM2 protein expression in the AC16 cells without significant interleukin release (Fig.13C-D).

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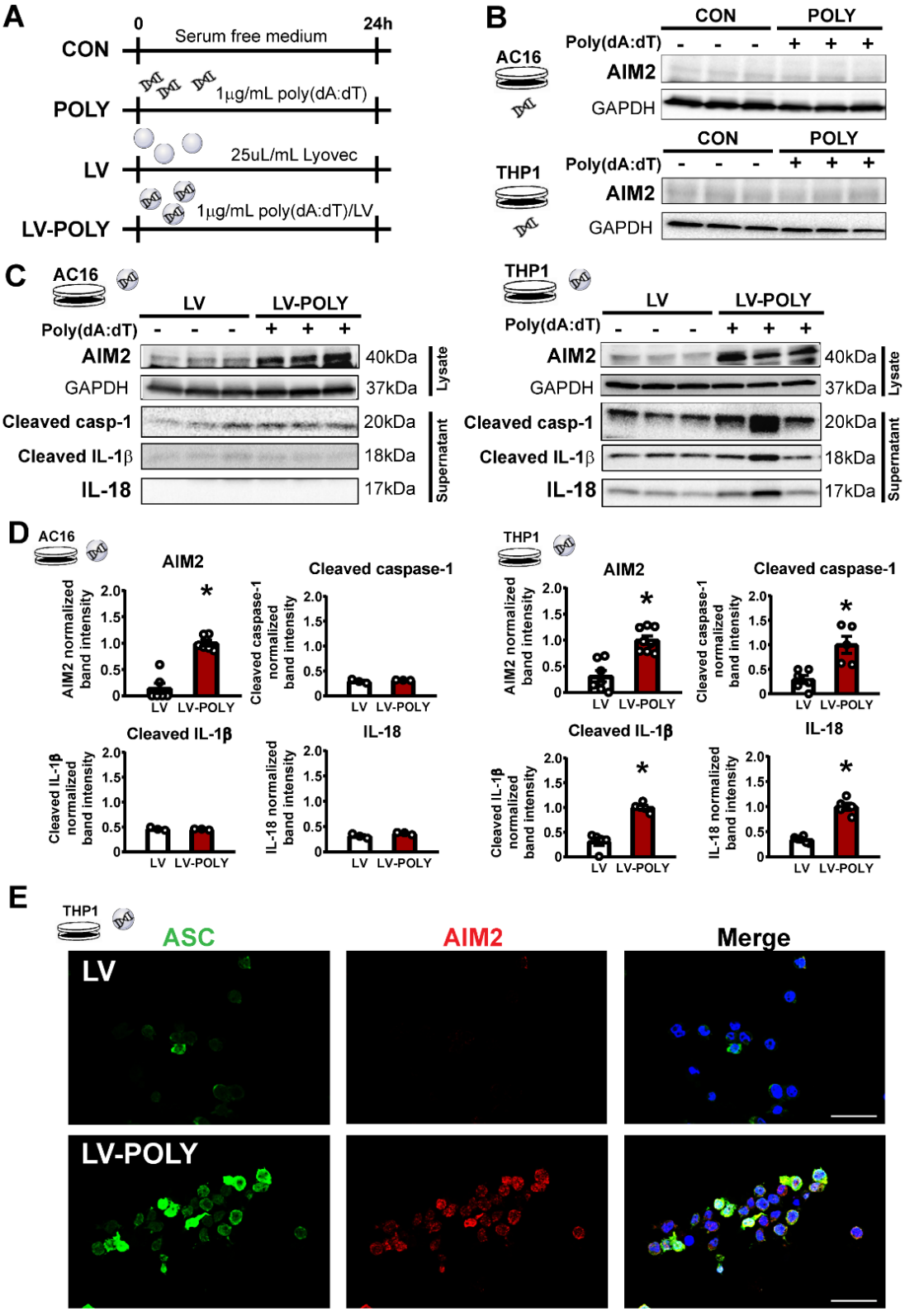

Figure 13 - Liposome encapsulated poly(dA:dT) induced the expression of AIM2 and inflammasome activation in vitro. (A) Experimental protocol for AIM2 induction in human AC16 cardiac and THP-1 monocytic cell lines. (B) Representative Western blot images for naked poly(dA:dT) stimulus on AC16 and THP-1 cells. (C) Representative Western blot images for liposome encapsulated poly(dA:dT) on AC16

and THP-1 cell lines. (D) Quantification of Western blot analysis on poly(dA:dT)-induced AIM2 inflammasome activation in AC16 and THP-1 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 THP-1 cells. DAPI (blue) was used for counterstain. Scale bar: 50μm. (154)

3.3.2. Pannexin-1 channel inhibitor probenecid attenuated AIM2 inflammasome activation in THP-1 monocytic cell line *in vitro*

Inflammasome activation by NLRP3 or NALP1 has been shown to be strongly associated with the activation of purinergic signaling via P2X7 and hemichannel Panx1. However, it is unknown whether AIM2 inflammasomes and Panx1 have molecular 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 (Fig.14).

