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Characterization of NLRP3 in the Maintenance of Cardiac Tissue Homeostasis

by

Nathan Alex Bracey

A THESIS

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Abstract

Chronic cardiovascular diseases are characterized by tissue destruction and ongoing inflammation that impairs organ function. This non-microbial and chronic inflammation is mediated by pattern recognition receptors (PRRs). NLRP3 is one intracellular PRR that regulates pro-inflammatory cytokine secretion in response to endogenous signals from dying cells. While much in known regarding NLRP3 regulation of cytokine secretion, its broader function in diverse cell populations during cardiac injury has not been explored. Using murine models, we sought to characterize the role and expression of NLRP3 in chronic cardiovascular disease. We discovered that in addition to its established roles in regulating cytokine secretion, *Nlrp3*$^{-/-}$ mice were protected against Angiotensin II-induced hypertensive cardiac fibrosis despite negligible leukocytic infiltrate, apoptosis and cytokine processing. These results suggested the possibility of non-canonical signalling mechanisms in non-immune cells. We further explored the possibility of cytokine-independent roles for NLRP3 with the use of primary human and murine cell culture, microscopy and *in vitro* reporter systems. We began looking in cardiac fibroblasts, which displayed significant NLRP3 expression in left ventricular tissue samples from human patients with heart failure. NLRP3 was induced by pro-fibrotic signalling during cardiac fibroblast differentiation to myofibroblast phenotype by TGFβ. We found that NLRP3 augmented TGFβ-induced receptor associated Smad (R-Smad2/3) phosphorylation, nuclear accumulation and transcriptional activity in a Nucleotide Binding Domain-dependent mechanism. Interestingly, these phenotypes appeared independent from canonical NLRP3 signalling through caspase-1, IL-1β and IL-18. The ability of NLRP3 to regulate diverse cellular processes suggested a potentially broad function in cellular physiology. We went on to establish that endogenous NLRP3 localized to mitochondrial structures in primary human cardiac fibroblasts, macrophages and epithelial cell

lines. Finally, using live-cell imaging and flow cytometry we found that NLRP3 potentiated the production of mitochondrial reactive oxygen species (ROS), which are required for TGFβ signalling and differentiation. Our results propose for the first time a general function for NLRP3 in structural and professional immune cells. A role for NLRP3 upstream of ROS offers a potentially unifying explanation for NLRP3 cytokine-dependent and independent pathways, and provides novel insight into the pathogenesis of chronic inflammation and fibrosis in cardiovascular disease.

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I have to acknowledge my friends and family for their ongoing confidence in my abilities. They have collectively put up with my moody cyclothymic behaviour during my training, and for that I am eternally grateful. Finally, my parents have always stood by my decisions in life, and I am thankful for all of their help in my academic achievements. Their continued support has motivated me to keep striving for success.

To the enduring pursuit of black swans, painful as it may be.

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List of Symbols, Abbreviations and Nomenclature

αSMA	α-Smooth Muscle Actin
AngII	Angiotensin II
APDC	(2R, 4R)-4-aminopyrrolidine-2, 4-dicarboxylate
APS	Ammonium Persulfate
ASC	Apoptosis-Associated Speck-like Proteins Containing a CARD
ATI	Angiotensin Receptor Type 1
ATP	Adenosine Triphosphate
CAPS	Cryopyrin-Associated Periodic Syndromes
CARD	Caspase Recruitment Domain
CF	Cardiac Fibroblast
CLR	C-Type Lectin Family
CNTg	Calcineurin Transgene
COXIV	Cytochrome C Oxidase IV
CTGF	Connective Tissue Growth Factor
DAG	Diacyl Glycerol
DAMP	Danger Associated Molecular Pattern
DED	Death Effector Domain
EDTA	Ethylendiaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FGF-2	Fibroblast Growth Factor-2
FLAG	Flag Octapeptide

FS	Fractional Shortening
GFP	Green Fluorescent Protein
H&E	Hematoxylin and Eosin Stain
HEK-293T	Human Embryonic Kidney 293 Cells Containing SV-40 Antigen
HeLa	Cervical Epithelial Cell Line
HF	Heart Failure
IκB	Inhibitor kappa B
IL-1β	Interleukin-1-β
IL-1R	Interleukin-1 Receptor
IL-1ra	Interleukin-1 Receptor Antagonist
IP3	Inositol Triphosphate
LV	Left Ventricle
LVIdD	Left Ventricular Internal Dimension in Diastole
LVIDs	Left Ventricular Internal Dimension in Systole
LVPWd	Left Ventricular Posterior Wall Dimension
LRR	Leucine Rich Repeats
MMP-2	Matrix Metaloproteinase-2
NAC	N-AcetylCysteine
NACHT	NAIP, CIITA, HET-E and TP-1
NB-ARC	Nucleotide Binding and Oligomerization Region
NBD	Nucleotide Binding Domain
NFκB	Nuclear Factor kappa-Light Chain-Enhancer of Activated B Cells
NLR	Nucleotide-Binding Domain and Leucine Rich Repeat Containing

NLRP	NLR Family, Pyrin Domain Containing Protein 3
PAMP	Pathogen Associated Molecular Pattern
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PRR	Pattern Recognition Receptor
PYD	Pyrin Domain
Redox	Reduction and Oxidation
RLR	Rig-Like Helicase Receptor
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Smad	Mothers Against Decapentaplegic Homolog
TEMED	Tetramethylethylenediamine
TGFβ	Transforming Growth Factor β
TGFRI	Transforming Growth Factor Receptor
THP-1	Human Monocytic Cell Line
TIR	Toll Interacting Region
TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor α
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
WA	Walker A Motif
WT	Wild Type

Chapter One: **Introduction**

1.1 The Maintenance of Tissue Homeostasis

The diversification of cellular life approximately 543 million years ago with the Cambrian explosion brought about the origins of multicellular, complex living systems in the Metazoan kingdom[1]. Perhaps the greatest change that arose from cellular and tissue diversification was the need for protection and maintenance of a stable physiological state. Specialized cellular processes of secretion, replication and aspects of eukaryotic cellular biology rely on the regulation of electrical, ionic, pH, redox and many other spatiotemporal gradients. Injurious stimuli that disrupt these gradients pose threats to the cells that comprise tissues, which is why organisms have evolved complex detection and effector mechanisms to combat these threats. The ways by which cells detect and respond to injurious stimuli is collectively referred to as immunity, with the ultimate function of maintaining cellular, tissue and organismal homeostasis[2]. Two distinct components operate in vertebrates: the phylogenetically ancient innate immune system, and the more recent adaptive system[3]. While the adaptive immune system operates by somatic gene rearrangements to generate direct antigen-targeting specificity, the innate immune system incorporates diverse cell types with sets of germline-encoded proteins, secreted substances and barriers[4].

1.1.1 Injury and Repair

In order to understand how immunity functions in health and disease, it is useful to imagine three potential outcomes of a theoretical "injury" (Figure 1A). Assuming the injury is of sufficient intensity to induce tissue damage, the resulting destruction could be completely repaired with no lasting residual changes. Alternatively, restorative processes could lead to complete functional recovery with changes in tissue architecture only. Lastly, repair mechanisms

may be altogether insufficient, with a resulting degree of both functional impairment and remodelled architecture. This third scenario underscores the basis of human chronic diseases, because with time the residual functional impairment following longstanding injurious stimuli feedback to act as additional injuries themselves, creating cyclical damage and non-resolving inflammation[5].

The type of injury must also be considered, because not all injuries appear to impart the same degrees of damage or elicit the same immune activating pathways. Three categories can occur: pathogenic infection, acute non-microbial injury, and chronic non-microbial injury (Figure 1B). During infection, conserved microbial elements called Pathogen Associated Molecular Patterns (PAMPs) are detected by host cells of the innate immune system[6]. In general, these motifs are structurally unique and critically vital for pathogen survival, ensuring a targeted response on their recognition. Identification of PAMPs results in the production of soluble factors called cytokines that recruit and activate additional cellular mediators into the damaged tissue, initiating an inflammatory cascade. This ability of organisms to detect and eliminate pathogens confers a tremendous evolutionary advantage because it represents the capability of a cell to identify harmful environments. However it is now appreciated that pathogenic factors are not the sole activators of inflammatory processes. Endogenous host signals are also able to activate innate immune pathways in the absence of microbes[7-9]. Acute non-microbial injuries are mediated by the release of such host factors termed Danger Associated Molecular Patterns (DAMPs). Similar to PAMPs, DAMPs are typically substances that are sequestered from the extracellular environment, such as ATP, nucleic acids and

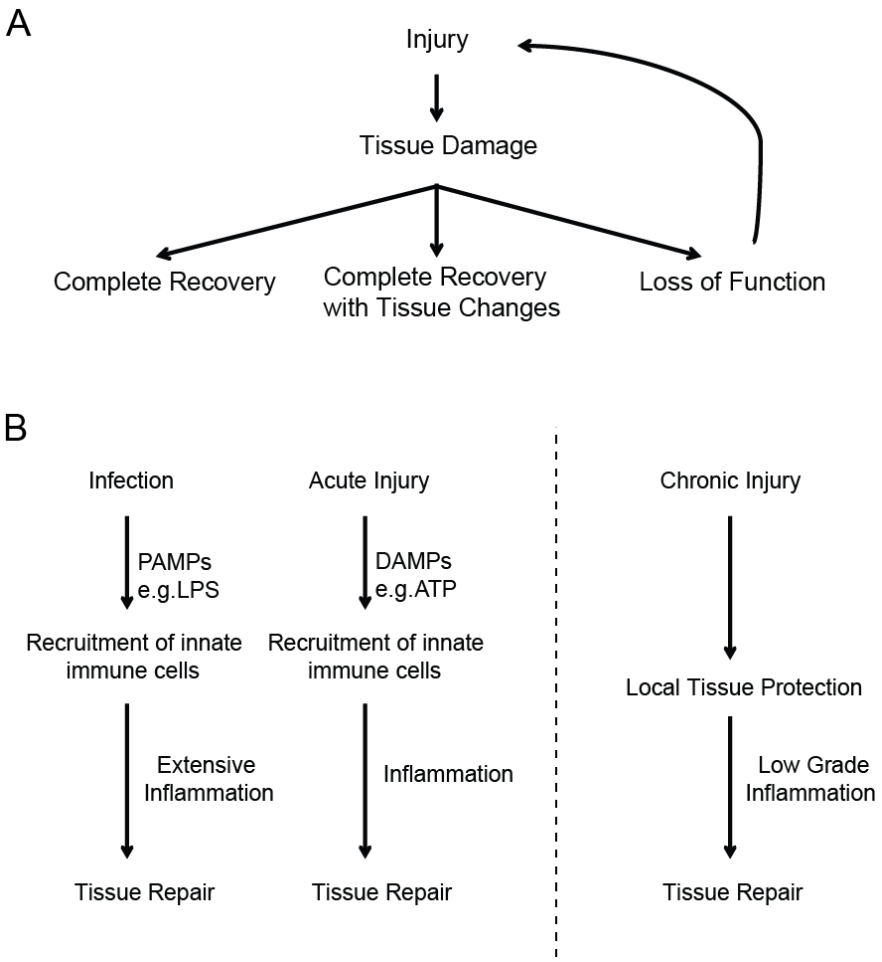

Figure 1. Patterns of injury and repair.

A. Three potential phenotypes following hypothetical injury. When wound-healing pathways are all together insufficient, loss of function occurs. Impaired organ function can persist and cause cyclical damage by acting as a non-resolving injury, giving rise to chronic disease. **B.** Infection and acute non-microbial injury operate through classical pattern recognition, with resulting inflammation and subsequent resolution and repair. In contrast, chronic injury involves hierarchical mechanisms that result in persistent low-grade inflammation, with predominant fibrosis and tissue remodelling.

metabolic products like monosodium urate[10,11]. Again, DAMP recognition by cells of the innate immune system leads to tissue infiltration by phagocytic cells, induction of proinflammatory cytokine production and initiation of tissue repair. One classic example of acute non-microbial injury is ischemia, which in the context of the coronary arterial system produces the acute coronary syndromes. When cardiac myocytes undergo necrosis in the absence of sufficiently oxygenated blood flow, release of DAMPs recruits and activates differential populations of leukocytes to initiate repair pathways[12]. These immune cells recognize host-derived factors to mount an appropriate and timely response, limiting the potential damage caused by cellular loss.

In contrast to pathogenic and acute non-microbial injuries, chronic injuries appear to induce distinct wound healing processes. For example, myocardial tissue from patients with chronic heart disease displays only mild cellular infiltrate and low-grade inflammatory cytokine production[13,14]. Instead, histology generally shows predominant fibrosis and extensively remodelled architecture, reminiscent of the cyclical damage scenario previously described[15]. Although the inflammation observed during chronic injury is far different from microbial and acute non-microbial injuries, isolated cells and tissue specimens from patients with chronic diseases still display many significant abnormalities- cytoplasm becomes vacuolated, the membrane architecture changes, organelles are remodelled and the extracellular environment is restructured[16]. These findings likely reflect differential capabilities of resident cells and professional immune cells at maintaining homeostasis during injurious settings. Thus it is clear that the mechanisms governing innate immunity in disease rely not only on the inciting source, but also on the duration and extent by which an injury occurs.

1.1.2 Principles of Defence

There are clearly many diverse types of injuries that can impact an organism, and in turn, many ways in which the organism can combat such insults. In theory, an organism can manage injurious threats by way of three potential actions: avoidance, resistance, and tolerance[17]. Avoidance is characterized by behavioural modifications that reduce exposure to potentially adverse stimuli. Subjectively unpleasant odours of bacterial amino acid metabolic products cadaverine and putrescine prevent prolonged exposure of humans to decaying flesh, thereby offering protection by reducing contact with pathogenic environments[18]. Once contact has been made, resistance then functions as the specialized mechanisms by which an ongoing injury is directly modified by the host. The expression of diverse sets of receptors that recognize PAMPs and DAMPs allow for the targeted identification of pathogens that have breached host barriers, facilitating production of soluble agents that directly neutralize invaders. Given their overt nature and potential for collateral host damage, resistance mechanisms have classically been the most widely studied aspects of immunology[19]. In contrast, tolerance reflects the ability of an organism to maintain homeostasis through the alterations of structure and function, independent from the absolute burden of injury[20]. For example, humans harbouring the sickle cell anemia mutation in the hemoglobin beta chain show reduced susceptibility to malarial neuropathology[21]. This has been recently shown to result from an induction of heme-oxygenase by damaged erythrocytes, conferring protection despite the same absolute degree of plasmodium infection. Tolerance can therefore be regarded as a time-dependent plasticity of organ systems, remodelling to adapt to changing environments.

1.1.3 Wound Healing Mechanisms in the Cardiovascular System

1.1.3.1 Avoidance and Resistance Measures

We now recognize that an organism can manage an injurious stimulus either by avoiding dangerous scenarios, targeting the injury itself as in resistance, or rendering itself less susceptible to injury as in tolerance[22,23]. The examples listed above reflect our recently improved understanding of these concepts in pathogen immunity; however the same principles can be applied to non-microbial injuries and chronic diseases. For example, variable cardiac ischemia and local myocardial stress result in a symptom complex of tachycardia, chest pain, dyspnea, diaphoresis and nausea that are unpleasant deterrents for the activities precipitating increased oxygen demand[24]. Ascending cardiac sympathetic fibers transmit nociceptive information from the heart to the spinal cord, which manifests pain. These fibers can be remodelled with altered sensitivity in longstanding heart disease, reflecting the importance of avoidance behaviour in longstanding cardiac dysfunction.

Resistance mechanisms are also important in the response to non-microbial injury. During cardiovascular disease, injuries mostly result from hemodynamic alterations that cause the death of cardiac myocytes by necrosis[25]. Unregulated cell death results in the release of DAMPs, which are sensed by peripheral leukocytes that migrate into the tissue and secrete local factors aimed at modulating metabolism, limiting cell death and removing cellular debris[26]. In the heart, the recruitment of innate immune cells occurs in distinct stages. Initially, marginated neutrophils migrate into the myocardium in response to established chemokine gradients[27]. Activated neutrophils release factors like Annexin A1 and lactoferrin that limit further neutrophil influx[28]. Neutrophils are short-lived cells, and their death releases chemokines that recruit

additional phagocytic cells called monocytes. When monocytes extravasate across capillary endothelium into the tissue, they differentiate into professional phagocytic and cytokine secreting macrophages. The phagocytosis of dying neutrophils stimulates macrophages to produce anti-inflammatory cytokines like IL-10 and TGFβ, facilitating progression from the initial inflammatory phase to a proliferative and finally resolution phase of wound healing[29]. When properly coordinated, the consequences of leukocyte recruitment directly limit on-going cell death and injury[30]. They represent classical resistance measures by the host to detect a harmful stimulus and mount an appropriate response. The downstream changes in tissue architecture that result, however, can be separated from these pathways, and suggest that similar to pathogen immunity, tolerance also plays a critical role in homeostasis.