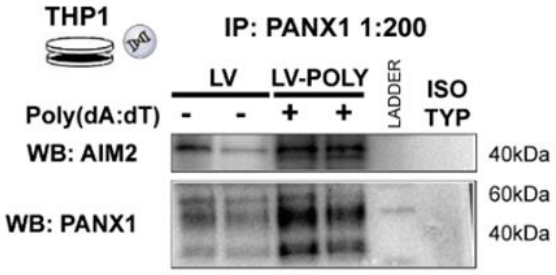

Figure 14 - Pannexin-1 channel is associated with AIM2 inflammasome sensor by co-immunoprecipitation. Representative Western blot images for co-immunoprecipitation from control and poly(dA:dT)-stimulated THP-1 cell lysate. Panx1 is shown as a loading control. Isotype anti-rabbit control was used as negative control. (154)

As the opening of Panx1 channels modulate immune responses by releasing of "find me" signals, we tested the effects of probenecid, a clinically used uricosuric and also known to be a potent Panx1 inhibitor, on AIM2 inflammasome activation *in vitro* (Fig.15). Treatment of different concentrations of probenecid (1-100μM) 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 (Fig.15B-C, Fig.16). By detecting downstream effectors, high dose probenecid was shown to be capable of attenuating AIM2 inflammasome activation *in vitro* (Fig.16).

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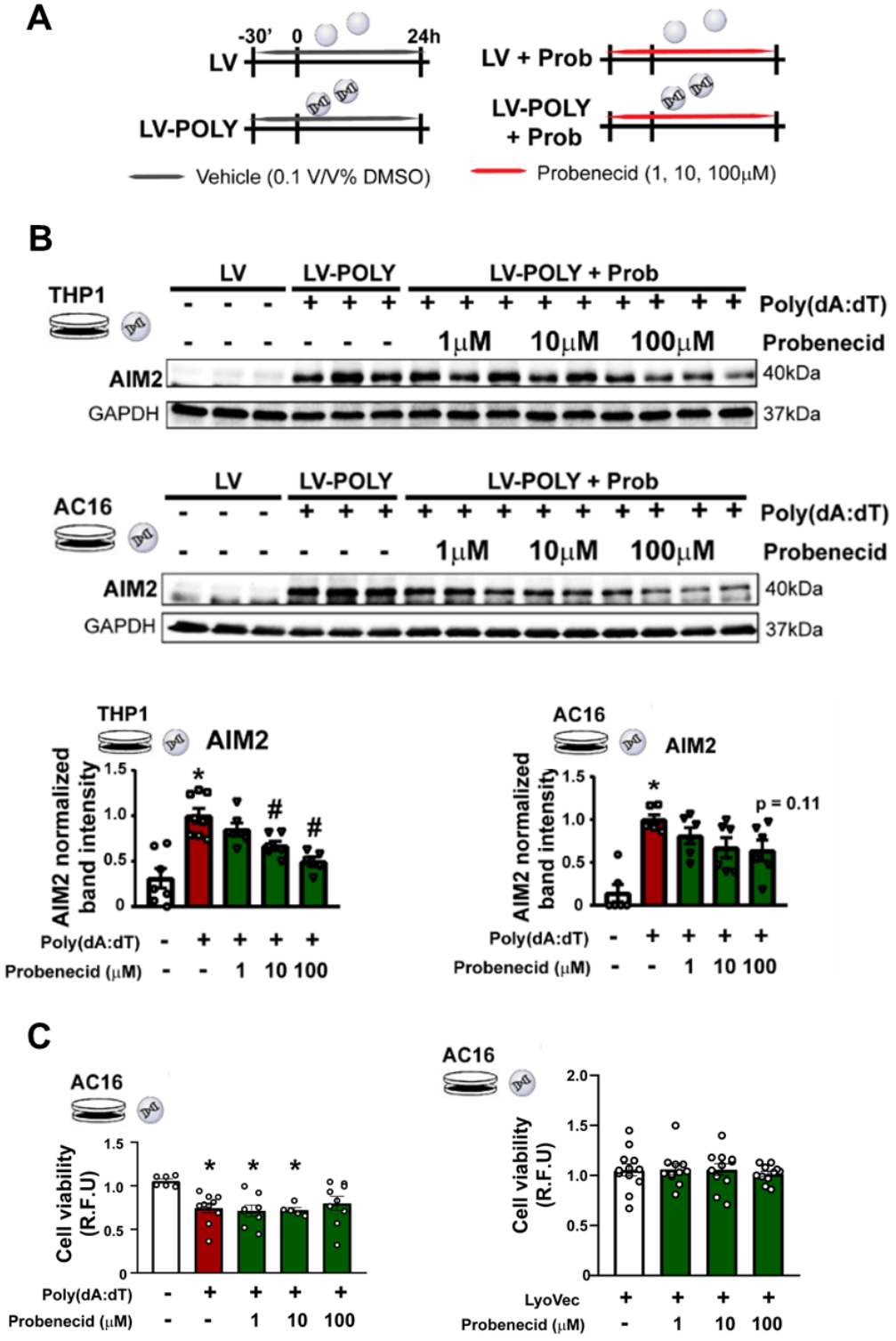

Figure 15 - Pannexin-1 channel inhibition attenuates AIM2 inflammasome expression in vitro. (A) Experimental protocol for testing the Panx1 blocker probenecid in cell model for AIM2 inflammasome activation on human AC16 and THP-1 cell lines. (B) Western blot analysis of AIM2 protein expression on poly(dA:dT)-stimulated THP-1 monocytic and AC16 cardiac 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. (C) Cell viability of poly(dA:dT)-stimulated (left) or liposome-treated (right) 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. (154)

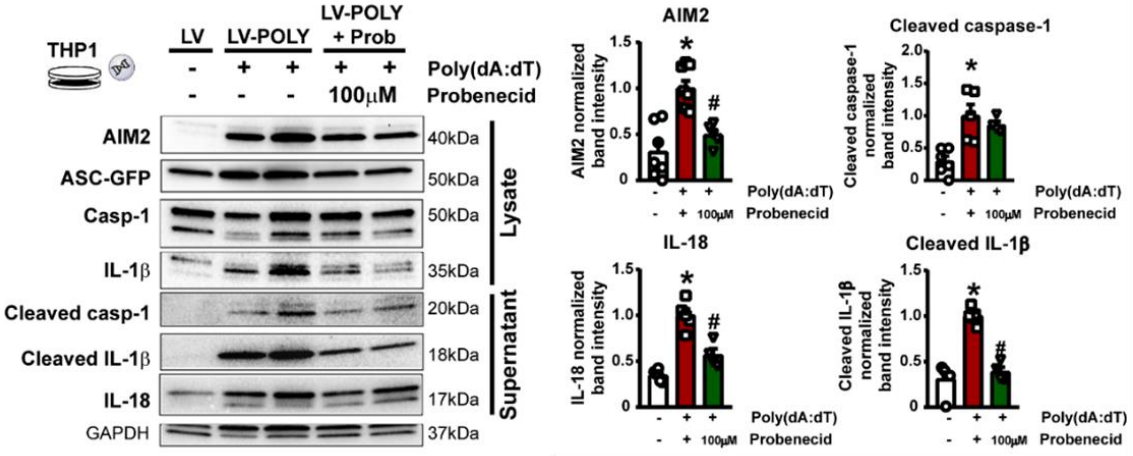

Figure 16 - Pannexin-1 channel inhibition by probenecid inhibits AIM2 inflammasome activation in vitro. Western blot analysis of downstream signaling of AIM2 inflammasome activation in cell lysate and supernatant of poly(dA:dT)-stimulated THP-1 cells in the presence of 100μM probenecid. *p<0.05 vs control; #p<0.05 vs poly(dA:dT) without probenecid; one-way ANOVA; n=5-6. (154)

Interestingly, the expression of Panx1 showed no significant differences in Panx1 levels between healthy and failing hearts with high individual variability (Fig.17).