1.1.3.2 Tolerance Measures

While the restoration of hemodynamic parameters and abrogation of cell death are critical in regulating homeostasis in the cardiovascular system, vertebrates have also adapted mechanisms to render tissue less susceptible to ongoing damage. For example, chronic elevations in systemic blood pressures and low-grade ischemia lead to remodelling within the heart by the diffuse deposition of extracellular matrix scar tissue called reactive fibrosis[31,32]. Fibrosis increases the tensile strength of damaged tissue, limiting the effects of impaired cellular function on integrity[33,34]. Importantly, the protective benefits of modified tissue architecture proceed somewhat independently from the various initial damaging stimuli that result in its formation, again supporting its classification as a tolerance pathway. Fibrosis thus represents a conserved measure that is displayed in many organ systems including the heart, kidney, liver, gut and lungs[35]. The importance of fibrosis as a mechanism of tolerance during injury is underscored by

genetic studies that disrupt conventional pathways regulating scar formation. Experimental myocardial infarction in mice lacking key pathways in fibrotic signalling display increased incidence of cardiac rupture, impaired infarct healing and increased mortality[36,37]. However the formation of scar tissue is not without long-term consequence- fibrosis in human patients with heart failure predicts for worse survival, and diffuse myocardial fibrosis in longstanding heart disease further impairs cardiac function by cyclical damage[38]. It is clear that fibrosis, like other aspects of innate immunity in immunopathology, is initially adaptive, becoming maladaptive when dysregulated over time. Tolerance thus imparts only short-term benefit, because the inciting injury is ultimately left to proceed in the background, resulting in further activation of resistance pathways. The inability of tolerance measures like fibrosis to provide long-term protection likely gives rise to the decompensation phenotype often displayed by patients with chronic diseases, such as in heart failure (Figure 2).

1.2 Heart Failure as a Chronic Disease

1.2.1 The Clinical Syndrome of Heart Failure

As previously described, vertebrates maintain homeostasis in response to ongoing injury by avoidance, resistance and tolerance. Ultimately, neurohumoral adaptations and

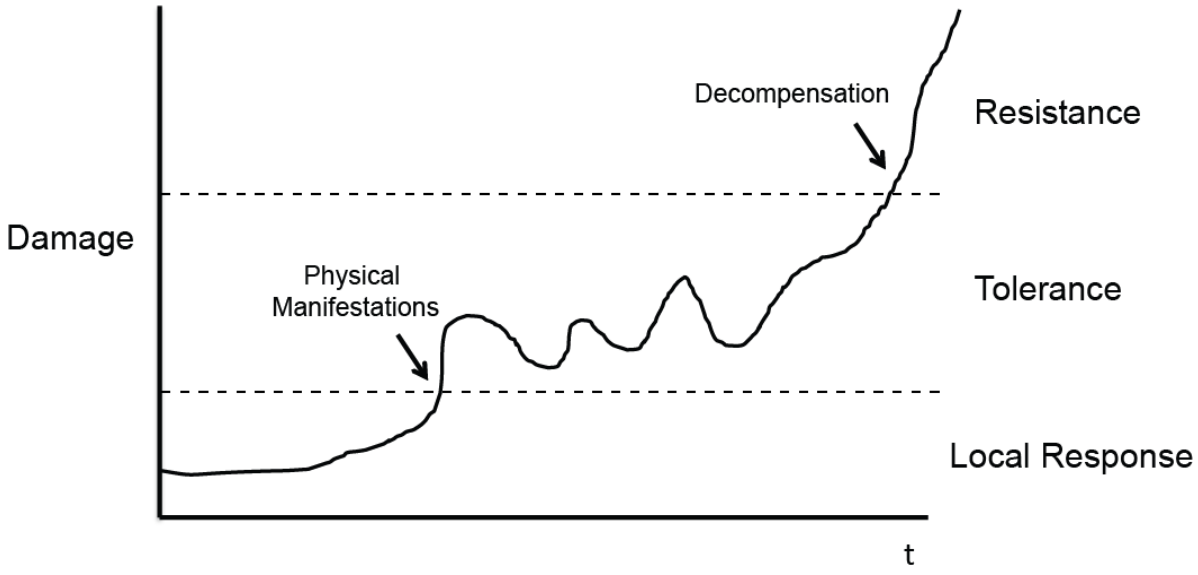

Figure 2. Time-dependency of chronic injury and repair.

During chronic injury, increasing tissue damage gives rise to hierarchical repair pathways. Initially, low levels of damage are maintained by alterations in resident cellular function. Persisting injury ultimately breaches arbitrary thresholds and activates local tissue tolerance responses, such as reactive fibrosis and remodelling. These are often accompanied by physical manifestations, reflective of altered organ function. When tolerance pathways are no longer sufficient, decompensation occurs, with the final initiation of overt inflammatory and resistance measures.

recruitment of innate immune cells act as resistance measures, while fibrosis induces tissue tolerance to produce a spectrum of wound healing pathways that result in lasting changes to structure, function or both. Therefore, any persistent injury that acts on the heart over time gives rise to a similar sequence of events to produce chronic organ impairment. The clinical constellation of signs and symptoms observed in patients with chronic heart disease are collectively referred to as heart failure (HF)[39]. Regardless of etiology, diverse injurious stimuli within the heart lead to a final common pathway towards impaired cardiac function with a well-described clinical presentation. Evidence from the Framingham Heart Study demonstrated that the lifetime risk of developing HF is one in five, and 50% of patients will not survive past 5 years[40]. Approximately 23 million people around the world are affected by HF and it remains the most common cause for admission to hospital in North America[41,42]. Accordingly, HF is a tremendous clinical problem that finely illustrates how longstanding tissue damage gives rise to different wound healing mechanisms in attempts to maintain homeostasis.

Historically, cardiac hemodynamic function has been depicted using analogies to a pump. The heart is effectively a 4-chambered muscular structure consisting of two connected but semi-independent pumps[43]. The left ventricle (LV) normally accepts oxygenated blood at relatively low filling pressures during diastole, and subsequently propels the bolus of blood forward at higher pressures during systole. It is intuitive that cardiac dysfunction can occur via two mutually exclusive processes- those that impair cardiac ejection (systolic HF) and those that impair cardiac filling (diastolic HF)[44]. The heart failure syndrome therefore encompasses both of these processes, defined as a symptom complex resulting from the inability of the heart to pump blood efficiently to peripheral tissues, or the ability to do so only under the requirements of elevated filling pressures. Approximately two thirds of all patients with symptomatic disease

have echocardiographic evidence of impaired LV systolic function with a reduced ejection fraction. These patients typically present with symptoms related to pulmonary congestion and reduced forward flow, including dyspnea (orthopnea and paroxysmal nocturnal dyspnea), pulmonary congestion, lower extremity edema and elevated jugular venous pressures[45-47]. In contrast, the remaining third of patients present with similar symptoms in the context of a preserved ejection fraction, and instead demonstrate elevated diastolic filling pressures with reduced ventricular compliance related to underlying fibrosis[48]. The prevalence of diastolic HF increases with age and is more common in females in the setting of hypertension and diabetes[49,50]. Systolic HF, in contrast, is more commonly seen in males with ischemic heart disease following myocardial infarction. It is currently hypothesized that systolic and diastolic disease exist as different stages in a uniform entity, and that overt systolic decompensation takes place in the context of chronically elevated filling pressures and cardiac remodelling processes through a host of cellular and molecular mediators[51].

1.2.2 Cardiac Fibrosis and Resistance/Tolerance Crosstalk

1.2.2.1 Mechanisms of Fibrosis

Pathological studies examining tissue from patients with end-stage heart disease have continued to support the importance of fibrosis in wound healing and disease pathogenesis. The cardiac extracellular matrix (ECM) is a structurally complex mixture of fibrillar collagen, elastin, laminin, fibronectin and other proteoglycans that anchor cells into a three dimensional network[52]. It is a dynamic microenvironment by which diverse cell types within the heart signal and respond to each other[53]. For instance, in response to chronic hypertension, increased deposition of fibrous

collagen has been observed in both animal models and human tissue samples, with proliferation and differentiation of non-myocyte cell populations within fibrous regions[54]. Importantly, the localization of ECM remodelling in chronic processes occurs in a diffuse interstitial pattern, in contrast to the classically localized scar formation following acute injury[55]. Cardiac fibroblasts comprise approximately 40-60% of the total cellular compartment in the adult human heart[56]. Within the myocardium, resident fibroblasts respond to mechanical and soluble stimuli to change morphology and differentiate into specialized wound healing cells called myofibroblasts. These cells actively participate in reparative processes and ECM remodelling, increasing the tissue tolerance to on-going damage[57]. Myofibroblasts produce increased amounts of collagen and exert weakly contractile forces on adjacent tissue due to a specialized 'fibronexus' structure that anchors cell adhesion proteins with dense α-Smooth Muscle Actin (αSMA) containing bundles of cytoplasmic stress fibres[58]. Interestingly, many of the anti-inflammatory and resolution cytokines secreted by macrophages following inflammatory wound healing stages have been shown to activate fibroblasts and induce myofibroblast differentiation, reflecting the cross talk that occurs amongst different defence paradigms. Myofibroblasts have been identified within pathological tissue specimens and in cell culture by their increased expression of proteins such as αSMA, the discoidin domain-containing receptor 2 (DDR2/CD167b) and vimentin. Their exclusive appearance following injury within multiple tissue types and organ systems indicates that myofibroblasts are the chief regulators of remodelling in heart disease[33,59,60]. As a result, the signalling pathways regulating their differentiation and activation have become a critical topic of investigation.

1.2.2.2 TGFβ and Angiotensin II

Myofibroblast cell biology has been routinely studied in culture systems through treatment of primary cardiac fibroblasts with the pro-fibrotic mediators Angiotensin II (AngII) and Transforming Growth Factor-β (TGFβ)[61]. AngII and TGFβ are both highly expressed within diseased cardiac tissue, and their signalling pathways have been shown to mediate transformation of cardiac fibroblasts into myofibroblast phenotype both *in vivo* and *in vitro* using extensive crosstalk.

TGFβ is a pro-fibrotic and anti-inflammatory cytokine produced by diverse cell types in diseased tissue. It exists in at least 3 isoforms and is synthesized/secreted as a precursor protein coupled to the Latent TGF Binding Protein (LTBP) and Latency Associated Peptide (LAP)[62]. The active form is produced following cleavage of the associated proteins by serum proteases[63]. A central role for TGFβ in cardiac fibrosis has been firmly established with the use of knockout/transgenic mouse studies. Abrogation of TGFβ signalling with neutralizing antibodies reduced fibrosis and chamber dysfunction following myocardial pressure overload[64]. Interestingly, TGFβ is highly expressed during the transition from stable HF to decompensated disease, further supporting the importance of fibrosis in longstanding cardiac dysfunction[65]. TGFβ signalling is well described to proceed through canonical activation of the Smad pathway, with resulting increases in fibrotic gene expression (collagen, αSMA, vimentin) (Figure 3)[66]. Binding of TGFβ to the TGF type II receptor (TGFRII) induces hetero-oligomerization and

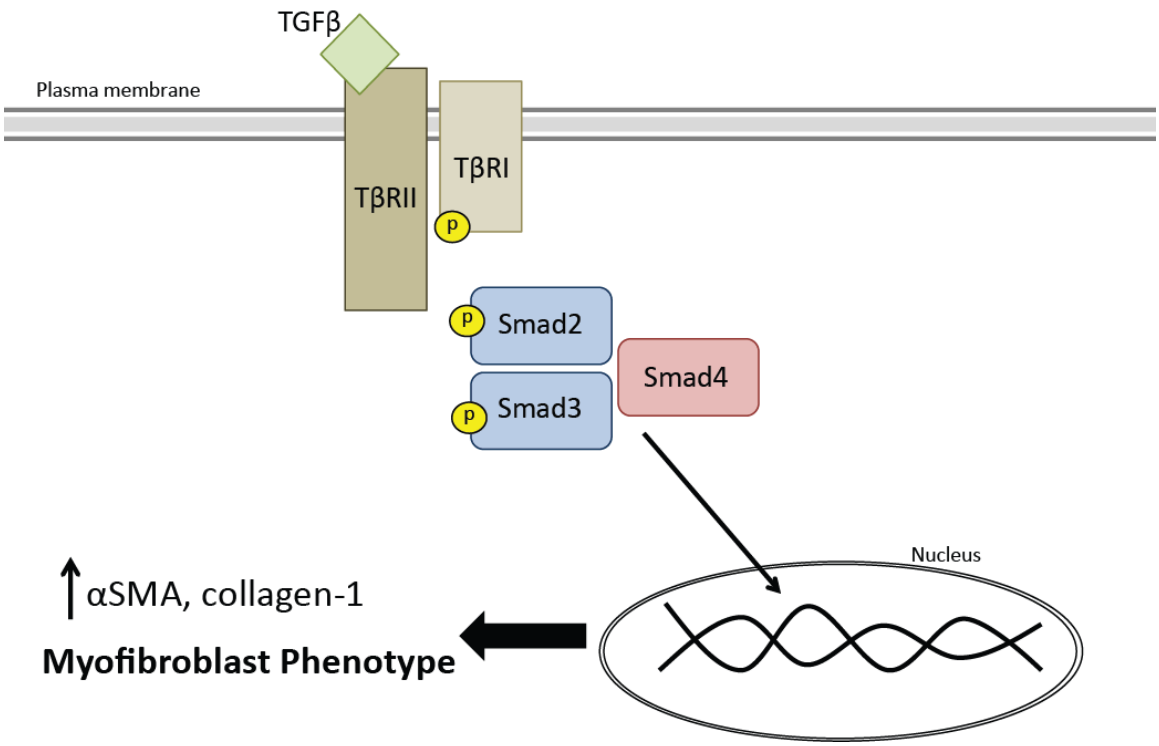

Figure 3. The canonical R-Smad pathway.

Binding of TGFβ to TβRII results in heterodimerization of TβRI/TβRII, with phosphorylation of the intracellular TβRI domain. This recruits the receptor-associated Smads (R-Smad2/3) to the membrane complex, which are phosphorylated on serine/threonine residues. Phosphorylated Smad2/3 go on to associate with the co-Smad Smad4 to form a transcription factor complex that translocates to the nucleus and regulates fibrotic gene expression programs.

phosphorylation of the type-I receptor by the intracellular serine/threonine kinase domain of TGFRII[67]. The phosphorylation of TGFRI results in the subsequent recruitment and phosphorylation of serine/threonine sites on the receptor-associated Smads, Smad2/3. In a highly organized and regulated pathway, the TGFRI/II activated phospho-Smad2/3 complex is endocytosed, resulting in the release of phospho-Smad2/3[68]. This results in the recruitment of coSmads (Smad4), forming the functional transcription factor Smad2/3/4 that acts on Smad binding elements (SBE) to regulate fibrotic gene expression. The persistent activation of TGF signalling has been shown to promote myofibroblast phenotype and induce epithelial-mesenchymal transition in various organ systems including the heart, kidney, lung, liver and skin[69]. It has been established as a critical factor in the development of both innate immune dysfunction and tissue fibrosis in animal models and human patients[70,71].

Angiotensin II (AngII) is a vasoactive decapeptide that is the key mediator of the Renin Angiotensin System (RAS). In response to reduced perfusion pressures and lowered serum osmolality, juxtaglomerular cells in the kidney secrete the enzyme renin, which cleaves hepatically produced Angiotensinogen into Angiotensin I. Angiotensin I is then further processed by the Angiotensin Converting Enzyme (ACE) to produce AngII. The importance of AngII in cardiovascular disease is demonstrated by the utility of ACE inhibitors and angiotensin receptor blockers (ARBs) used clinically, as these agents are among few drugs shown to reduce mortality in patients with HF[72]. In addition to its modulation of systemic blood pressures within the vasculature, AngII exerts pro-fibrotic tolerance effects on cardiac fibroblasts via two mechanisms. First, as a hypertensive factor, AngII increases systolic pressure, resulting in pressure-mediated local production of other fibrotic factors such as TGF and CTGF[73]. However AngII is also capable of inducing myofibroblast phenotype on its own, upregulating αSMA in

isolated cardiac fibroblasts and renal tubular epithelial cells even in the absence of paracrine signalling. Indeed, treatment with AngII has been shown to increase Smad2/3/DNA binding in tubular epithelial cells, and induces a bi-phasic phospho-Smad3 response in vascular smooth muscle cells, indicating both early direct and late paracrine effects[74]. The AT1 receptor is a classical Gq protein coupled receptor, signalling through phospholipase C (PLC) mediated production of diacylglycerol (DAG) and inositol triphosphate (IP3)[75]. IP3 acts at the IP3 receptor on the endoplasmic reticulum to increase cytosolic calcium, while DAG facilitates downstream signalling through protein kinase C (PKC), ultimately altering gene expression programs to regulate differentiation, proliferation and migration. AngII has also been found to signal downstream of the AT1 receptor through non-canonical crosstalk involving Smad3 in both vascular and renal tissue[74,76,77]. Vascular samples from hypertensive patients with atherosclerosis demonstrated increased Smad2/3 transcriptional activity[78]. In animal models, infusion of mice with AngII results in a progressive cardiac injury that results from combined local effects with TGFβ, in addition to chronic hypertension[65,79]. Importantly, studies have shown that this animal model of heart failure occurs in a largely TGFβ/Smad dependent mechanism, reliant on functional Smad2/3 signalling[80]. It is evident then that the activation of Smad signalling downstream of both TGFβ and AngII axes represents a final common pathway towards tissue fibrosis, combining to regulate the degree of damage in response to diverse injurious stimuli.

1.3 Molecular Mechanisms of Innate Immunity

1.3.1 Construction of Immune Sensors Through Conserved Domains

Clearly the pathophysiology of chronic heart disease is complex, with diverse cellular players interacting to facilitate architectural and structural changes. Both innate immune cell recruitment and local resolution/fibrosis by myofibroblasts act in concert to maintain homeostasis in the failing heart. The nature of these responses in chronic disease and cyclical injury represents potentially shared signalling paradigms. To better understand these pathways, it is therefore critical to explore innate immune proteins that are jointly expressed by both infiltrating immune cells and resident populations.

The Pattern Recognition Receptors (PRRs) are germline encoded proteins that mediate innate immune cell activation by danger signals like PAMPs and DAMPs[4,81]. PRRs are modular proteins containing domains that are expressed throughout the proteome. Invertebrates rely solely on PRRs for immunity, and the conservation of their structures across multiple organisms suggests that they evolved from an ancient defence mechanism that has since developed into a complex network[82]. Activation of PRRs in professional innate immune cells results in pleiotropic effects, including the downstream mobilization of soluble factors, induction of co-stimulatory molecules to activate adaptive immunity, and facilitation of other specialized defence processes by transcriptional activation of NFkB or Interferon (IFN) signalling[83,84].

The PRR superfamily consists of 4 subfamilies- the Toll-like Receptors (TLRs), C-type lectin receptors (CLRs), RIG-1 (Retinoic-acid-inducible gene 1)-like receptors (RLRs) and the Nucleotide binding domain containing leucine-rich repeats (NLRs). The classification of these proteins is largely based on their underlying structural similarities. There are eight key domains

that rearrange in different combinations to assemble all PRRs and ultimately a reductionist view of the innate immune system[85,86]. These include the Toll Interacting Region (TIR), Leucine Rich Repeat (LRR), Caspase Recruitment Domain (CARD), Pyrin Domain (PYD), Nucleotide Binding Domain (NBD), RNA Helicase, C-Type Lectin Domain (CTLD) and Immunoreceptor Tyrosine based Activation Motif (ITAM). While the CLRs and RLRs are generally involved in antimicrobial immunity, the TLRs and NLRs respond to wide range of DAMPs and PAMPs and are expressed in structural cells in addition to professional immune cells. Their contributions to human disease have been extensively explored recently[87-89]. Accordingly, much attention has gone into better understanding the modular components that assemble these families, the TIR, LRR, NBD, CARD and PYD domains.

1.3.1.1 TIR Domain

The TIR domain is a shared signalling structure present in all TLRs, the IL-1 receptor (IL-1R) and intracellular signalling adaptors MyD88, Mal, TRIF and TRAM[90]. As a component of plasma membrane bound receptors, the TIR domain facilitates dimerization of TLRs and the IL-1R following ligand binding[91]. The close proximity of dimerized receptors then recruits TIR-containing adaptors to the membrane receptor complex, resulting in the downstream activation of kinase cascades leading to NFkB activation[92]. The conservation of TIR domains amongst the IL-1R and TLRs is a critical mediator underlying the historical observations that both lipopolysaccharide (LPS) and Interleukin-1β (IL-1β) produce similar cellular responses. Moreover, the identification of homotypic TIR-TIR interactions to facilitate downstream signalling and recruitment of additional TIR-containing adaptors has served as a model for subsequent paradigms in the study of apoptosis and inflammation[93].

1.3.1.2 LRR

In addition to the TIR domain, the TLRs contain the 20-29 residue LRR domain that is also present in NLRs[94]. It has been established that in TLRs, the LRR is responsible for generating PRR diversity. Its presence in jawless fish in the Variable Lymphocyte Receptors (VLRs) produces genomic rearrangements from a single germline encoded gene, offering unique comparison to the somatic rearrangements of the immunoglobulin domain of vertebrates used for antigen specificity[95,96]. In the TLRs, the LRR is an extracellular region that surveys the extracellular environment for PAMPS and DAMPs.

1.3.1.3 NBD

The NBD family contains distinct subfamilies: NACHT (NAIP, CIITA, HET-E and TP1), NB-ARC (Nucleotide Binding adaptor shared by Apaf-1, R proteins and CED-4) and NOD[97]. Based on sequence comparison these families correspond to those involved in apoptosis (eg Apaf-1 NB-ARC ATPases) and those involved in stress resistance or inflammatory signalling (eg NACHT found in NLRs)[98,99]. As a group of P-Loop NTPases, the NACHT domain of NLRs has been shown to contain the two strongly conserved Walker A and Walker B motifs to bind β and γ phosphates of nucleotides in the presence of Mg^{2+}[100]. The Walker A motif has a characteristic GxxxxGK[ST], and is conserved among all NLRPs. It is believed that in response to nucleotide binding, the NACHT domain facilitates self-oligomerization that is required for further NLR signalling via homotypic interactions and recruitment of additional proteins[101,102]. Many of the NLR NACHT domains are predicted to preferentially bind ATP/dATP over GTP, with the exception of CIITA. The overall conservation of the NBD family in general implies

structural importance, and its mutation in various NLRs gives rise to a range of genetic diseases, further establishing its importance in protein function.

1.3.1.4 CARD/PYD

The CARD and PYD domains are structurally related members of the Death Fold Domain superfamily, which also includes the Death Domain (DD) and Death Effector Domain (DED) members involved in apoptosis[103]. The defining feature of Death Fold Domains is the inclusion of six alpha helices that have been shown to associate through homotypic interactions. The CARD is found in a variety of proteins, including the Caspase family, facilitating their association and oligomerization following activation of cell death and inflammatory signals[104,105]. The PYD is only weakly associated with the Death Fold Domain family, though its' structural similarity has been verified by NMR[106]. Interestingly, it lacks the H3 helix that is important for homotypic interactions by the other members, raising the question of whether PYD-PYD interactions are structurally different than the other members[107]. Protein mining for PYD and CARD-containing proteins helped establish the NLR family, an array of intracellular proteins involved in unique cellular processes.

1.4 The NLR family of Pattern Recognition Receptors

While the TLR family of PRRs are membrane bound and survey the extracellular environment for danger signals, the NLRs are a family of intracellular proteins that share a general tripartite structure[108]. The C-terminus contains the LRR, with a central NBD and N-terminal effector domain containing either a PYD or CARD. Based on the homology and identity of the N-terminus, the NLR family can be sub-classified into 4 groups: The acidic transactivating

domain-containing NLRA (NAIP), the BIR-containing NLRB, CARD-containing NLRCs, and PYD-containing NLRPs[109] (Figure 4). Alternatively, phylogenetic sub-classification produces 3 categories: The NODs, NLRPs and IPAF/NLRC4[97,110]. Interestingly, one member, NLRX1, does not contain an N-terminal effector domain, but instead has a mitochondrial targeting sequence, placing it into a distinct category of its own[111,112].

With the exception of the NODs, very little is known regarding how some NLRs respond to DAMPs or PAMPs. While initial reports suggested that the NLRPs were capable of binding to PAMPs such as muramyl dipeptide and bacterial flagellin, the concept that they actually utilize their LRR domains to bind ligands in the classical concept of a "receptor" is not consistently supported, and has since fallen out of favour[113,114]. Instead, the NLRPs generally appear to regulate the cytoplasmic environment, facilitating downstream consequences of diverse microbial and non- microbial injurious stimuli to modulate cytokine production, signal transduction and ultimately cellular fate.

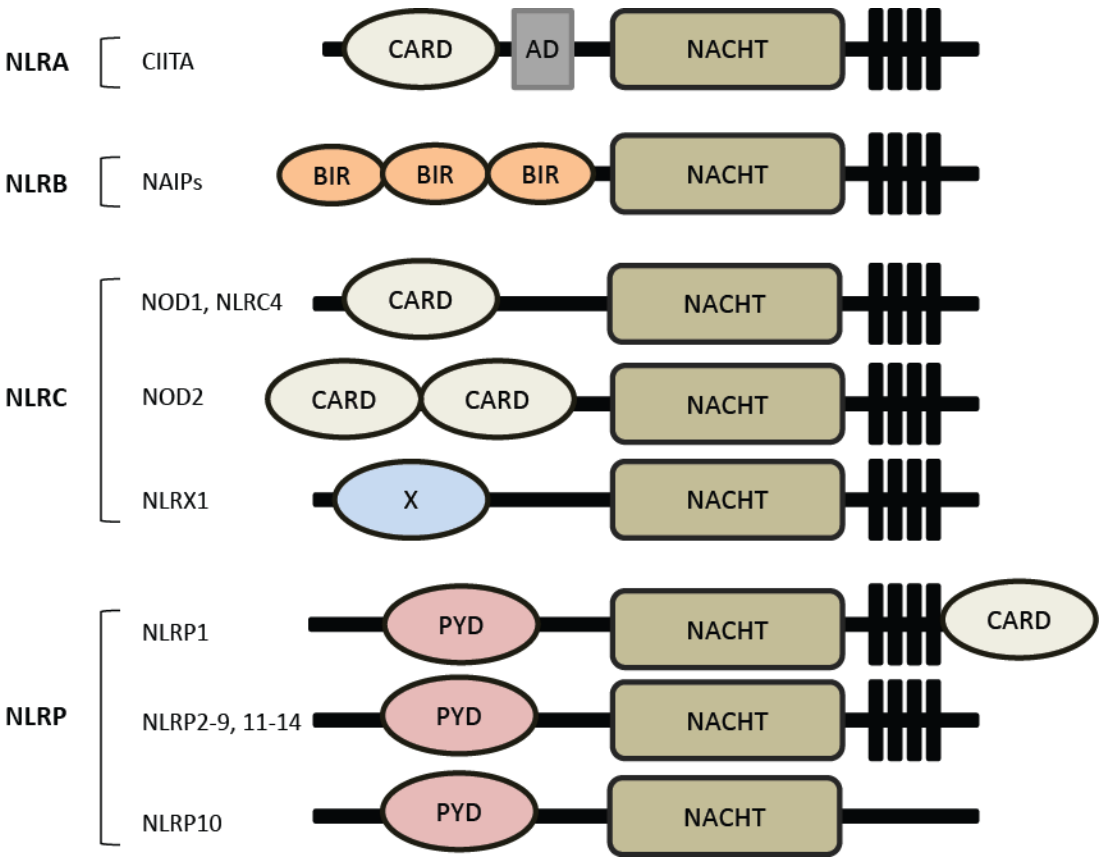

Figure 4. The NLR family of pattern recognition receptors.

CARD caspase recruitment domain; AD activating domain; NACHT NAIP, CIITA, HET-1, TP-1; BIR baculovirus IAP repeats; PYD pyrin domain.

1.4.1 NLRs and IL-1β-Mediated Systemic Inflammatory Disorders

IL-1β is a key proinflammatory-signalling molecule that is a member of the IL-1 family of cytokines. There are 11 members of the IL-1 family, including IL-1β, IL-18 and IL-1α[115]. As an endogenous pyrogen, IL-1β exerts its effects with high potency and induces fever at only 1-10 ng/kg[116]. The cellular consequences of IL-1β release by innate immune cells are diverse and ultimately impact proliferation, differentiation, migration and apoptosis. In human monocytes, IL-1β mRNA and protein rise rapidly during stimulation with LPS and other TLR ligands inducing NFkB activation[117]. IL-1β is atypical, in that once induced, it is synthesized as a 31 kDa precursor protein that lacks an N-terminal signal sequence and is retained within the cytoplasm in the absence of ER to Golgi transport[118]. Pro-IL-1β is only weakly active, and localizes within perimembrane vesicles following translation. Following its cytoplasmic accumulation, the activation and processing of IL-1β proceeds following a second stimulus such as extracellular ATP or perforating ionophore toxins like nigericin[119]. These stimuli facilitate the enzymatic cleavage of pro-IL-1β into the 17.5 kDa active form, which is then secreted in a poorly characterized mechanism. IL-1β exerts its proinflammatory effects on diverse cell types through binding to the IL-1 receptor (IL-1R), which displays similar domains and downstream consequences to the TLRs. Once secreted, binding of IL-1β to the IL-1R extracellular region results in the recruitment of the IL-1AcP co-receptor chain, forming a receptor-ligand complex at the cell membrane[120]. This results in the dimerization of receptors and recruitment of cytosolic adaptor MyD88 through TIR-TIR homotypic interactions, ultimately leading to kinase cascades that activate NFκB regulation of inflammatory transcriptional pathways and gene expression. IL-1β secretion is also accompanied by the co-release of a soluble IL-1R antagonist (IL-1ra), an

endogenous molecule that is believed to negatively regulate the highly potent effects of IL-1β signalling[121].

The significance of IL-1β has been most appreciated through its role in inflammatory diseases. In contrast to the autoimmune diseases that are typically caused by dysregulated adaptive immunity, autoinflammatory diseases are caused by defects in innate immunity and display periodic courses that relapse and remit[122]. The NLR family has also been implicated in clinically apparent chronic inflammatory diseases through the identification of various genetic associations. For example, mutations within the LRR domain of NOD2 have been associated with Crohn's disease, revealing possible roles in regulating the innate immune response to mucosal enteric pathogens[123]. While far less common than the inflammatory bowel diseases, a group of autoinflammatory disorders have revealed the importance of other NLRs in directly regulating pro-inflammatory cytokine production. The periodic fever syndromes are characterized by fevers, arthralgias, abdominal pain and intensely pruritic skin rashes. These include the cryopyrin associated periodic syndromes, which are caused by autosomal dominant missesnse mutations in the NACHT domain of the NLRP member NLRP3[124,125]. Interestingly, these syndromes are characterized by the unregulated constitutive release of IL-1β by peripheral blood mononuclear cells. Targeted antagonism of IL-1β ameliorates the symptoms in these patients, further demonstrating that the NLR family, and NLRP3 in particular, are critical regulators of inflammatory cytokine production[126].

1.4.2 NLRPs

Genomic database mining of PYD-containing proteins identified 14 members of the NLRP family that are characterized by their conserved N-termini[103]. NLRP1 is unique in that in addition to both CARD and PYD domains, it contains a distinct FIIND (function to find) domain of undetermined significance[97]. The human *NLRP* genes are located in two clusters on chromosomes 11p15 (6, 10, 14) and 19q13.4 (2, 4, 5, 7, 8, 9, 11, 12, 13). *NLRP1* is located on 17p13 and *NLRP3* on chromosome 1q44[127]. The *NLRP* genes also appear to cluster differentially in other species, suggesting that recent duplication events initially formed the NLRP sub-groups, giving rise to multiple combinations of PYD/CARD and NACHT containing proteins. Despite the presence of known orthologs across diverse species and their similarity to plant R proteins involved in the so-called effector triggered immunity, little is known about the overall function of NLRPs in vertebrate immunity. Moreover, while their expression profiles have not been extensively characterized, initial work has demonstrated the presence of some NLRs in both immune and solid organ tissues, suggesting potential fundamental roles in cellular biology. For example, NLRP1 has been found in the heart, thymus, spleen, kidney, liver and lung in addition to peripheral circulating leukocytes[128,129]. NLRP3 has consistently been found in circulating monocytes, heart, kidney, lung and liver. Clearly the NLRs within these tissue types displays a functional significance, however no unifying theories for NLRP function have been proposed in professional and non-professional immune cells.

1.4.3 Multi-Protein Caspase Activating Platforms

Despite the diverse tissue expression of some NLRs, their highest levels are found within circulating innate immune cells. Taken in association with the NLRP mutations in

autoinflammatory disorders, there is a clear role for the NLRPs in regulating pro-inflammatory cytokine production. Of particular importance are the PYD and CARD domains, which are members of the Death Fold Domain superfamily[130]. Cell death pathways have been well characterized to proceed through sequential Death Fold Domain recruitment of various proteins to ultimately produce multiprotein quaternary complexes and activate caspases. There are two types of apoptotic caspases, the initiators (caspase 2, 8, 9, 10) and effectors (caspase 3, 6, 7)[131,132]. Interplay of these different cysteine proteases mediate responses to cell death signals. For example, FasL binds to the Fas receptor, resulting in intracellular dimerization of Fas DD domains[133]. This leads to the recruitment of FADD, a DED-containing adaptor protein that also has a CARD. FADD-Fas interaction via DED domains exposes the CARD domain on FADD, recruiting and activating caspase-8 to initiate the process of cell death[134]. Caspase-8 activation of the effector caspase-3 characteristically results in DNA fragmentation, PARP cleavage and ultimately apoptotic cell death. The Fas/FADD/Caspase-8 complex is called the death inducing signalling complex, or DISC[135-137]. It represents a multiprotein caspase-activating complex that regulates cellular fate through the formation of sequential homotypic interactions. In addition to the DISC, other complexes have also been described to regulate apoptosis. The intrinsic apoptotic pathway proceeds following mitochondrial damage and release of cytochrome c into the cytosol. Cytochrome c then binds to Apaf, an adaptor protein containing CARD and NBD domains that recruits and activates caspase-9. The cytochrome c/Apaf/caspase-9 complex was called the apoptosome, another quaternary structure regulating cell death[138-141].

In addition to the apoptotic CARD-containing caspases, there are distinct caspases (caspase 1, 4, 5, 11 in mice and humans) that regulate inflammatory cytokine production[132]. Caspase-1 was initially called the IL-1β Converting Enzyme (ICE)[142]. It is a cysteine protease

that is present as a 45 kDa inactive precursor zymogen, and forms self-activated tetramers consisting of two 10kD and two 20kD subunits during inflammatory stimulation[143,144]. Like the other caspases, caspase-1 contains an N-terminal CARD domain, suggesting that it too could be mobilized by homotypic interactions. Caspase-1 has been shown to be expressed highly in monocytes, where it regulates IL-1β processing in response to LPS and nigericin. Since the adaptor protein apoptosis associated speck-like protein containing a CARD (ASC) contains both PYD and CARD domains, early work suggested that ASC could be capable of bridging caspase-1 with the NLRPs to mediate IL-1β processing and secretion[145]. Using cell free systems, NLRP1 was first shown to proceed through this mechanism, governing the processing of IL-1β through interactions with ASC and caspase-1[146]. Mechanical disruption of LPS stimulated THP-1 cells gave rise to a large complex when subjected to size exclusion chromatography. The putative 10^6 Da complex containing NLRP1, ASC, caspase-1 and caspase-5 was termed the "inflammasome", in homage to its apoptotic counterpart. While NLRP1 was found to associate with caspase-1 through the adaptor protein ASC, its association with caspase-5 was independent, suggesting the potential for alternative functions of inflammatory caspases downstream of NLRs. Direct visualization of NLRP1 complexes was later reported by electron microscopy in cell free systems following stimulation with muramyl dipeptide, proposing a model by which the NLRPs function to regulate secretion of IL-1β[113]. Interestingly, these multiprotein complexes formed by NLRP1, ASC and caspase-1 have not been verified using endogenous expression in cell culture. This has previously been attributed to the fact that its putative members (IL-1β, IL-18, caspase-1) are secreted in an as of yet uncharacterized mechanism following its assembly. Moreover, specific ligands like muramyl dipeptide are not universally accepted as NLRP1 activators,

further complicating its functional relevance in the physiological regulation of IL-1β and caspase-1[147].