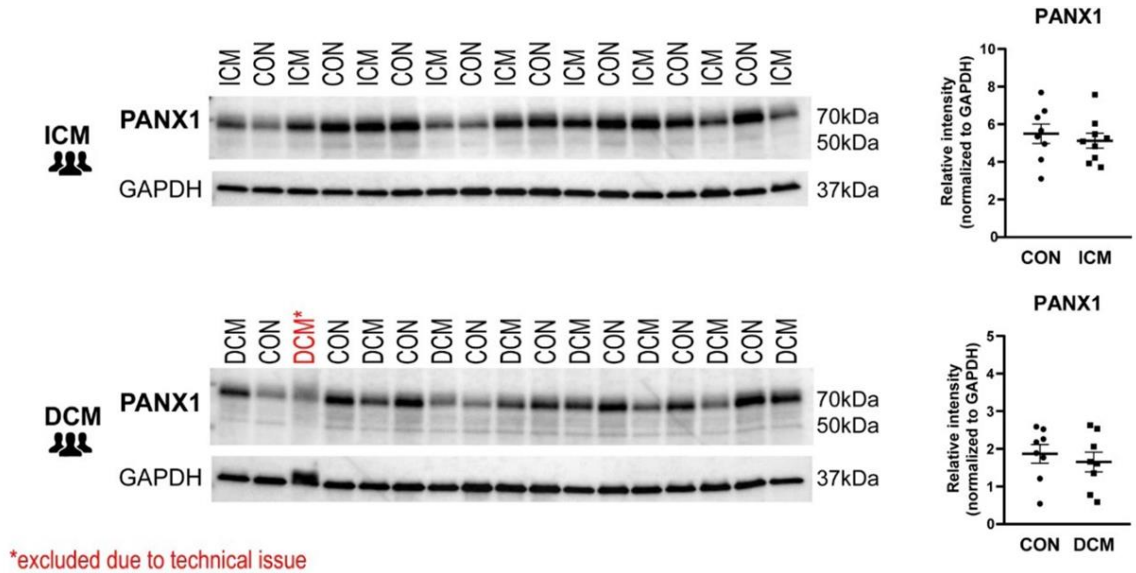

Figure 17 - Pannexin-1 protein expression did not change in human failing hearts. Western blot analysis and representative images of pannexin-1. GAPDH is shown as loading control. Results are expressed as mean ± SEM; p>0.05 vs. CON, Student's t-test, n=8-9. (154)

3.4. Oral probenecid treatment improved outcomes in pressure overload heart failure rat model *in vivo*

After our encouraging *in vitro* results, we investigated probenecid in the rat HF model induced by TAC to test if probenecid improves cardiac function *in vivo* (Fig.18, Table 4). 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/body weight kg/day) or vehicle (hydroxyethyl cellulose) control during the experiment (Fig.18A).

We monitored the mortality throughout the whole study. The group treated with vehicle and having TAC-induced HF showed an increased mortality rate compared with vehicle-treated sham operated rats as nearly half of the animals died until week 14 (Fig.18B). However, 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 (Fig.18B).

As observed before (Fig.10), TAC rats developed HF. 14 weeks after TAC, left ventricular ejection fraction was reduced significantly compared to 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 (Fig.18C). Oral probenecid treatment of rats with TAC significantly prevented deterioration of ejection fraction compared to vehicle treatment. In accordance, at 14 weeks after TAC, left ventricular end-systolic volumes increased more in the vehicle group compared with the probenecid treated group (Fig.18C, Table 4). In accordance with our previous results above (Fig.10), the protein levels of IL-1β and its mature form increased 14 weeks after TAC surgery, which was reduced by probenecid treatment (Fig.18D). In addition, treatment with probenecid prevented the development of left ventricular hypertrophy (Fig.18C, E-F). 14 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 (Fig.18C, E). This was further confirmed by analysis of pro-hypertrophic genes (*Nppa* and *Nppb*) and the pro-fibrotic factor *Ctgf* (Fig.18F). All these transcripts were significantly induced by TAC surgery, and their upregulation was prevented by probenecid (Fig.18F).

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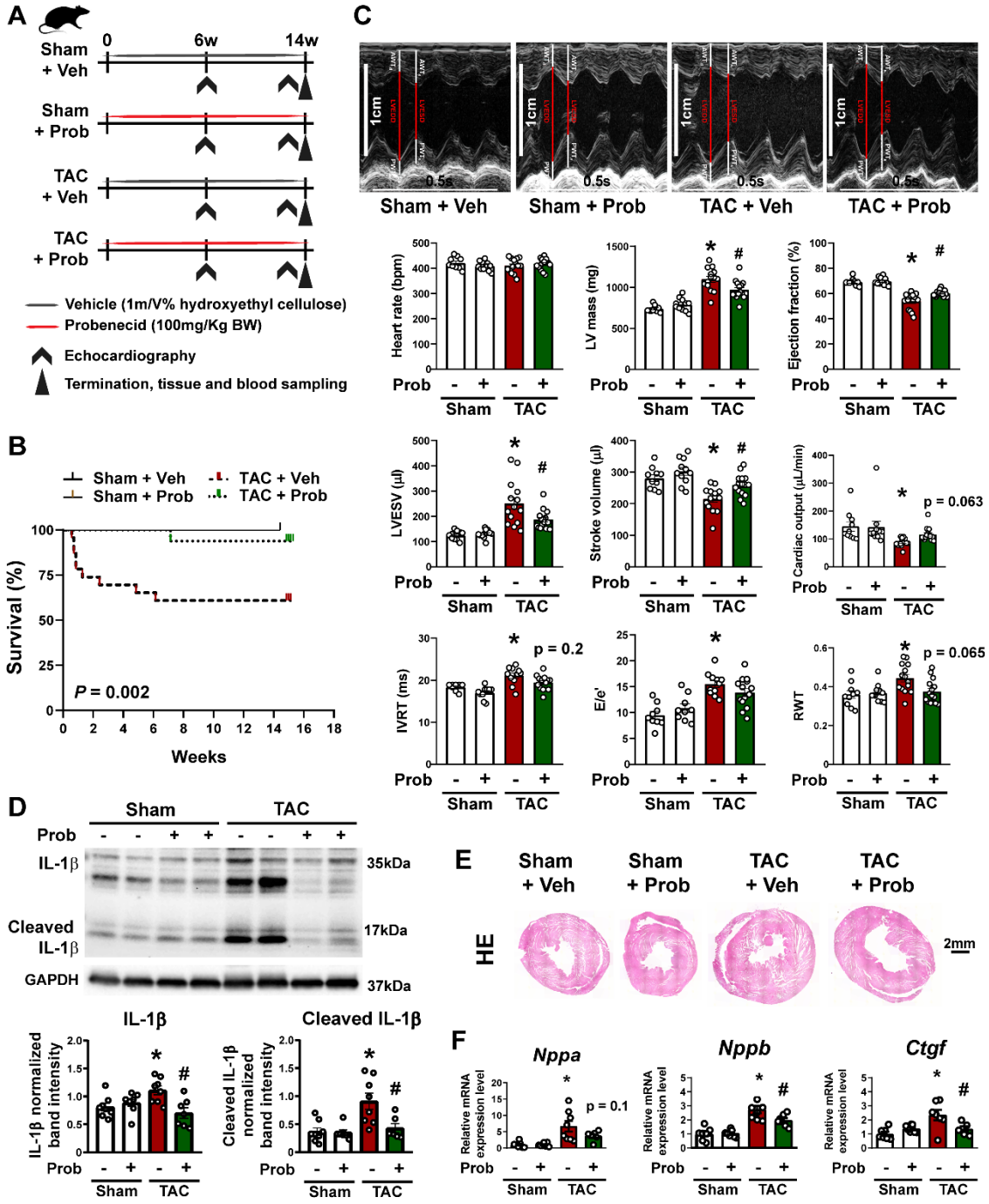