1.4.4 NLRP3 Regulation of IL-1β

The identification of NLRP1 as a caspase-1 activating NLR suggested that the other NLRPs could also form quaternary complexes. Since *NLRP3* mutations lead to the autoinflammatory disorders previously described with constitutive IL-1β release, it represents an important protein in pro-inflammatory cytokine regulation. There are approximately 23 distinct mutations in the NACHT domain of NLRP3 that cause autoinflammatory disease, the best characterized is the R260W found in patients with Muckle Wells Syndrome[125,148]. Macrophages from these patients secrete significantly more IL-1β both at baseline and in the presence of LPS stimulation compared to healthy controls, again substantiating the importance of NLRP3 in regulating inflammatory cytokine production. Additionally, its expression in diverse tissues and cell types implicates a potentially unique role amongst the NLRs in cellular physiology.

Like NLRP1, NLRP3 has also been proposed to activate caspase-1 through ASC in overexpression and cell free systems. Immunodepletion of NLRP3 abolishes caspase-1 activation during overexpression studies, and the precipitation of ASC from LPS stimulated lysates also co-precipitates NLRP3 and caspase-1[149]. NLRP3 was later shown to preferentially bind ATP/dATP and act as an ATPase in mediating IL-1β secretion[150]. Importantly, nucleotide binding is critical for "inflammasome" assembly, and proceeds directly through the Walker A and B motifs in the NACHT domain. Mutation of the Walker A sequence GIGKT to GIAAA impairs NLRP3 dependent IL-1β production, nucleotide hydrolysis and oligomerization/complex formation. Clearly there is substantial biochemical evidence in overexpression, ectopic expression and

reconstituted cell free systems that NLRP3 is a regulator of caspase-1 and IL-1β cleavage through its central nucleotide binding region. However the specific roles of the LRR domain, and physiological relevance of the PYD in signalling through ASC and caspase-1 has not been thoroughly validated.

1.4.5 Models of NLRP3 Cytokine Regulation

Despite the absence of endogenous evidence for the "inflammasome", experimental murine knockout models proceeded to reveal the general activating stimuli and paradigms mediating mobilization of NLRP3 to regulate IL-1β. Since extracellular DAMPs and PAMPs were known to activate TLRs, many of the same signals were explored in mediating NLRP3-dependent IL-1β processing and secretion. Indeed, NLRP3 has been shown to regulate caspase-1 and IL-1β processing in response to a growing list of activators, including ATP, monosodium urate, silica crystals, asbestos, nigericin, membrane lipids, viral products, intracellular bacterial pathogens, direct production of reactive oxygen species (ROS), mitochondrial DNA and more[151-156]. In theory, the concept of redundant pathways acting both at the cell membrane (TLRs) and cytoplasm (NLRs) could function as important measures to respond to overwhelming danger signals during extensive tissue injury. Organisms harbouring functionally redundant pathways would thereby have an evolutionary advantage in damaging environments. However the diversity of NLRP3 "activators" raises the question of whether it acts as a receptor at all, considering the observation that TLRs in contrast have demonstrated high specificity for ligand binding through their extracellular domains[93,157]. The LRR of NLRP3 likely does not bind to all activating stimuli directly, suggesting that the various signals could lead to a common sequence of events that ultimately converge on NLRP3. Moreover, the consequences of NLRP3 activation are drastic.

Following release of IL-1β, macrophages undergo a distinct form of inflammatory cell death that results in release of the intracellular contents called pyroptosis. While this has been linked to ASC dimerization and caspase-1 cleavage via formation of the pyroptosome, its effector mechanisms and endogenous physiological significance are unclear[158]. Moreover, it is unclear how the presence of ASC dimers/oligomers on SDS PAGE has been reported with the use of denaturing and reducing gels. Despite these important unresolved issues, it is generally well accepted that the activation of caspase-1 by NLRP3 represents a highly regulated event that requires multiple checkpoints in order to prevent unrestricted inflammation.

There are three theories that attempt to explain how various signals converge on NLRP3: potassium efflux, lysosomal destabilization and production of ROS[159]. The role of potassium has been implicated through the observation that many activators such as the pore forming toxins and crystalline substances require K^+ efflux in order to induce IL-1β secretion[160-162]. Pore forming toxins directly mediate efflux of K^+, and ATP induces P2X7/pannexin association to directly extrude K^+. Formation of the pyroptosome also requires low intracellular K^+, and alterations in extracellular K^+ appear to impact caspase-1 processing and IL-1β secretion. However K^+ also regulates diverse cellular processes non-specifically, and may only represent a general checkpoint on cellular physiology. For example, caspase-8 is also inhibited by increased extracellular potassium, indicating that K^+ gradients may influence caspases in a broader mechanism[163]. Additionally, there are a number of examples where potassium extrusion does not result in cell death or inflammatory signalling, such as cardiac myocytes and neuronal cells during the repolarization phase following an action potential. These cells express various components of the inflammasome including caspase-1 and NLRP3, yet modulation of K^+ occurs routinely, independent from their mobilization.

The observation that crystalline substances induce NLRP3-dependent caspase-1 and IL-1β processing raises the question of whether NLRP3 recognizes the stabilization state of lysosomes during phagocytosis. Disruption of lysosomes activates NLRP3 even in the absence of crystals, and stimuli such as pannexin activators and cathepsin B may also destabilize lysosomes[164,165]. Many other NLRP3 activating signals could theoretically also converge on the lysosome. For example, extracellular calcium was shown to mediate NLRP3-dependent IL-1β and caspase-1 processing[166]. While this mechanism was proposed to occur by specific signalling processes involving the calcium sensing receptor and cAMP, the low solubility of free calcium in solution cannot be ruled out as a confounding factor. It is possible that increased calcium concentrations lead to the formation of various precipitates, with the crystals being endocytosed in cell culture systems. It is possible that lysosomal rupture following destabilization of endocytosed particulate, crystals and microbial signals results in the cleavage of a conserved NLRP3 binding partner that activates a final common pathway towards cytokine processing by caspase-1; however this has yet to be shown experimentally.

Lastly, a number of NLRP3 activating stimuli appear to induce the production of ROS within the cell. Inhibition of ROS during stimulation with ATP, MSU, silica and asbestos crystals results in reduced caspase-1 processing and IL-1β release, indicating that reactive intermediates may converge on NLRP3 to facilitate downstream signalling[167,168]. Moreover, direct production of ROS by the pharmacological destabilization of the electron transport chain using rotenone and antimycin A results in NLRP3-dependent production of IL-1β and caspase-1 cleavage[156]. Despite the mounting evidence that ROS results in the activation of IL-1β and caspase-1, there is still no direct evidence that places NLRP3 directly downstream of ROS. NLRP3 could in principle also operate upstream of ROS, as these important baseline controls

have consistently been absent from previous reports. The production of ROS has also been shown to regulate a wide range of cellular processes, raising the question of its direct importance in "activating" NLRP3. The role of ROS in caspase-1 activation downstream of NLRP3 thus remains correlational, and further work is required to understand how ROS is regulated within the cell during inflammatory signalling.

1.5 NLRP3 in Chronic Disease

Despite the elusive mechanism of action, it is clear that NLRP3 regulates the production of IL-1β in professional immune cells. Most importantly, the growing list of endogenous danger signals that activate NLRP3-dependent cytokine production suggests that the pathway could be important in the pathogenesis of chronic human diseases. As previously mentioned, DAMPs that are released during acute and chronic non-microbial injuries activate a range of host innate immune responses. Inflammation is recognized to promote pathophysiological changes in multiple organ systems, including heart and kidney disease, atherosclerosis, inflammatory bowel disease, liver disease, rheumatoid arthritis, diabetes and obesity/metabolic syndrome[169]. Many such conditions have been linked to IL-1β production, and the notion of blocking inflammatory processes is an attractive and novel therapeutic option to clinicians[170-172]. Accordingly, targeting IL-1β and NLRP3 in chronic disease has been a popular topic.

1.5.1 NLRP3 in Models of Renal and Cardiovascular Disease

Chronic kidney disease and heart disease are similar, in that both proceed through the previously mentioned cyclical damage model of chronic non-microbial injury, with low-grade inflammation and intense remodelling/fibrosis[173,174]. The chronic nature of renal and

cardiovascular pathophysiology reflects the fact that the cells comprising the heart and kidney are highly specialized and terminally differentiated, unable to replicate. Since damaging stimuli induce cell death so gradually in these conditions, slow recruitment of innate immune cells takes place through the recognition of DAMPs released into the tissue microenvironment[175]. Given that NLRP3 appears to regulate cytokine production following endogenous danger signals, its role in chronic renal and cardiac injury could offer unique insight into disease pathophysiology with potential therapeutic utility. Moreover, NLRP3 has been found to be expressed both within infiltrating immune cells and resident parenchymal cells, indicating that it could participate in the crosstalk between resistance and tolerance measures to promote chronic disease pathogenesis.

In the kidney, NLRP3 genetic deletion in mice reduces damage induced by experimental ischemia reperfusion injury and unilateral ureteric obstruction[176,177]. The abrogation of NLRP3, but not ASC, resulted in reduced fibrosis, less apoptotic cell death and improved renal structure. The protected phenotype in the absence of cytokine production or significant inflammation suggested that the biology of NLRP3 might not proceed strictly through regulation of IL-1β and IL-18. Moreover, chimeric mice harbouring NLRP3 deletion restricted to the bone marrow compartment were not completely protected from injury, further suggesting that epithelial-expressed NLRP3 may play an important role in regulating homeostasis.

In the heart, the role of IL-1β in disease pathogenesis has been extensively explored. IL-1β has been shown to induce systolic dysfunction, impair myocyte calcium handling, alter sodium channel function, induce apoptosis and recruit additional innate immune cells for subsequent immunopathology[171,178,179]. Additionally, caspase-1 has been demonstrated to promote mortality and left ventricular remodelling after myocardial infarction[180-182]. These early studies using animal models paved the way for human clinical trials to assess the efficacy of IL-

1β antagonism following myocardial infarction[183-185]. Patients with ST-elevation myocardial infarction (STEMI) were randomized to receive either Anakinra (IL-1ra) or placebo for 14 days. Anakinra was found to be safe, and reduced adverse cardiac remodelling in the experimental group. Inflammatory markers (C-reactive protein, CRP) were also normalized following Anakinra, demonstrating the importance of IL-1β in the development of adverse cardiac changes following acute injury. Since IL-1β regulation has been shown to depend on caspase-1, ASC and NLRP3 in the context of many non-microbial injuries, these pathways have also been explored in animal models or cardiovascular disease. Genetic deletion of ASC resulted in reduced infarct development, fibrosis and inflammatory responses during ischemia reperfusion[186]. Chimeric mice harbouring ASC deletion restricted to the bone marrow were not completely protected, again supporting the importance of structural cells. Curiously, the production of IL-1β was proposed to occur via "inflammasome" activation in cardiac fibroblasts, but not macrophages or myocytes. Hypoxic injury stimulated the secretion of IL-1β from fibroblast cultures but not from myocytes, suggesting that cardiac fibroblasts could be important regulators of inflammatory signalling during acute injury. These studies, however, relied on very low levels of IL-1β that are potentially below the threshold detection limit for assay measurements.

Given the protective effects of ASC deletion in general, NLRP3 was also explored and proposed to form aggregates alongside ASC and activated caspase-1 in experimental murine myocardial infarction[187]. These apparent structures were mainly present within granulation tissue, suggesting a role for NLRP3 within innate immune cells during resolution/fibrosis. The knockdown of NLRP3 and the P2X7 receptor using siRNA *in vivo* prevented pathological changes, limiting infarct size and remodelling. However the expression of NLRP3 was said to critically depend on cardiac myocytes based on histological analysis. These studies proposed that

NLRP3-dependent activation of caspase-1 did not result in cytokine production, but rather in the activation of non-canonical cell death. In contrast to these findings, other groups reported that ischemia reperfusion injury in isolated hearts from WT and NLRP3 knockouts showed no changes in viability, IL-1β production or protection following cardiac preconditioning[188]. Instead, NLRP3 deletion only mildly exacerbated mechanical dysfunction following ischemic injury. This study must be taken in association with similar experiments that reported NLRP3 "inflammasome" expression in cardiac fibroblasts during ischemia[189]. Fibroblasts were again said to secrete IL-1β in response to extracellular ATP, this time in a NLRP3 dependent manner. However once again the proposed levels of cytokine released were extremely low, and its importance *in vivo* during canonical fibroblast-activating stimuli is uncertain. Clearly the underlying biology of NLRP3 in the heart remains to be explored. Its expression, localization, cellular distribution and function in different cell types have not been adequately addressed from a cell biology perspective in order to propose a global model in regulating homeostasis.

1.6 Rationale for this Thesis

NLRP3 and the "inflammasome" thus remain controversial topics in immunology. The current body of evidence in the literature is predominantly focussed on studying NLRP3 during acute injuries using animal models. However there are many unresolved issues regarding NLRP3 function not only in the heart but in professional immune cells in the regulation of cytokine processing. Given the complex wound healing mechanisms employed by vertebrates in regulating homeostasis, NLRP3 could operate in chronic cardiac injury in the context of resistance measures, tolerance pathways or both.

1.6.1 Role of NLRP3 in Chronic Non-Microbial Injury

As mentioned, NLRP3 has been significantly implicated in the pathogenesis of non-microbial inflammation and injury. However the disease models employed have largely relied on acute injuries as opposed to chronic, likely a result of the difficult nature in modelling chronic disease in animals. This is an important distinction; non-resolving inflammation involves the recruitment of diverse cell types within tissue microenvironments[5]. Before NLRP3 can be discussed as a drug target for clinical use, understanding its expression in the course of an injury and where it exerts effects is of critical importance. The observation that diverse parenchymal cells express NLRP3 raises the question of whether they are capable of secreting cytokine products. Most human chronic diseases are not characterized by profound cytokine involvement, but rather the slow, smouldering inflammation typically found in patients with heart failure[13]. Any involvement of NLRP3 and IL-1β in animal models would hopefully reflect these observations. Moreover, the role of NLRP3 in both systolic and diastolic HF and the decompensation of heart disease must be explored, given its putative roles in regulating underlying inflammatory processes.

1.6.2 Resolving NLRP3 Function in Different Tissue Types

Presumably a protein would not exert profoundly different biology in diverse cellular populations. Rather, proteins within families generally display similar functions, and the relative abundance, distribution or presence of binding/interacting partners determines regional changes in function. The observation that NLRP3 could differentially regulate caspase-1 to produce inflammation or apoptosis in distinct cell types requires further analysis. Instead, the simpler explanation is that NLRP3 functions in a broader mechanism to regulate some aspects of cellular

physiology. Such a general role would ideally explain its regulation of caspase-1 and IL-1β in addition to whatever distinct roles are observed in non-professional immune cells. However to date, no clear unifying theory for NLRP3 function has been explored.

1.6.3 NLRP3 Subcellular Localization

Clues to protein function generally reside in their subcellular localization. For example, the Death Fold Domain-containing proteins that are putatively similar to NLRP3 tend to reside in and around the mitochondria[190]. NLRP3 has recently been suggested to translocate to mitochondria, however the significance is not universally accepted[191]. A cellular biology approach to NLRP3 must be undertaken in order to truly understand its regulation of cellular physiology. While NLRP3 biochemistry and potential protein-interacting models have been proposed, none of these have been supported with validated microscopy techniques using endogenous proteins.

Accordingly, we sought to characterize NLRP3 in the regulation of cardiac tissue homeostasis. We undertook diverse approaches using whole animal, molecular, biochemical, cellular and physiological experimental methodologies to better define NLRP3 function during chronic cardiac injury. We hypothesize that NLRP3 exerts dual roles in professional and non-professional immune cells to facilitate wound healing and repair. These distinct roles reflect the same underlying biology, but differ through the presence and absence of cytokine substrates. This work proposes a novel paradigm for NLRP3 and the NLRPs, which could function not as specific pattern recognition receptors, but rather as intracellular danger sensors that facilitate generalized wound-healing measures in all cell types.

Chapter Two: **Experimental Methods and Procedures**

2.1 *in vivo* Mouse Models

2.1.1 Calcineurin Transgene Expression Behind The α-Myosin Heavy Chain

We employed a murine model of structural cardiomyopathy originally established by the Olsen Lab and previously used by the Duff lab[192,193]. Calcineurin is a type 2B Ca^{2+}/calmodulin-dependent protein phosphatase that dephosphorylates NF-AT3, allowing its nuclear translocation and association with GATA4[194]. Activation of calcineurin in cardiac myocytes results in the induction of fetal gene expression with resulting hypertrophy, electrical instability, dilated cardiomyopathy and inflammation/fibrosis[195]. The constitutively active calcineurin A catalytic subunit-containing cDNA was cloned into an expression linker containing the a-myosin heavy chain promoter and injected into fertilized oocytes derived from FVB mice[193]. Oocytes were then transferred to the oviducts of pseudopregnant ICR mice. Transgenic mice (line 37) were a gift from the Olsen lab, and on arrival were backcrossed onto the C57BL/6 background for more than 5 generations prior to the present studies. Transgenic mice (CNTg) and their WT littermates were used between the ages of 14-20 weeks to allow for significant progression of disease. The CNTg and wild-type (WT) mice were separated using genomic DNA and PCR analysis. Primer sequences used were: forwards 5′-GTCTGACTAGGTGTCCTTCT-3′ and reverse 5′-CGTCCTCCTGCTGGTATTAC-3′.

For the generation of *Nlrp3*$^{-/-}$CNTg mice, CNTg and *Nlrp3*$^{-/-}$ were crossed. Their heterozygous progeny were then bred further to obtain mixed *Nlrp3*$^{+/-}$CNTg/*Nlrp3*$^{-/-}$ CNTg groups, which were used to assess cardiac function compared with wild-type (n=4–5 per group). Colonies were maintained through mating of *Nlrp3*$^{-/-}$CNTg homozygotes.

For drug treatments, osmotic pumps (Alzet, Cupertino, CA, USA) were filled with either drug-containing solution or sterile saline (n = 5 per group) and surgically implanted into the dorsum under general anaesthesia with isoflurane delivered by inhalation. A small 1–2 cm incision was made in the skin and separated using blunt dissection of subcutaneous fascia. After implantation of the pump the skin was closed with a 7–0 silk suture and mice were allowed to recover with standard food and water. The recombinant soluble IL-1 receptor antagonist (Anakinra) was used to treat mice at 10mg/kg/day for 14 days.

Echocardiograms were obtained in conscious, restrained mice to assess cardiac structure and function using a SONOS 5500 from Agilent Technologies (Andover, MA, USA) with a 15 MHz pediatric probe. Physiological data were independently reviewed by a cardiologist in a blinded fashion.

2.1.2 Angiotensin II-Induced Hypertensive Heart Disease

Administration of Angiotensin II (AngII) by osmotic pump results in hypertensive disease phenotype with resulting cardiac fibrosis[196]. We modified this model to a 28-day infusion in WT and *Nlrp3*$^{-/-}$ animals.

Osmotic Pumps- All experiments were performed under the approval of the Animal Care Committee at the University of Calgary and animals were housed in a specific pathogen free facility. Male C57BL/6 mice at age 10-12 weeks were used for all studies and *Nlrp3*$^{+/+}$ (n=7) compared to *Nlrp3*$^{-/-}$ (n=8) littermates as previously described[177]. For osmotic pump implantation, animals were anesthetized with isoflurane mixed with oxygen by mask inhalation and a 1 cm incision was made with a sterile blade. Osmotic pumps (Durect Corporation,

Cupertino CA) were filled with recombinant human Angiotensin II (Sigma Aldrich, 1.5mg/kg/day) or sterile saline, implanted into the dorsum and the incision closed with an 8-0 silk suture. Mice were allowed to recover with access to standard food and water.

Echocardiograms were performed on conscious, restrained mice to assess cardiac structure and function using a SONOS 5500 from Agilent Technologies (Andover, MA) with 15 MHz pediatric probe. Serial blood pressure recordings were performed using the BP2000 Visitech Systems tail cuff apparatus. Clinical parameters were independently reviewed by a cardiologist blinded to the identity of the samples.

2.1.3 Collection of Human Cardiac Tissue

Human right atrial appendages were obtained from consenting patients undergoing cardiac surgery and left ventricular tissue was taken from consenting patients undergoing surgical placement of left ventricular assist devices at the Foothills Medical Centre, Calgary, Alberta Canada. Tissue specimens were collected under an ethics protocol approved by the University of Calgary. Tissue was taken from the surgical suite in PBS, brought to the lab and prepared for primary cell isolation or histological assessment.

2.2 *in vitro* Experimental Methods

2.2.1 Cell Culture

2.2.1.1 Cell Lines

The following cell lines were used in experiments: HeLa human cervical epithelial cells, 293T human embryonic kidney cells and THP-1 human monocytic cells. For adherent cells, monolayers were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 100 µg penicillin/ml, 100 µg streptomycin/ml and 2 mM *L*-glutamine. Cells were grown at 37°C in a 5% CO2 incubator and passaged by digestion in 0.05% trypsin EDTA for 4 minutes. After neutralization in complete media, plates were diluted to 10 ml suspensions and passaged at 1:5 and 1:10 in 10 cm sterile plates. For THP-1 culture, cells were grown in T75 flasks containing RPMI supplemented with 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 1X penicillin/streptomycin and 10% FBS. Cells were serially passaged at 1:8 and 1:4 dilutions.

2.2.1.2 Primary Cell Culture

2.2.1.2.1 Primary Neonatal Cardiac Myocyte and Fibroblast Mixed Cultures

For neonatal cardiac cell isolation, neonatal mice (8-15) were taken at P0-P3, euthanized by cervical dislocation and sprayed in 70% ethanol. The thorax was exposed with sharp scissors and the beating hearts removed into a sterile dish containing DMEM wash solution. Hearts were sequentially rinsed and transferred to separate sterile dishes containing wash solution, minced

with fine scissors and transferred to 5ml small beakers containing 2-3 ml fresh enzyme solution (DMEM solution 30 ml, BSA 300 mg, Taurine 68 mg, collagenase 7 mg, protease 0.6 mg for 50 ml volume). Minced tissue in enzyme solution was placed in a dry bath heater with a magnetic stir bar and incubated at 37°C. The enzyme solution was continuously replaced every 5 minutes. Cells were maintained in DMEM containing 10% FBS and centrifuged at 1000Xg for 5 minutes. The supernatant was discarded, and the pellet of cells re-suspended in 10 ml growth medium. Cell suspensions were passed through a 50 µm mesh filter and incubated overnight. For separation of fibroblast and myocyte populations, fibroblasts were allowed to adhere for 4-6 hours in the incubator, and the myocyte-containing media was removed and re-plated. The following day media was changed and cells displayed spontaneous beating.

2.2.1.2.2 Primary Adult Cardiac Fibroblasts

Ventricular tissue from 1-2 mice were removed, cleaned of valvular, pericardial and atrial structures and sequentially rinsed in sterile PBS. Tissue was finely minced in 1 ml DMEM containing 10% FBS and 100 µg penicillin/ml, 100 µg streptomycin/ml and 2 mM *L*-glutamine. Finely minced tissue was plated in 2X 10 cm sterile collagen coated plates. Tissue was suspended in complete DMEM as above and allowed to adhere. The following day, media was changed. Fibroblasts migrated from tissue and proliferated on the plates over the course of 1-2 weeks. Media was replaced every 3-5 days until confluent areas were seen. Cells were then passaged using 0.05% trypsin EDTA and used for experiments at P1-2 when homogenous populations emerged.

2.2.2 Antibodies and Plasmids Used

During the course of experiments, several antibodies and plasmid constructs were routinely used, as shown in Tables 1 and 2.

Table 1. Commonly used primary antibodies.

Antibody	Catalogue	Species	Diluent	Dilution
hNLRP3	Adipogen Cryo2	mouse	PBST	1:1000
ASC	Enzo AL177	rabbit	PBST	1:1000
mCaspase-1 p10	SC514	rabbit	PBST	1:500
hCaspase-1	SC515	rabbit	PBST	1:500
mIL-1β	R&D	goat	PBST	1:500
Smad2/3	BD	mouse	PBST	1:1000
pSmad2	SC	rabbit	PBST	1:500
pSmad3	SC	rabbit	PBST	1:500
FLAG M2	Sigma	mouse	PBST	1:2500
β-Tubulin	Sigma	mouse	PBST	1:2500
αSMA	Sigma	mouse	PBST	1:2000

Table 2. Plasmids routinely used.

Plasmid	Vector
eGFP	pHEF
hFLAG-NLRP3	pCR3
hNLRP3	pcDNA3.1
TGFRII	-
SBE4Luc (Firefly)	-
TK Luc (Renilla)	-
hFLAG-NLRP9	pCMV6
hFLAG-NLRP10	pCMV6
hNLRP3 FLAG- PYD	pCR3
hNLRP3 FLAG-NACHT	pCR3
hNLRP3 FLAG-LRR	pCR3
hNLRP3 FLAG-PYD-NACHT	pCR3
hNLRP3-GFP	pCMV6

2.2.3 Transient Transfection

293T cells and HeLa cells were transiently transfected with plasmid DNA constructs for further studies on protein interactions, signal transduction and subcellular localization. 293T cells were used for their ease of transfection using the calcium phosphate method. They demonstrated consistently high transfection efficiency of 50-70% with ease of growth and manipulation. These properties make 293T cell transfections ideal for use in protein interaction studies, signalling assays and flow cytometry.

On the day before transfection, one 10 cm plate of 293T cells (70-90% confluent) was diluted into a 10 ml cell suspension. 0.5 ml of the suspension was further diluted into 12 ml complete DMEM growth media, and the cells were plated at 0.5 ml/well in a 24 well plate for approximately $5X10^4$ cells/well. For use in 60 mm plates, the 10 ml cell suspension was plated at 1:12-18 for approximately $8X10^5$ cells/plate in 5 ml/plate. The following day, cells should be 70% confluent.

For 24 well plates, 0.5 µg of plasmid DNA was used/well. Experiments were performed in triplicate. For three 24-well samples, DNA was diluted in 112.5 µl H_2O, 125 µl 2X HBS in 1.5 ml eppendorf tubes. In separate tubes, 12.5 µl 1.5 M $CaCl_2$ was distributed. The DNA/HBS/H_2O mixture was then added drop-wise to the calcium solution and incubated at room temperature for 15 minutes to allow a fine DNA/calcium phosphate precipitate to form. The tubes were mixed by shaking throughout the incubation. 75 µl of the precipitate-containing solution was then added to the 500 µl media/well (for n=3). The cells were incubated for 8 hours and the media was changed to fresh complete growth medium. The following day cells were used for experiments.

Table 3. Solutions for calcium phosphate transfection.

<u>24 well plates: for n=3 wells</u>

0.5 µg DNA

112.5 µl H_20

125 µl 2X HBS

12.5 µl 1.5M $CaCl_2$

75 µl/well

<u>60 mm plates: (double for 10 cm plate)</u>

4 µg DNA

250 µl 2X HBS

225 µl H_20

25 µl 2.5 M $CaCl_2$

500 µl/plate

Solutions for calcium phosphate transfection cont.

$CaCl_2$ 2.5 M

183.7 g $CaCL_2$-$2H_20$ (Sigma; tissue culture grade)

H20 to 500 ml

Filter sterilize through 0.45 µm nitrocellulose filter

Store @ -20°C in 10 ml aliquots

HEPES-buffered saline (HBS), 2X

16.4 g NaCl (0.28 M final)

11.9 g HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; 0.05 M final)

0.21 g Na_2HPO_4

800 ml H_20

Titrate to pH 7.05 with 5 N NaOH (*N.B.* pH of 7.05-7.12 is critical)

Add H_20 to 1 L

Filter sterilize through 0.45 µm nitrocellulose filter

Store at -20°C in 50 ml aliquots

The calcium phosphate method described above works in select cell lines only. For HeLa cell transfection we employed a commonly used chemical method using lipid reagent (Lipofectamine 2000, Life Technologies). HeLa cells display excellent cellular morphology, grow in flattened monolayers and characteristically demonstrate prominent organelle structure (ribbon Golgi stacks, linear mitochondria). Accordingly, they are ideal for imaging experiments, confocal microscopy and localization studies.

On the day before transfection, HeLa cells were plated at $5X10^4$ cells/well in 24-well plates in complete growth DMEM medium. The following day the cells should appear 70% confluent. On the day of transfection, 0.5 µg of DNA/well was combined with 25 µl OPTIMEM (Life Technologies) in eppendorf tubes. In separate tubes, 2 µl lipofectamine lipid reagent was combined with 25 µl OPTIMEM. The DNA and lipid reagent mixtures were then combined and incubated at room temperature for 15 minutes, and 50 µl was then added drop wise to 500 µl of media containing cells/well. Cells were incubated for 24 hours before experiments were performed. Lipofectamine 2000 provides approximately 15-30% transfection efficiency in HeLa cells using this protocol in our experience.

Table 4. Transfection volumes for Lipofectamine 2000 protocol (Life Technologies).

Culture Vessel	Surface Area/Well	Plating Volume	DNA (µg) and Volume	Lipofectamine 2000 and Volume
96-well	0.3	100 µl	0.2 in 25 µl	0.5 µl in 25 µl
24-well	2	500 µl	0.8 in 50 µl	2.0 µl in 50 µl
12-well	4	1 ml	1.6 in 100 µl	4.0 µl in 100 µl
35 mm	10	2 ml	4.0 in 250 µl	10 µl in 250 µl
6-well	10	2 ml	4.0 in 250 µl	10 µl in 250 µl
60 mm	20	5 ml	8.0 in 0.5 ml	20 µl in 0.5 ml
10 cm	60	15 ml	24 in 1.5 ml	60 µl in 1.5 ml

2.2.4 Protein Analysis

Luciferase Assay and Transfection- Luciferase activity was determined using the Stop and Glo Dual Luciferase assay kit (Promega) according to the manufacturer's protocol. Luciferase activity was measured using a Monolight 3010 luminometer. The day before transfection, 293T cells were plated to 60% confluency in 24-well plates. On the day of transfection, 0.5 µg of control peGFP or pCR3-hNLRP3 constructs were mixed with 0.4 µg pCR3-TBRII and 100 ng R-Smad activated SBE4-Luciferase (Firefly)/Thymidine-Kinase-Luciferase (Renilla) constructs in a 10:1 ratio. Media was changed after 4-6 hours, and cells were stimulated with TGFβ (10 ng/ml) for 6 hours. Firefly luciferase activity was normalized to Renilla luciferase and data expressed as fold induction over mock-treated cells.

2.2.4.1 SDS-PAGE

For tissue samples, tissue was rinsed in PBS and placed in ice-cold NP-40 lysis buffer on ice. Samples were minced with fine scissors and homogenized with a handheld rotor until no tissue remained. The samples were then cleared by centrifugation at 4°C, >12000Xg for 15 minutes. The supernatants were collected and diluted into 3X Sample Buffer (1/3 volume of the lysate for final concentration of 1X) containing fresh DTT and heated for 5 minutes at 95°C in a dry bath heater. The samples were allowed to cool and centrifuged again at 12000Xg for 2 minutes before being subject to SDS PAGE.

Cells in culture were placed on ice, media aspirated and rinsed in PBS. Plates were then lysed in NP-40 lysis buffer (50 µl/24 well, 100 µl/6well, 200 µl/6 cm plate). Plates were shaken on ice for 15 minutes to allow complete lysis, scraped with a rubber policeman and collected into eppendorf tubes. Lysates were cleared by centrifugation at 4°C, >12000Xg for 15 minutes. The

supernatants were collected and diluted into 3X Sample Buffer (1/3 volume of the lysate for final concentration of 1X) containing freshly added 100 mM DTT and boiled for 5 minutes at 95°C in a dry bath heater. The samples were allowed to cool and centrifuged again at 12000Xg for 2 minutes before being subject to SDS PAGE.

Samples were then loaded into 1mm or 1.5 mm 8%, 10% and 15% polyacrylamide gels containing SDS for protein separation and 180 V was applied for appropriate resolution. The protein was then transferred to nitrocellulose membranes for 1 hour at 100 V for identification by immunoblotting.

Table 5. Solutions used for SDS-PAGE.

Lower Gel Buffer: @ 4C

91 g Tris

10ml 20% SDS

pH to 8.8 w HCl

ddH20 to 500 ml

Upper Gel Buffer: @ 4C

30.5g Tris

10ml 10% SDS

pH to 6.8 w HCl

ddH20 to 500 ml

Pyronin Red:

0.5 g in 50 ml ddH20 (Pyronin Y Sigma 213519-5G)

Lysis Buffer: (50 ml) @ -20C, 5ml aliquots

0.5 ml NP-40 (1% Final)

2.5 ml 1 M Tris pH 7.4

1.5 ml 5 M NaCl

0.5 ml 0.5 M EDTA

0.5 ml 100 mM NA3VO4 (1 mM)

100 mM NaF

Protease Inhibitor Tabs (EDTA free, Roche)

0.5 ml 0.1 M PMSF

0.5 ml 100X Apopeptin

0.5 ml 100X Leupeptin

43 ml ddH20

3X Sample Buffer (100ml)

37.5 ml upper gel buffer

34.4 ml glycerol

6 g SDS

0.03 g phenol red

DTT to 100 mM (add fresh) from 1 M stock

ddH20 to 100 ml

Ponceau Stain (100 ml)

3% TCA

0.05% Ponceau (0.05 g)

H20 to 100 ml

Table: SDS PAGE Gel Preparation

Table 6. Casting gels for SDS-PAGE.