Figure 18 - Pannexin-1 channel inhibitor probenecid improves survival and cardiac function in vivo (A) Study design for investigating the effects of probenecid (Prob) in a rat model for chronic heart failure (TAC). (B) Kaplan-Meier analysis of overall mortality. p<0.05, log-rank (Mantel-Cox) test; n=11-23. (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. *(Representative echocardiographic images were taken by Alex Ali Sayour, Heart and Vascular Center, Semmelweis University, Hungary*.) (154)

Table 4 – Echocardiography data on vehicle- or probenecid-treated rats with pressure overload induced heart failure. Abbreviations: s – systolic; d – diastolic; Veh – vehicle; TAC – transverse aortic constriction; Prob - probenecid; LV – left ventricle; Cor – corrugated; LVAW – left ventricular anterior wall thickness; LVPW - left ventricular posterior wall thickness; MV – mitral valve; IVRT - isovolumic relaxation time; RWT – relative wall thickness. *(Echocardiography and data analysis were performed by Alex Ali Sayour and Mihály Ruppert, Heart and Vascular Center, Semmelweis University, Hungary*.) (154)

		Parasternal LONG AXIS (PSLAX) B-MODE									
Group ID		**Heart Rate**	**Area;s**	**Area;d**	**Volume**	**Volume;s**	**Volume;d**	**Stroke Volume**	**Ejection Fraction**	**Fractional Shortening**	**Cardiac Output**
Sham + Veh	Mean	421,16	39,77	82,42	124,77	124,77	405,34	280,57	69,19	22,95	145,04
	SD	23,36	3,48	6,13	17,81	17,81	46,97	34,30	2,75	1,86	53,39
Sham + Prob	Mean	409,10	41,27	86,71	130,75	130,75	433,36	302,60	69,75	22,33	142,54
	SD	17,98	3,45	5,60	16,80	16,80	49,80	40,46	2,97	2,02	71,97
P vs. Sham + Veh		*0,223*	*0,333*	*0,112*	*0,439*	*0,439*	*0,200*	*0,193*	*0,660*	*0,473*	*0,928*
TAC + Veh	Mean	410,23	51,02	82,62	251,17	182,21	397,13	214,92	53,96	15,82	87,78
	SD	28,29	5,46	6,50	88,48	30,23	48,58	38,90	6,58	2,90	15,21
P vs. Sham + Veh		*0,326*	***0,000***	*0,941*	***0,000***	***0,000***	*0,682*	***0,000***	***0,000***	***0,000***	***0,008***
TAC + Prob	Mean	417,38	48,92	86,71	187,05	171,76	430,91	259,15	60,17	18,76	116,18
	SD	22,37	5,34	7,33	37,12	27,60	59,03	33,80	2,29	1,53	23,54
P vs. Sham + Veh		*0,702*	***0,000***	*0,128*	***0,000***	***0,000***	*0,242*	*0,140*	***0,000***	***0,000***	*0,135*
P vs. TAC + Veh		*0,459*	*0,304*	*0,123*	***0,022***	*0,341*	*0,103*	***0,003***	***0,004***	***0,003***	***0,001***