4X 1 cm Gels:	15%	10%	8%	Stacking
Acrylamide	10 ml	6.65 ml	5.32 ml	1.125 ml
Lower Buffer	5 ml	5 ml	5 ml	-
Upper Buffer	-	-	-	2.08 ml
ddH_2O	5 ml	8.35 ml	9.68 ml	5.08 ml
APS	125 µl	125 µl	125 µl	83.5 µl
TEMED	12.5 µl	12.5 µl	12.5 µl	10.41 µl
Pyronin Red	-	-	-	10 µl

2.2.4.2 Immunoblotting

Following transfer, nitrocellulose membranes were blocked in phosphate buffered saline (PBS) or Tris buffered saline (TBS) with Tween-20 (0.05-5%) containing either 5% skim milk powder or albumin where appropriate. Blocking was performed at room temperature for 30 minutes with gentle rocking. After blocking, membranes were incubated in appropriate antibodies diluted into blocking solution and rocked at 4°C overnight. The following day, membranes were rinsed in PBST/TBST in 5 washes of 5 minutes at room temperature. The membranes were then incubated in appropriate horseradish peroxidase (HRP) conjugated secondary antibodies at 1:5000 dilution for 45 minutes at room temperature with gentle rocking. The secondary antibodies were washed, and detected using chemiluminescence.

2.2.4.3 Immunoprecipation

Immunoprecipitation of protein from cells in culture was performed to detect protein interactions and post-translational modifications. For overexpression experiments, 60 mm plates were lysed in 200 µl NP-40 lysis buffer and shaken at 4°C for 15 minutes. Samples were cleared by centrifugation at 12000Xg for 15 minutes, transferred to eppendorf tubes containing 10ul of 6B sepharose beads and pre-cleared for 4 hours by rocking at 4°C. Beads were centrifuged at 4°C, 6000Xg and supernatants collected. 30 µl of sample was removed, and 170 µl remaining was added to 10 µl protein G coated sepharose beads containing 1ug primary antibody. Samples were rocked overnight at 4°C. The following day, beads were centrifuged at 6000Xg, supernatants collected and the beads rinsed 5 times in ice-cold lysis buffer. Beads were finally eluted with 45 µl 3X sample buffer containing freshly added 100 mM DTT and boiled at 95°C for 5 minutes.

2.2.5 Immunofluorescence

2.2.5.1 Fluorescent Confocal Microscopy

Cells were seeded for microscopy on 1.5 mm circular glass coverslips in 24 well plates at 70% confluency. Coverslips were kept in 70% ethanol and flame sterilized prior to use. Coverslips were rinsed in complete growth medium containing 10% FBS to enhance cell attachment. On the day of fixation, cells were treated with Mitotracker Red (Life Technologies) at 400 nM for 20 minutes at 37°C. Cells were rinsed in sterile PBS and fixed in 4% paraformaldehyde diluted in PBS for 20 minutes at 37°C. Cells were again rinsed in PBS, and free aldehydes quenched in 50 mM ammonium chloride for 10 minutes at room temperature. Coverslips were rinsed twice and cells permeabilized in 0.1% Triton X-100 with 0.05% SDS for 5 minutes at room temperature. Cells were then blocked in PBS containing 0.02% gelatin in 5X five minute washes at room temperature. Coverslips were incubated in 30 µl primary antibodies diluted into blocking solution at 1:50-1:500 face down on parafilm for 1 hour at room temperature. Coverslips were then rinsed in 3X five minute washes with PBS and placed in appropriate fluorescent secondary antibodies at 1:500 in blocking solution for 45 minutes at room temperature. Coverslips were again rinsed 3X five minutes in PBS and mounted on glass slides in ProLong Gold containing DAPI reagent (Life Technologies) and cured at room temperature overnight.

Confocal images were taken using a Laser Scanning Microscope under 40X and 63X oil objective lenses with LSM software (Zeiss). Images were analyzed and semiquantitative data generated using Volocity software (PerkinElmer).

2.2.5.2 Live-Cell Confocal Imaging Reactive Oxygen Species

Primary cardiac fibroblasts (50,000 cells/plate) were seeded in sterile 35 mm tissue culture plates with glass slide bottoms on the day prior to experiments. The following day, cells were treated with 400 nM Mitosox Green (Life Technologies) and rinsed in HBSS with calcium and magnesium in the absence of phenol red. Plates were then transferred to a 37°C stage at the confocal microscope and MitoSOX Red (Life Technologies) was added at final concentration of 5 µM in a 2 ml final volume. Images were taken at 0, 3, 6, 9, 12 and 15 minutes in 488 nm for Mitotracker green and 555 nm for MitoSOX Red. MitoTEMPO (SantaCruz Biotechnology) was used as a pretreatment at 500 µM for 10-12 hours diluted into complete medium.

2.2.5.3 Fluorescent Confocal Immunohistochemistry

Mice were sacrificed by cervical dislocation and tissue was removed, rinsed in saline and embedded in OCT reagent for cryosectioning followed by confocal fluorescent immunohistochemistry. Briefly, slides were blocked for 1 hour in PBS containing 5% goat serum and 0.1% Triton X-100. Primary antibodies to FGF-2 and αSMA (Santa Cruz Biotechnology, Santa Cruz, CA), were diluted 1:200 into blocking solution and incubated overnight at room temperature. Following washing in PBS, appropriate secondary antibodies were diluted 1:400 in blocking solution and incubated 1 hour at room temperature. For histological analysis, tissue was fixed in formalin and embedded in paraffin followed by standard staining with haematoxylin and eosin (H&E). Fibrosis was assessed both by Masson Trichrome and Musto stains. Embedding, sectioning and staining of all tissue was performed under the standard protocol by Calgary Laboratory Services (CLS), and the samples reviewed by a pathologist blinded to the identity of the samples.

Human tissue was taken from consenting patients undergoing surgical placement of cardiac left ventricular assist devices at the Foothills Medical Centre, Calgary, Alberta Canada. Tissue was rinsed in saline, formalin fixed and embedded in paraffin. Slides were de-paraffinized overnight in xylene washes, hydrated in ethanol and placed in 95°C citrate buffer for 40 minutes for antigen retrieval. Slides were blocked in PBS containing 5% goat serum, 0.5% Triton-X100 for one hour, followed by antibody incubation as described for immunocytochemistry.

2.2.6 Flow Cytometry

Primary adult murine ventricular cardiac fibroblasts were seeded in sterile 60mm culture dishes overnight, rinsed in saline and loaded with MitoSox (5uM, Invitrogen) in HBSS at 37^{o}C for 10 minutes. Cells were then trypsinized, centrifuged at 1000Xg and resuspended in phosphate buffered saline and placed on ice. 293T cells were seeded in 24 well plates, transfected with 0.5 μg of indicated plasmids, loaded with Mitosox, rinsed and collected from plates in phosphate buffered saline. All flow cytometry and analysis were performed by the flow cytometry facility at the University of Calgary Centre for Advanced Technology.

2.2.7 Molecular Biology

2.2.7.1 Site Directed Mutagenesis

NLRP3 Walker-A mutant constructs were generated using the Quick Change kit according to the manufacturers protocol (Stratagene). The following primers were used: NLRP33 F1 5’GGGCGGCAGGGATTGCGGCAGCAATCCTGGCCAGGA3’, NLRP3 R1 5’TCCTGGCCAGGATTGCTGCCGCAATCCCTGCCGCCC3’.

2.3 Statistical Methods

Statistical analyses were performed using Microsoft Excel software with the Analysis ToolPak plugin. The results were analyzed for statistical variance using unpaired student's t-test or one-way ANOVA with post hoc analysis, where appropriate. Statistically significant data was considered at $p < 0.05$. Data are presented as mean +/- standard deviation, unless otherwise specified.

Chapter Three: **The Role of NLRP3 in Animal Models of Cardiovascular Disease**

Parts of this chapter have been previously published as “The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through Interleukin-1β” (Bracey *et al.*, 2013 J Exp Physiol 98(2): 462-472)

Overview

While caspase-1 and its cytokine substrates IL-1β and IL-18 have been implicated in the pathogenesis of heart disease, the specific role of NLRP3 in chronic cardiac injury has not been adequately evaluated. All prior animal models examining the role of IL-1β and caspase-1 have employed acute cardiac insults such as coronary artery ligation or ischemia reperfusion[180,185]. These models do not represent the truly chronic nature of human heart failure, such as the ongoing and cyclical injury of diverse etiologies. End stage human heart disease is best characterized by progressive ventricular hypertrophy that leads to cardiac dilatation and the development of electrical instability with atrial and ventricular arrhythmias[15]. Increased ventricular wall thickness initially attempts to limit tension, as illustrated by the Law of Laplace: T=pr/(2t) where T=tension, p=pressure, r=radius and t=wall thickness[197]. However with time, the increased wall tension is unsustainable and manifests as myocardial wall stress, resulting in the loss of functional myocytes and interstitial cells by a spectrum of apoptosis and necrosis. Therefore myocardial hypertrophy is of sufficient chronic injury itself to induce wound-healing responses through the production of endogenous DAMPs, putative NLRP3 activating ligands. Many patients with longstanding heart disease also develop changes in cardiac structure and interstitial cell death in the absence of overt ischemia, especially in the pathogenesis of diastolic dysfunction following longstanding hypertension[198]. Therefore exploring the role of NLRP3 in different models of chronic cardiac injury may better reflect its function in wound healing processes than acute injurious stimuli alone. Finally, nobody to date has looked at the expression of NLRP3, ASC or caspase-1 in isolated cardiac cells or human cardiac tissue from patients with heart failure. We have found that various NLRs are expressed at the mRNA level in murine cardiac tissue, including NLRP3 (Figure 5A). Moreover, in agreement with others we have

shown that isolated cardiac myocyte and fibroblast cultures each contain NLRP3 and pro-caspase-1 protein, as do the murine atrial cardiac myocyte-like HL-1 cell line and NIH 3T3 fibroblasts (Figure 5B)[187]. These preliminary results indicate that NLRP3 may be present within the heart during diseased states in addition to infiltrating leukocytes and innate immune cells with distinct functional significance.

As a proof of principle for its *in vivo* relevance, we looked to establish whether NLRP3 participated in the development of chronic non-ischemic heart disease using diverse models that represent the underlying pathophysiology of human heart failure. Using a mouse model of severe structural systolic heart disease, we showed that elevated IL-1β production was associated with cardiac interstitial cell death and inflammation in a largely NLRP3-dependent pathway. However the genetic deletion of NLRP3 also protected mice from developing fibrosis in the less severe model of Angiotensin II-induced diastolic cardiac disease. Interestingly, reduced fibrosis and architectural remodelling in NLRP3-deficient animals proceeded in the absence of cytokine production, extensive inflammation or cell death during AngII infusion, suggesting that NLRP3 may exert unique functions in cardiac cells. Finally, to determine its relevance in humans, we obtained left ventricular tissue samples from patients with end stage heart disease and explored the localization and expression of various inflammasome components. Together, these initial results identify putative roles for NLRP3 in the development of different stages of chronic cardiac injury, and serve as a starting point to further identify specific functions in different cellular populations.

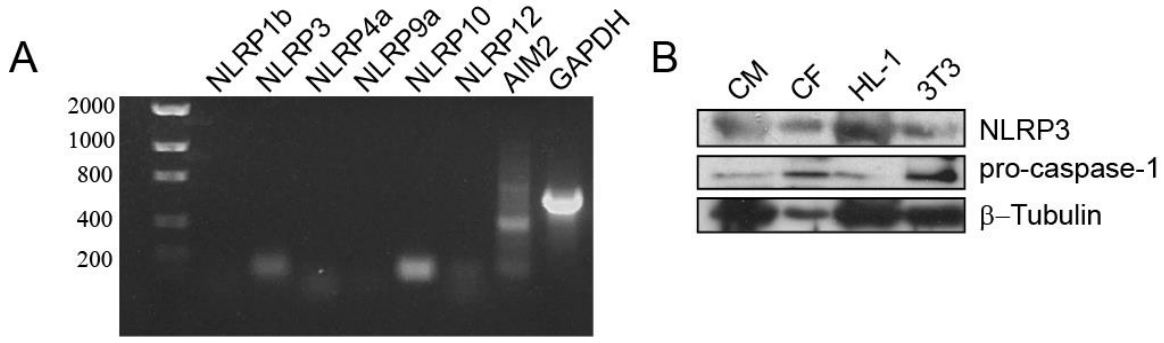

Figure 5. NLR expression in heart.

A. Reverse transcriptase PCR for various NLRs in murine ventricular cardiac tissue. **B.** Immunoblot for NLRP3 and pro-caspase-1 in primary murine neonatal cardiac myocytes (CM), cardiac fibroblasts (CF), the atrial myocyte-like HL-1 cell line or NIH-3T3 murine fibroblasts.

3.1 NLRP3 in Calcineurin Transgene-Induced Non-Ischemic Cardiomyopathy

Calcineurin is a calcium dependent serine/threonine phosphatase that is involved in diverse aspects of cellular physiology[193]. In response to increased cytosolic calcium within cardiac myocytes, calcineurin dephosphorylates the serine-rich N-terminus of NF-AT3 and GATA4 transcription factors that subsequently translocate to the nucleus. Activation of GATA4 and NF-AT3 induces fetal gene expression profiles that result in cardiac hypertrophy[195]. Cardiac myocyte-specific expression of a calcineurin transgene (CNTg) under regulation of the α-myosin heavy chain promoter results in massive cardiac hypertrophy in mice, progressing to ventricular dilatation, electrical instability, myocardial inflammation/apoptosis and ultimately death. We began by establishing this disease model and assessed cardiac function by echocardiography in consciously restrained animals. By 14 weeks of age, CNTg mice display markedly impaired left ventricular systolic function, evidenced by significantly reduced fractional shortening (Figure 6A). Tissue from CNTg mice displayed increased relative size of myocytes under longitudinal section, with an increased cellularity consistent with previously described inflammatory infiltrate (Figure 6B). The inflammation is of particular importance to disease pathogenesis, and has previously been shown to occur in part through iNOS-mediated nitric oxide mechanisms in both cardiac myocytes and infiltrating innate immune cells[192]. The CNTg model therefore represents a mode to assess non-microbial inflammation during the development of chronic non-ischemic cardiomyopathy.

Low-grade serum cytokine levels have previously been detected in human patients with chronic heart disease. IL-1β has been shown to be elevated to varying degrees in patients with longstanding heart failure, particularly in those with end stage disease[13]. We detected elevated levels of IL-1β in the serum from CNTg animals by 14 weeks of age, a time where they display

extensive hypertrophy and begin to develop significant arrhythmias and sudden death. At this time, NLRP3 mRNA was also increased in CNTg hearts compared to WT, suggesting that NLRP3 activation of inflammatory caspases could be responsible for increased inflammatory cytokine production (Figure 6C, D). Consistent with this, caspase-1 was present in its cleaved form in whole heart lysates from CNTg mice, but not in WT hearts as shown by the detection of the p10 band by immunoblotting (Figure 6E). Together, these data implicate a potential role for NLRP3 in the production of IL-1β, which appears in association with extensive cardiac dysfunction and altered structure.

Since IL-1β exerts its effects at very low concentrations, we hypothesized that the detected levels seen in CNTg mice were of significant physiological consequence. To test this, we treated CNTg mice with the recombinant human IL-1R antagonist (IL-1ra, Anakinra) for 14 days. Echocardiographic assessment of structure and function was performed, and mice were sacrificed by 14 days for histology. CNTg mice administered saline continued to substantially decline in systolic function during the 14-day course (Figure 7A). Interestingly, infusion of IL-1ra resulted in a relative preservation in cardiac function, as fractional shortening only declined modestly during 14 days. We did not detect changes in other cardiac structural parameters, such as septal wall thickness, indicating that the benefits in systolic function were not a direct result from reduced hypertrophy. Rather, cardiac tissue from IL-1ra-treated CNTg mice showed a reduced inflammatory infiltrate compared to saline treated controls on H&E staining, as indicated by a pathologist blinded to the specimen identities (Figure 7B). Since IL-1β has been

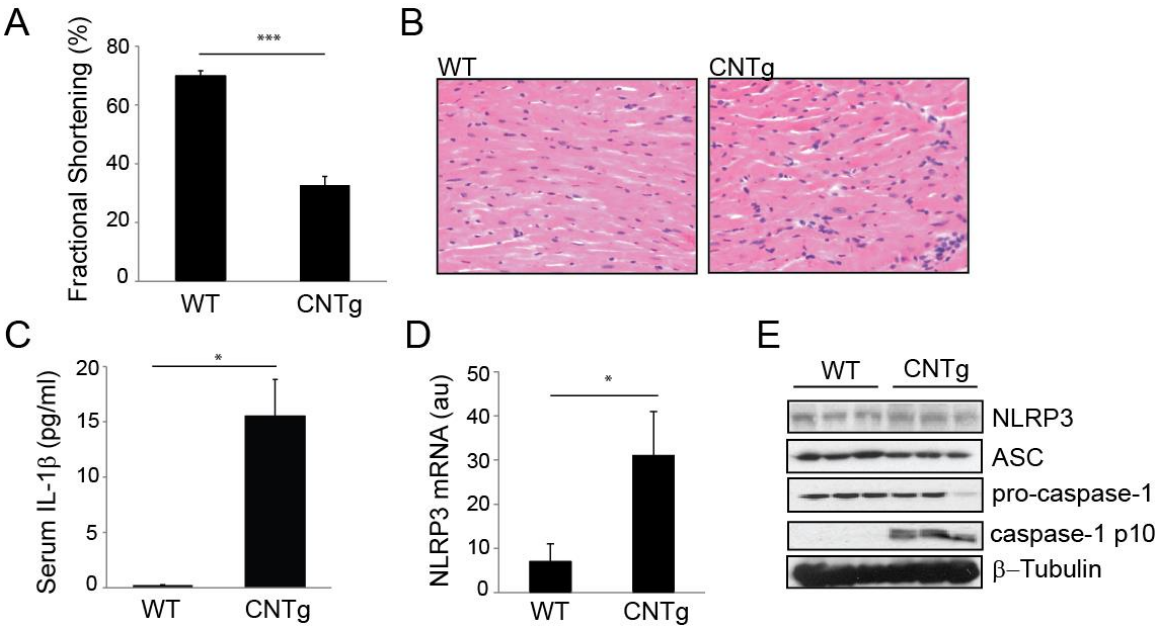

Figure 6. Cardiac phenotype of CNTg mice.

A. Echocardiographic assessment of left ventricular systolic function (fractional shortening) in conscious, unrestrained CNTg mice at 14-16 weeks of age (n=4). **B.** H&E stained tissue sections of WT and CNTg myocardium, 40X objective. **C.** IL-1β measurement in WT and CNTg serum by Luminex. **D.** Measurement of NLRP3 mRNA from WT and CNTg hearts by real time PCR. **E.** Representative immunoblot showing expression of inflammasome components in WT and CNTg animals. (*$p<0.05$, ***$p<0.001$).

shown to promote cell death, we went on to assess apoptosis by TUNEL staining (Figure 7C)[199]. Consistent with preserved cardiac histological architecture, myocardium from IL-1ra-treated CNTg mice contained significantly fewer TUNEL positive cells, further suggesting a protective benefit with IL-1β blockade.

Since NLRP3 mRNA was elevated with increased conversion of caspase-1 within the hearts of CNTg mice, we next looked to test whether the increased IL-1β was a result of NLRP3 driven processes. To that end, we bred CNTg mice onto a NLRP3 deficient background and compared them to WT, *Nlrp3*$^{-/-}$ and CNTg$^{+/+}$ counterparts for cardiac assessment (Figure 8A). *Nlrp3*$^{-/-}$ animals showed no differences at baseline compared to WT with respects to cardiac size, ventricular dimension, systolic function or other hemodynamic parameters. While CNTg mice again showed impaired systolic function, *Nlrp3*$^{-/-}$CNTg mice demonstrated significantly preserved fractional shortening comparatively, though still depressed compared to WT and *Nlrp3*$^{-/-}$ animals (Figure 8B). Moreover, tissue from *Nlrp3*$^{-/-}$CNTg mice further showed preserved cardiac architecture compared to CNTg, consistent with a putative role in the regulation of IL-1β (Figure 8C). This included substantially fewer regions of myofiber fallout, and reduced inflammatory infiltrate. In association, we were unable to detect IL-1β in the serum of *Nlrp3*$^{-/-}$CNTg animals, indicating that NLRP3 is critically required for its induction during disease progression in the CNTg model (Figure 8D). Cardiac tissue from *Nlrp3*$^{-/-}$CNTg mice also showed reduced cleavage of caspase-1, again supporting a role for NLRP3 in regulating pro-inflammatory IL-1β during non-ischemic cardiomyopathy (Figure 8E). Taken together, these results implicate NLRP3 as an important regulator of both IL-1β production and caspase-1 processing during the pathogenesis of the CNTg model of chronic cardiac injury.

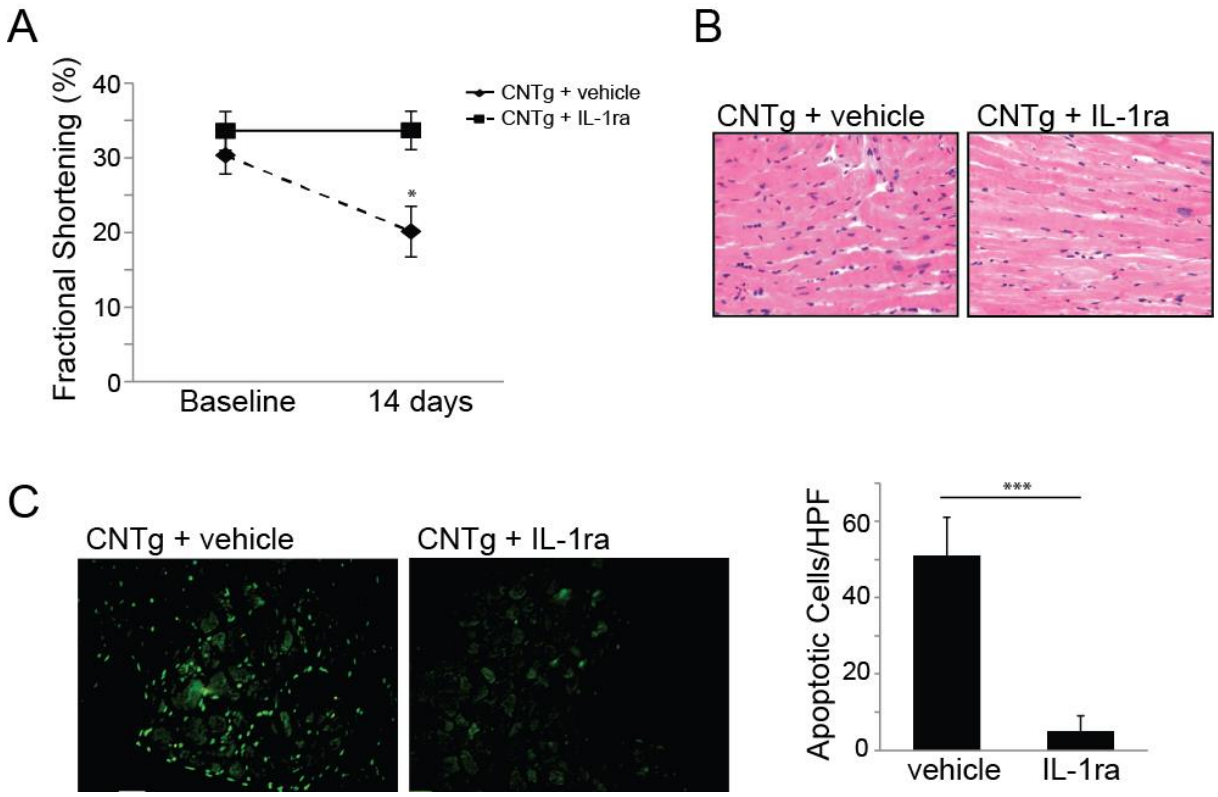

Figure 7. Effects of IL-1ra on cardiac parameters in CNTg mice.

A. Echocardiographic assessment of CNTg mice treated with saline or IL-1ra (n=5, 10mg/kg/day) for 14 days. **B.** H&E stain of myocardium from saline or IL-1ra-treated CNTg mice. **C.** TUNEL stain showing apoptotic cells from myocardium of saline and IL-1ra-treated CNTg mice. (*$p<0.05$, ***$p<0.001$).

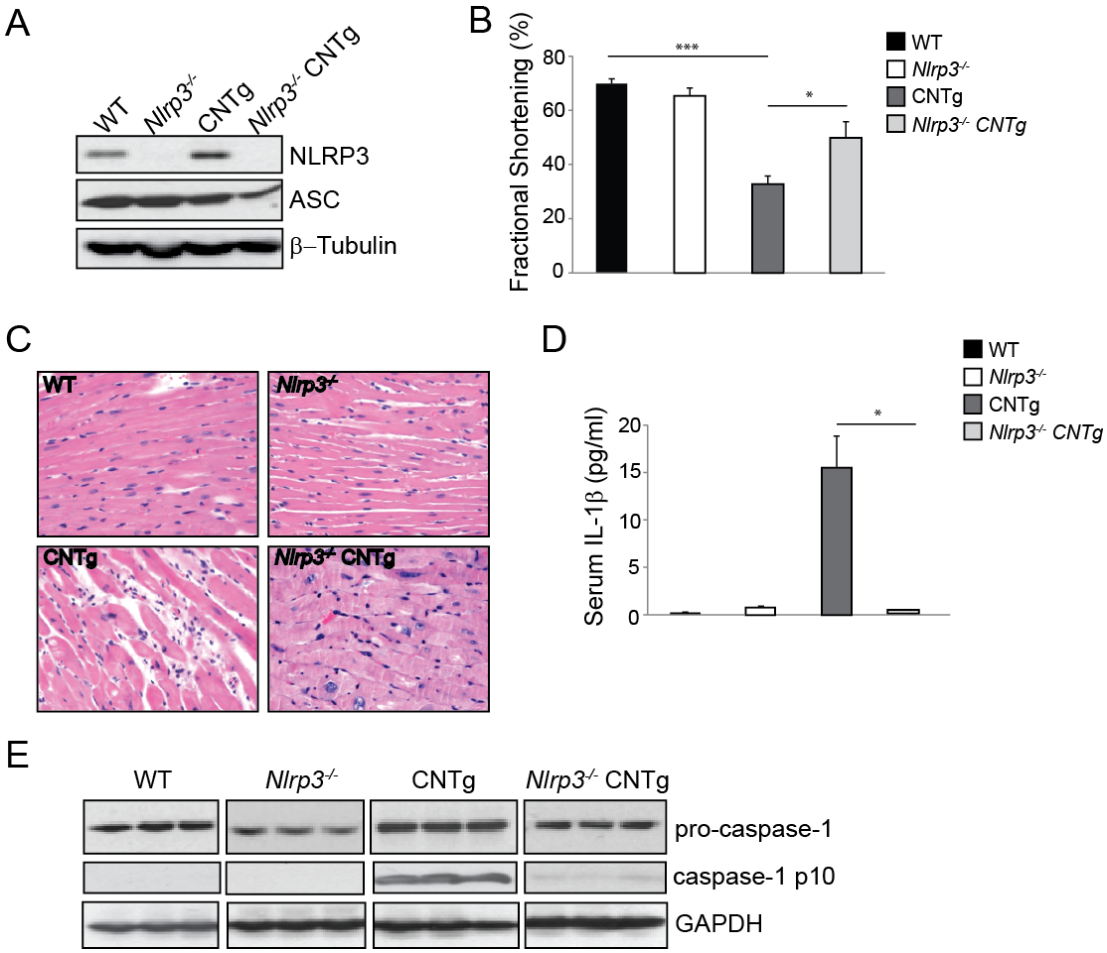

Figure 8. Cardiac phenotype of *Nlrp3*$^{-/-}$CNTg mice.

A. Immunoblot for NLRP3 and ASC in WT, *Nlrp3*$^{-/-}$, CNTg and *Nlrp3*$^{-/-}$CNTg mice. **B.** Echocardiographic assessment of left ventricular systolic function in conscious, unrestrained mice. **C.** H&E stained cardiac tissue from WT, *Nlrp3*$^{-/-}$, CNTg and *Nlrp3*$^{-/-}$CNTg mice. **D.** Serum IL-1β measurement by Luminex. **E.** Immunoblot for pro-caspase-1 and the p10 cleavage product in WT, *Nlrp3*$^{-/-}$, CNTg and *Nlrp3*$^{-/-}$CNTg hearts. (n=3-6/group, *p<0.05, ***p<0.001).

3.2 NLRP3 in Angiotensin II-Induced Hypertensive Heart Disease

The CNTg model represents an end-stage systolic disease that is driven genetically, with significant inflammatory elements and structural changes indicative of a complex underlying pathophysiology. Since NLRP3-deletion appeared to impart a partially protective phenotype through the regulation of IL-1β in CNTg animals, we next looked to explore its potential involvement in the early development of less severe diastolic cardiac injury. Chronic infusion of AngII in mice has been reported to result in hypertension and ventricular hypertrophy with fibrosis that proceeds in a TGFβ-dependent mechanism[65,80]. Mild hypertrophy and fibrosis are hallmarks of human diastolic heart disease[50]. Fibrosis represents the resolution of inflammation, indicative of tissue repair processes that are activated in response to cellular stress. Accordingly, we looked to determine whether NLRP3 participated in these phenotypes in the AngII model.

WT and *Nlrp3*$^{-/-}$ mice were infused with recombinant AngII for 28 days, and their hemodynamic parameters were routinely assessed by echocardiography. Consistent with previous reports, AngII induced the early development of systolic hypertension, which proceeded in tandem with concentric hypertrophy of the left ventricle (Figure 9A)[80,196]. We observed the progressive increase in ventricular septal wall thickness in association with decreased left ventricular chamber dimension during diastole (LVIDd). Importantly, these changes are similar to those reported in human patients with longstanding hypertension, reflecting potential clinical utility from this disease model[197]. Consistent with our findings in CNTg mice, both WT and *Nlrp3*$^{-/-}$ mice developed similar degrees of hypertension and hypertrophy in response to AngII, again indicating that NLRP3 does not participate in the myocyte hypertrophic growth response or AngII hypertensive signalling within vascular smooth muscle cells. Moreover, the infusion of AngII did not impair systolic cardiac function in WT or

Nlrp3$^{-/-}$ mice as reflected by the preservation of fractional shortening, supporting its use as a model of diastolic dysfunction.

In the CNTg model, structural changes proceeded in association with elevated pro-inflammatory cytokine levels, similar to those seen in patients with late stage heart disease. However patients with diastolic heart failure in response to longstanding hypertension typically display only minimal inflammatory changes within the myocardium, with prominent fibrotic remodelling[200]. To fully characterize the structural and functional changes and potential inflammatory signalling pathways induced by AngII infusion, we collected serum from WT mice and measured cytokine expression by Luminex (Figure 9B). Interestingly, there was only a modest, low-level induction of pro-inflammatory cytokines and chemokines including G-CSF and CXCL-1, indicating a predominantly neutrophilic inflammatory process. While IL-6 was slightly elevated, IL-1β was not, again reflecting the mild dysfunction induced in this diastolic model compared to CNTg. Consistent with these findings, H&E staining of AngII treated WT myocardium showed only mildly increased cellularity and inflammation (Figure 9C). Instead, fibrosis was prominent. Masson Trichrome staining revealed perivascular radiating and reactive fibrosis that resulted in large changes to the cardiac interstitial architecture (Figure 9D). These lesions were found to be associated with vascular structures that contained extensive vascular smooth muscle proliferation, suggesting that they developed in response to pressure-mediated changes. In contrast, hearts from AngII-treated *Nlrp3*$^{-/-}$ mice contained both fewer and less severe fibrotic areas, reflecting that NLRP3 could participate in fibrotic signalling distinct from inflammatory mechanisms.

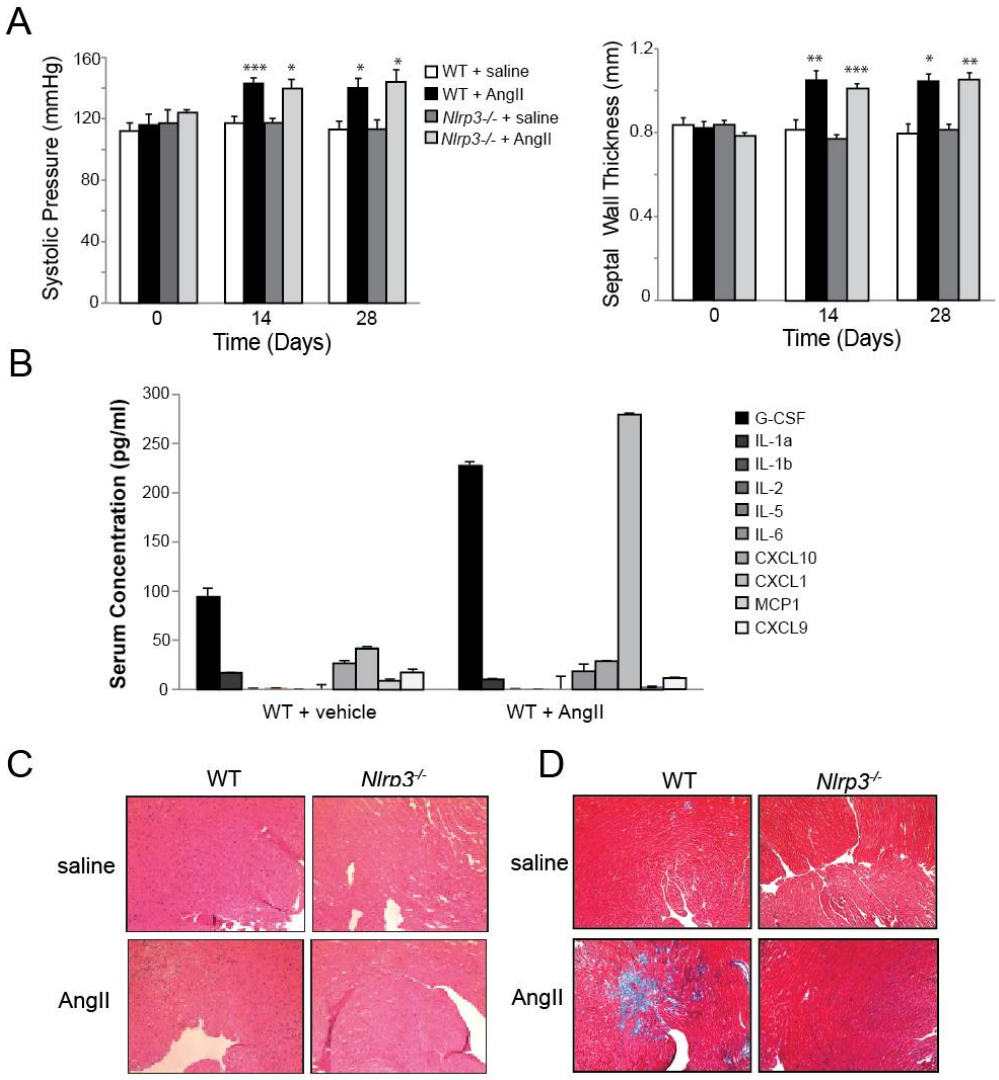

Figure 9. Effects of AngII on cardiovascular structure and function in WT and *Nlrp3*$^{-/-}$ mice.