		SHORT AXIS (SAX) M-MODE									
Group ID		**Volume;s**	**Volume;d**	**Ejection Fraction**	**Fractional Shortening**	**LV Mass**	**LV Mass Cor**	**LVAW;s**	**LVAW;d**	**LVPW;s**	**LVPW;d**
Sham + Veh	Mean	304,93	231,95	76,24	46,50	739,74	591,80	2,95	1,53	2,50	1,35
	SD	44,58	30,92	2,78	2,68	41,39	33,11	0,26	0,20	0,21	0,16
Sham + Prob	Mean	302,91	231,51	76,58	47,09	792,01	633,61	3,05	1,66	2,50	1,38
	SD	48,33	39,34	5,78	5,62	76,74	61,39	0,21	0,18	0,26	0,10
P vs. Sham + Veh		*0,922*	*0,978*	*0,864*	*0,760*	*0,067*	*0,067*	*0,358*	*0,144*	*0,979*	*0,630*
TAC + Veh	Mean	324,03	206,11	63,59	35,91	1098,95	879,16	3,22	1,98	2,70	1,72
	SD	30,28	22,81	3,61	2,74	137,70	110,16	0,24	0,18	0,35	0,26
P vs. Sham + Veh		*0,258*	***0,040***	***0,000***	***0,000***	***0,000***	***0,000***	***0,018***	***0,000***	***0,094***	***0,000***
TAC + Prob	Mean	362,48	239,08	66,44	38,37	969,36	775,49	3,04	1,68	2,59	1,52
	SD	59,64	34,44	6,15	4,90	111,84	89,47	0,30	0,19	0,27	0,15
P vs. Sham + Veh		***0,011***	*0,595*	***0,000***	***0,000***	***0,000***	***0,000***	*0,421*	*0,082*	*0,330*	***0,018***
P vs. TAC + Veh		***0,038***	***0,005***	*0,139*	*0,106*	***0,010***	***0,010***	*0,085*	***0,000***	*0,377*	***0,017***

		SHORT AXIS (SAX) B-MODE				MV FLOW					RWT
Group ID		**Area;s**	**Area;d**	**Fractional Area Change**		**E'**	**IVRT**	**MV E**	**MV E/E'**		
Sham + Veh	Mean	13,34	37,64	64,53		-84,86	18,36	796,62	-9,48		0,36
	SD	1,71	3,02	2,60		12,94	0,86	169,95	2,24		0,06

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Sham + Prob	Mean	14,58	42,42	65,74		-77,17	17,02	779,38	-10,86		0,37
	SD	3,64	5,54	7,34		21,79	1,46	103,32	2,50		0,05
P vs. Sham + Veh		*0,329*	***0,025***	*0,618*		*0,359*	***0,029***	*0,799*	*0,236*		*0,731*
TAC + Veh	Mean	21,38	44,79	52,13		-61,57	21,08	943,02	-15,51		0,44
	SD	3,33	5,20	5,91		10,64	2,09	140,11	2,20		0,07
P vs. Sham + Veh		***0,000***	***0,000***	***0,000***		***0,001***	***0,002***	*0,055*	***0,000***		***0,006***
TAC + Prob	Mean	20,58	46,54	55,83		-77,03	19,44	1046,29	-13,87		0,38
	SD	3,11	4,46	4,49		10,98	1,75	150,14	2,88		0,06
P vs. Sham + Veh		***0,000***	***0,000***	***0,000***		***0,154***	*0,073*	***0,002***	***0,001***		*0,552*
P vs. TAC + Veh		*0,514*	*0,343*	*0,071*		***0,002***	*0,054*	*0,090*	*0,119*		***0,011***

4. DISCUSSION

In this present work, we detected increased expression of the AIM2 inflammasome sensor and its downstream signaling in failing hearts harvested from human patients. This finding was confirmed in different small and large animal models of chronic HF, highlighting the importance of chronic inflammatory reactions in these conditions. Enhanced NLRC4 expression and decreased activity of ECS-related biosynthetic and hydrolytic enzymes were observed in human failing hearts as well. We showed that dsDNA is capable of inducing the AIM2 inflammasome activation in both human monocytic and cardiomyocyte cell lines, suggesting that apoptotic or necrotic DNA might be the major trigger of the AIM2 inflammasome in the chronic phase of HF. 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. Chronic treatment with probenecid improved outcomes including survival, cardiac function and failure markers of pressure-overload induced HF. These novel anti-inflammatory properties of probenecid could facilitate potential repurposing and use of this drug in chronic heart failure.

The role of inflammatory signaling pathways in cardiac diseases has been extensively studied over the last decades; nevertheless clinical translation of these findings was rather mixed and controversial as it was demonstrated by large prospective studies e.g. RENEWAL, CIRT or REDHART (35, 112). However, the CANTOS trial highlighted that significant reductions can be achieved in incidence of major cardiovascular adverse events of specific subpopulation of postinfarction patients by neutralizing IL-1β with canakinumab (113). These results confirm the active role of IL-1β in the pathomechanism of certain cardiovascular diseases. Nevertheless, there are limitations of the use of canakinumab (e.g.: high cost, infectious side effects) that led to the rejection by FDA in cardiovascular indications. Apart from this failure, modulating IL-1β-related pathways in cardiovascular diseases including chronic heart failure might be of major therapeutic importance.

Our human and translational animal data provides evidences for elevated expression of AIM2 and NLRC4 inflammasome sensors as well as significant inflammasome activation in chronic heart failure. Increasing number of evidences has indicated that AIM2 inflammasome activation is a key player of various cardiovascular diseases including

diabetic and ischemic cardiomyopathy (20, 21), atherosclerosis (85, 86) and ischemic stroke (11). It is hypothesized that the major inducer of AIM2 inflammasome activation might be double stranded DNA of mitochondrial and nuclear origin which is released as a result of permanent cellular damage and low degree of apoptosis and necrosis during cardiac remodeling (20, 85).

The elevated expression of NLRC4 in failing hearts is more surprising. The most characterized trigger of NLRC4 is flagellin of Gram negative bacteria (159). It is believed that HF-associated global hypoperfusion induces dysbiosis and increased gut permeability, promoting a chronic systemic inflammatory state, which is supported by showing gut microbiome modulation as an interesting target to alleviate the systemic inflammatory state in human HF (160). This hypothesis might provide an explanation for increased NLRC4 expression in human failing hearts. Of note, it is unknown whether significant gut hypoperfusion could have developed in our animal models. Furthermore, a similar co-activation pattern of AIM2 and NLRC4 has been described in animal models of stroke and ischemic cardiomyopathy previously, suggesting that the activation of these two inflammasomes might be linked (11, 20). We also show that co-activation of AIM2 and NLRC4 inflammasomes is a possible phenomenon, suggesting that single inflammasome targeting may not be sufficient in case of cardiovascular diseases including atherosclerosis and chronic heart failure.

The complex interplay between endocannabinoid signaling and inflammation including inflammasomes is under intense research recently (161). CB_1R upregulation can be observed with pro-inflammatory milieu e.g. in atherosclerosis or ischemic injury (145, 147). In our study, a subgroup of ischemic patients showed elevated expression of CB_1R as well as endocannabinoids but not of CB_2R. These results are in line with previous observation partially; however, CB_2R may be also increased in certain types of cardiomyopathies indicating the influence of other factors such as etiology (162). Another important point of our study was the decrease in the activity of endocannabinoid-degrading enzyme MGLL in the subgroup of ischemic patients. This finding confirms recent observation on impaired activity of MGLL as well as the detrimental effects of AEA and 2-AG in ischemic myocardium (163). However, the heterogeneity of the patient population observed in our study highlights the need for further research to identify the major factors leading to controversial results.

The complex pathways converging to inflammasome activation and signaling involve a series of triggers and modulators that may influence inflammasome activity and assembly. The best characterized triggers are the classic mediators promoting inflammasome priming through various PRRs e.g. TLR4, TLR9, TNF-α and interleukin receptors and inflammasome oligomerization, which is modulated by purinergic and pannexin channels (164). Panx1 channels have been known as modulators of NALP1, NLRP3 as well as of non-canonical inflammasome activities via ATP release (165-168). We have shown that Panx1 channels associate to the AIM2 inflammasome as well, and showed a notable anti-inflammatory effect of the Panx1 channel inhibitor probenecid *in vitro*. We have seen a reduction in the expression of AIM2 and its downstream signaling *in vitro* in both dsDNA stimulated monocytes/macrophages and cardiac cells. The anti-inflammatory effect of probenecid was mediated by decreasing Il-1β level in a rabbit sepsis model (169). In addition to AIM2 inflammasome inhibition, Panx1 channels may play a role in leukocyte migration and in modulation of the NF-κB pathway (170). A recent study has also confirmed that probenecid improves cardiac function at early phase of post-infarction heart failure via inhibiting endothelial Panx1 channels and consequential leukocyte infiltration (171). Therefore, we propose that probenecid might be a `broad-spectrum` inflammasome inhibitor and anti-inflammatory agent besides its well characterized uricosuric properties in gout. Probenecid has been proven to improve outcome in an animal model of ischemic HF with a shorter 4-week follow-up period by exerting positive inotropic effects via transient receptor potential cation channel subfamily V member 2 (TRPV2), and the positive inotropic effect was confirmed in a small number of patients with HF (172). We showed that probenecid is able to prevent adverse cardiac remodeling 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 in our study which should be acknowledged as a limitation. Nevertheless, the already published positive inotropic effects through TRPV2 and the novel anti-inflammatory effects might explain the recently observed clinical benefits of probenecid use in patients suffering from heart failure, as well as the epidemiological observation, that patients receiving probenecid therapy for gouty arthritis have better cardiovascular outcomes (172-174). Thus, we

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believe that probenecid or potential derivatives of it might be useful therapeutic tools and adjuvants for the management of chronic heart failure.

5. 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 which displayed increased expression of CB_1R as well as reduced expression and activity of some hydrolases responsible for the degradation or biosynthesis of endocannabinoids. Additionally, activity-based protein profiling was proven to be a potent tool to examine the activity of ECS-related enzymes in cardiovascular conditions. Human findings 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 pattern. Our results highlight the importance of specific inflammation patterns and the involvement of multiple pathways. Thus, it might facilitate the 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.

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6. SUMMARY

Inflammatory mechanisms and related pathways including inflammasomes or endocannabinoid signaling are important pathogenic factors in cardiovascular diseases. The CANTOS trial has proven new evidences that anti-inflammatory therapy by inhibiting IL-1β reduces effectively the incidences of major cardiovascular events and prevents complications. Therefore, IL-1β, inflammasomes and associated pathways are promising therapeutic targets in these cardiac diseases. In this recent study, we aimed to assess inflammasome activation and ECS-related pathways in chronic heart failure to identify potential new targets.

In human, the expression of the inflammasome protein AIM2 and NLRC4 as well as downstream signaling increased in failing hearts regardless of the etiology (ischemic or dilated cardiomyopathy) while the NALP1 and NLRP3 inflammasome showed no change. AIM2 expression was primarily detected in monocytes/macrophages of failing hearts. The mRNA expression of endocannabinoid 2-AG-related biosynthetic and hydrolytic enzymes decreased in a subgroup of ischemic cardiomyopathy, while the mRNA level of *CNR1* increased in the same subgroup. Translational animal models of HF, i.e. pressure- or volume-overload, and permanent coronary artery ligation in rat, as well as ischemia/reperfusion-induced HF in pigs, demonstrated an activation pattern of AIM2 similar to that of observed in end-stages of human HF. *In vitro* AIM2 inflammasome activation in human THP-1 monocytic cells and human AC16 cells induced by specific double-stranded DNA was significantly reduced by pharmacological blockade of pannexin-1 channels by probenecid, a clinically used uricosuric drug. Probenecid was also able to reduce pressure overload-induced mortality and restore indices of disease severity in a rat chronic HF model *in vivo*.

In summary, AIM2 and NLRC4 inflammasome activation contribute to chronic inflammation in heart failure. The activity of ECS-related enzymes was shown to be specific of the disease or its stage. Furthermore, pannexin-1 channel inhibitor probenecid alleviates chronic HF by reducing inflammasome activation. The present results suggest the possibility of repositioning of probenecid as for HF indications.

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8. LIST OF OWN PUBLICATIONS

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

I. **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

II. 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.

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∑IF of dissertation-related publications: 18.445

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

III. **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.

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VII. Brenner, GB, Makkos, A, Nagy, CT, **Onódi, Z**, Sayour, NV, Gergely, TG, Kiss, B, Görbe, A, Sághy, É, Zádori, ZS, Lázár, B, Baranyai, T, Varga, RS, Husti, Z, Varró, A, Tóthfalusi, L, Schulz, R, Baczkó, I, Giricz, Z, and Ferdinandy, P (2020). "Hidden Cardiotoxicity of Rofecoxib Can be Revealed in Experimental Models of Ischemia/Reperfusion." Cells 9(3).

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∑IF: 54.114

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NYILATKOZAT EREDETISÉGRŐL ÉS SZERZŐI JOGRÓL

a PhD disszertáció elkészítésére vonatkozó szabályok betartásáról

Alulírott Gulyás-Onódi Zsófia jelen nyilatkozat aláírásával kijelentem, hogy az **Immune-inflammatory targets in chronic heart failure: inflammasomes and the endocannabinoid system** című PhD értekezésem önálló munkám, a dolgozat készítése során betartottam a szerzői jogról szóló 1999. évi LXXVI tv. vonatkozó rendelkezéseit, a már megjelent vagy közlés alatt álló közlemény(ek)ből felhasznált ábra/szöveg nem sérti a kiadó vagy más jogi vagy természetes személy jogait.

Jelen nyilatkozat aláírásával tudomásul veszem, hogy amennyiben igazolható, hogy a dolgozatban nem saját eredményeimet használtam fel vagy a dolgozattal kapcsolatban szerzői jog megsértése merül fel, a Semmelweis Egyetem megtagadja PhD dolgozatom befogadását, velem szemben fegyelmi eljárást indít, illetve visszavonja a már odaítélt PhD fokozatot.

A dolgozat befogadásának megtagadása és a fegyelmi eljárás indítása nem érinti a szerzői jogsértés miatti egyéb (polgári jogi, szabálysértési jogi, büntetőjogi) jogkövetkezményeket.

Tudomásul veszem, hogy a PhD értekezés nyilvánosan elérhető formában feltöltésre kerül az Országos Doktori Tanács honlapjára.

Budapest, 2021.09.21.

Gulyás-Onódi Zsófia