A. Assessment of systolic blood pressure and septal wall thickness in WT and *Nlrp3*$^{-/-}$ mice treated for 28 days with saline (n=4) or AngII (n=8, 1.5 mg/kg/day). **B.** Serum cytokines in saline or AngII-treated mice by Luminex. **C.** H&E stained myocardial tissue from saline or AngII-treated mice. **D.** Masson Trichrome stained myocardial tissue from saline or AngII-treated mice. (*p<0.05, **p<0.01, ***p<0.001).

Since *Nlrp3*$^{-/-}$ mice were protected from fibrosis independently from cardiovascular hemodynamic parameters, we next looked to better characterize the fibrotic structures in hopes of identifying any potential cellular mediators. The development of cardiac fibrosis in response to AngII proceeds predominantly by resident cardiac fibroblasts[59]. Cardiac fibroblasts differentiate into myofibroblasts during disease states in response to pro-fibrotic signals such as AngII[57]. These professional wound-healing cells express abundant αSMA within contractile organelles and produce extensive amounts of interstitial collagen to remodel the extracellular matrix[79]. Myofibroblasts are only found in diseased and fibrotic tissue, supporting their classification as reparative cells that are critical for tissue homeostasis[201,202]. We looked to directly visualize whether myofibroblasts were present in AngII-treated myocardium demonstrating fibrosis. We performed fluorescent immunohistochemistry to detect αSMA-expressing myofibroblasts in WT and *Nlrp3*$^{-/-}$ treated tissue. While saline treated hearts showed little αSMA, AngII-treated WT hearts contained abundant αSMA-positive interstitial non-myocyte cells that displayed similar organization to fibrotic structures seen on Masson Trichrome staining (Figure 10A). These αSMA positive cells additionally contained the fibroblast growth marker FGF-2, supporting their classification as fibroblasts as opposed to vascular smooth muscle or endothelial cells[35]. Interestingly, these regions were not observed in tissue from *Nlrp3*$^{-/-}$ mice following AngII infusion, suggesting that NLRP3 may play a prominent role within cardiac fibroblasts during fibrotic stress signalling. To further quantify the reduced fibrotic development in *Nlrp3*$^{-/-}$ mice, whole ventricular tissue was processed and fibrotic markers were measured by immunoblotting (Figure 10B). Both αSMA and collagen-1 were increased following AngII infusion in WT hearts. In contrast, *Nlrp3*$^{-/-}$ tissue contained significantly less

αSMA and collagen after AngII, again supporting a role for NLRP3 in regulating fibrosis during experimental chronic diastolic cardiac injury.

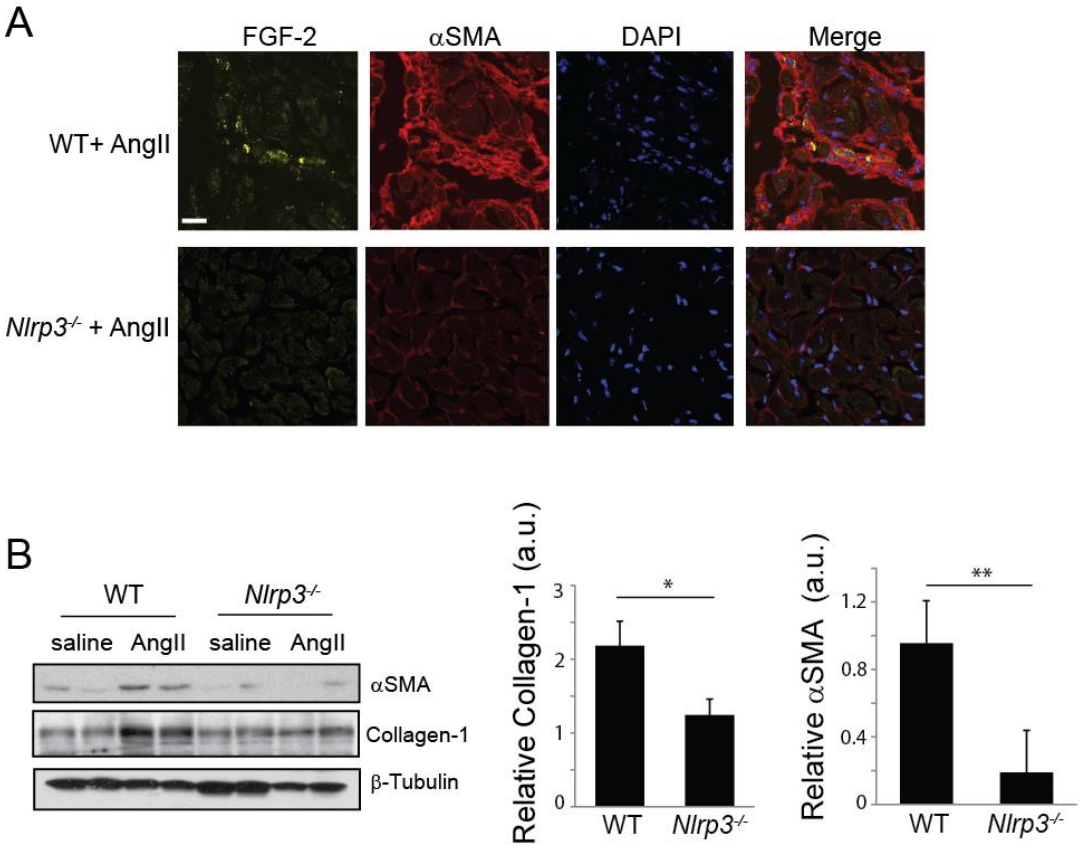

Figure 10. NLRP3 regulates AngII-induced fibrosis *in vivo*.

A. Confocal fluorescent immunohistochemical analysis of αSMA and FGF-2 in saline (n-4) and AngII-treated (n=8) WT and *Nlrp3*$^{-/-}$ mice. **B.** Representative immunoblot and semiquantitative analysis for αSMA and collagen-1 in myocardium from saline or AngII-treated mice. (*$p<0.05$, **$p<0.01$).

3.3 NLRP3 in Human Heart Disease

In contrast to the aforementioned animal models, human heart disease demonstrates varying degrees of injury resulting from complex combinations relating to hypertension, ischemia and hemodynamic alterations[197]. Since we previously found NLRP3 to play significant roles in the pathogenesis of both CNTg systolic dysfunction and AngII-induced diastolic injury in mice, we looked to explore its direct potential relevance in human heart samples from patients with heart failure. The late stage clinical management of heart failure requires direct augmentation of cardiac hemodynamic function. In suitable patients, this involves the surgical implantation of left ventricular assist devices (LVAD) in order to mechanically augment cardiac output and limit hemodynamic load as a bridge to transplant[203]. The surgical procedure involves the excision of a transmural piece of left ventricular tissue from the cardiac apex. We obtained these samples from 5 patients undergoing LVAD placement at the time of surgery, and processed them for protein and histological assessment to detect whether NLRP3 and other inflammasome components were present in human heart. As in mice, NLRP3, ASC and pro-caspase-1 were all found to be present at the protein level in all 5 patients (Figure 11A). Histological evaluation of human samples confirmed that end stage myocardium contained extensive inflammation and fibrosis (Figure 11B).

To determine which cell types predominantly expressed NLRP3 in human heart, we performed fluorescent immunohistochemistry on LVAD patient samples. Control right atrial appendage myocardium demonstrated highly organized sarcomeric structure as evidenced by titin localization connecting the M-line to the Z-line (Figure 11C). In contrast, myocardium from patients with late stage disease demonstrated prominent loss of titin and disorganized myocyte structure, consistent with a significant loss of function[204]. Moreover, in association with

picrosirius staining showing increased interstitial fibrosis, end-stage disease samples also showed disorganized interstitial collagen-1 compared to normal appearing atrial controls under fluorescent confocal microscopy. Interestingly, we detected NLRP3-positive, small, spindle shaped cells within these fibrotic areas that were not present in atrial control tissue. Importantly, these NLRP3-expressing cells did not display myocyte morphology or contain organized titin, and likely represented cardiac fibroblasts or myofibroblasts. Together, these data from human patients with HF supports a putative role for endogenous NLRP3 within the heart during fibrotic signalling, again suggesting that NLRP3 could exert as of yet unknown functions within resident cardiac cells.

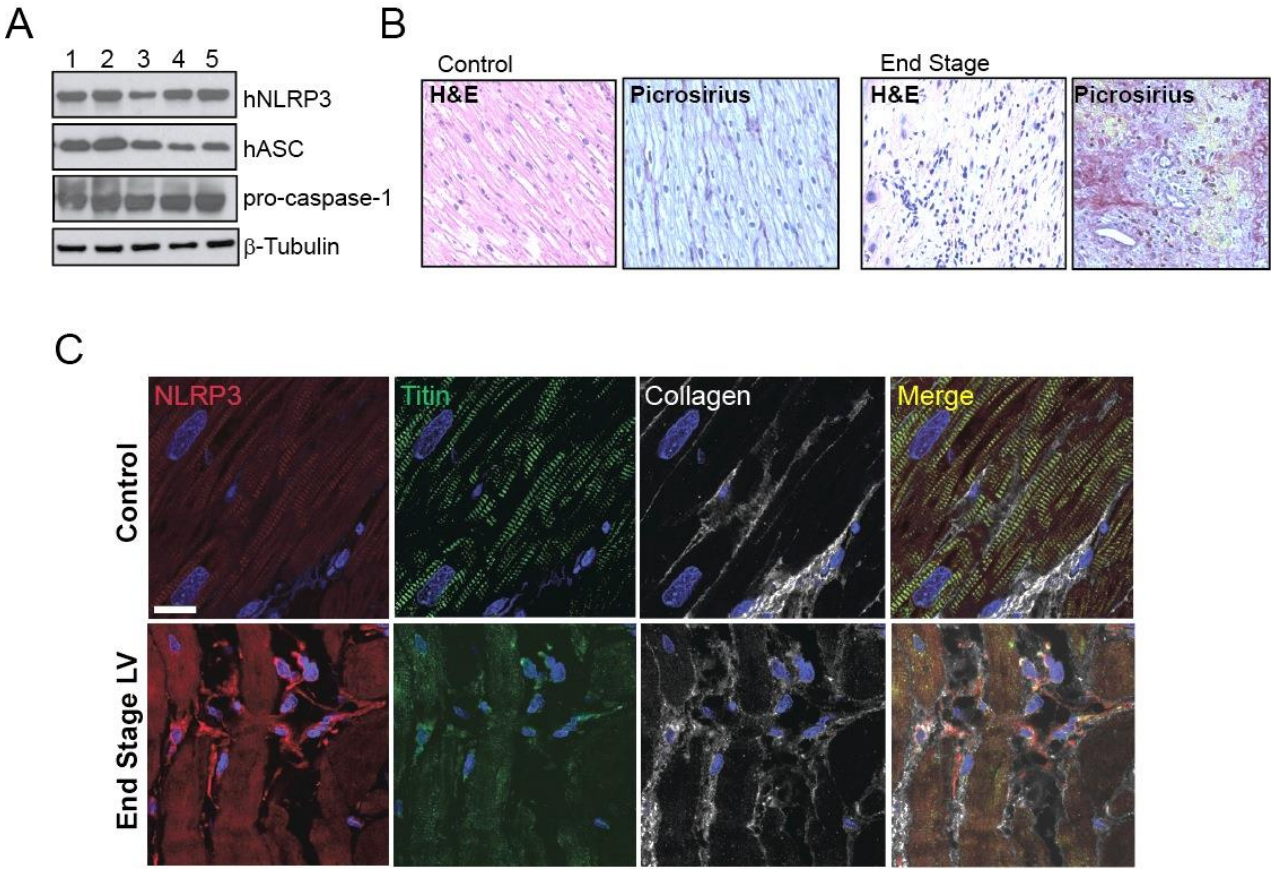

Figure 11. NLRP3 in human heart failure left ventricular tissue.

A. Immunoblot for NLRP3, ASC and pro-caspase-1 in human left ventricular myocardium from 5 patients with end-stage disease. **B.** H&E and picrosirius red stained left ventricular myocardium from patients with heart failure or normal appearing control right atrial tissue. **C.** Confocal fluorescent immunohistochemical analysis of NLRP3, titin and collagen-1 in control atrial and diseased left ventricular myocardium.

3.4 Discussion

3.4.1 NLRP3 and IL-1β in Late Systolic vs Early Diastolic Disease

In human HF, the distinction between diastolic and systolic disease likely reflects underlying remodelling processes within the heart following hypertrophy, inflammation and fibrosis[51]. Since NLRP3 is expressed endogenously in cardiac tissue, it may regulate one, all or none of these processes. Little is known regarding the precise cellular and molecular mechanisms responsible for the decompensation of heart disease. Accordingly, we looked to explore the relevance of NLRP3 in a spectrum of disease models and in human HF.

IL-1β has previously been shown to be present at very low levels in patients with late stage heart failure[205]. The observation of significant levels in the serum from CNTg animals supports this finding, and indicates that these mice are also at an end stage of disease. It is therefore not surprising that antagonizing IL-1β with the IL-1ra provided a functional response by improving systolic performance. IL-1β induces calcium dysregulation and impairs electrophysiological function alone in isolated cardiac myocytes. It induces apoptosis of myocytes and fibroblasts in cell culture, and infusion into mice *in vivo* results in widespread inflammation and cardiac depressant effects[171]. These results, in association with our findings continue to support the classification of IL-1β as a key proinflammatory cytokine during cardiac injury. Importantly, the antagonism of IL-1β using the IL-1ra (Anakinra) is already under experimental clinical use following acute ST-elevation myocardial infarction. Clinical trials have reported that early use of IL-1ra following STEMI resulted in improved systolic function and resolution of inflammatory markers, despite no changes in hypertrophy or cardiac dimensions[183,184]. These findings are consistent with our studies in CNTg mice, supporting the

potential utility of IL-1β blockade in response to acute stimuli and late stage disease. However the role of IL-1β in the early development of HF is controversial[206]. We did not detect a profound inflammatory response or elevated IL-1β in mice treated with AngII for 28 days, despite elevated blood pressure and concentric hypertrophy with fibrosis. These results indicate that the degree of inflammation likely parallels disease severity, and may promote decompensation of diastolic HF into systolic disease.

While NLRP3 regulated IL-1β production and caspase-1 processing in the CNTg model, distinctly different mechanisms of protection were evident in the AngII model of diastolic dysfunction. Instead of overt cytokine involvement, NLRP3 appeared to regulate the degree of fibrosis, which we found to be associated with reduction in the presence of interstitial myofibroblasts. It is tempting to speculate that the protected phenotype could be a result of low-level paracrine secretion of IL-1β locally within the heart, as previously reported[171]. However additional groups have described similar discrepancies in their studies regarding NLRP3 and ASC. Moreover, in both renal and cardiovascular models of injury, the targeted deletion of NLRP3 or ASC restricted to the bone marrow compartment alone does not provide complete protection compared to global knockout[177,186]. These results together suggest that NLRP3 could exert alternative roles in parenchymal and structural cells within organ systems independently from the classical concept of "inflammasome".

3.4.2 NLRP3 in Resistance and Tolerance Pathways

In additional to potentially novel roles for NLRP3 in cardiac injury, our *in vivo* findings also elaborate on the differential wound healing processes that take place during overt systolic

disease versus subtle diastolic dysfunction. There was a profound inflammatory infiltrate and extensive systemic inflammation present in CNTg mice, indicating an overwhelming mode of chronic injury. In the AngII mode though, only mild infiltrate was present, and instead extensive reactive fibrosis with the differentiation of local resident myofibroblasts occurred. These data support the notion that in response to chronic injuries, both resistant (leukocytic infiltrate) and tolerant (fibrotic) mechanisms differentially operate to regulate homeostasis. While resistance measures involve the outside recruitment of innate immune cells, tolerance pathways appear to be mediated by local structural cells. While we have not explicitly addressed these temporal aspects experimentally, it is likely that tolerance measures are first employed by resident fibroblasts to remodel tissue in response to subtle injurious stimuli. When these injuries become more elaborate and substantial, resistance measures are subsequently employed by the recruitment of additional more specialized cellular compartments. Given our findings of cytokine-dependent and independent effects, NLRP3 may participate in both of these processes.

3.4.3 NLRP3 in Fibrosis and Cardiac Fibroblasts

One consistent finding in both CNTg and AngII models is that NLRP3 appears to exert its protective effects independently from the development of cardiac hypertrophy. Indeed, these findings were paralleled by other groups in response to experimental myocardial infarction, with knockdown of NLRP3 using siRNA[187]. Hypertrophic growth responses in cardiac myocytes proceed largely in result to mechanical stimuli and secreted soluble factors like AngII and TGFβ, involving the addition of new sarcomeres either in parallel or in series with the existing

contractile units[200]. While cardiac myocytes probably do express NLRP3, we cannot comment on its functional significance in response to injury, and they have not been an explicit focus.

In contrast, our findings support a novel role for NLRP3 in cardiac fibroblasts and fibrosis. Previous groups have also implicated NLRP3 in the development of fibrosis and fibroblast biology. NLRP3 mRNA was found to be upregulated in skin biopsies from patients with systemic sclerosis, and the genetic deletion of NLRP3 also protected mice in a model of bleomycin-induced dermal fibrosis, with reduced myofibroblast differentiation[207]. Additionally, members of our group have previously reported that NLRP3-deficient mice do not develop renal fibrosis in a model of unilateral ureteric obstruction[177]. Importantly, protection was also observed in association with limited cytokine involvement, supporting our conclusions for additional NLRP3 roles in diverse cell types.

In conclusion, our *in vivo* findings have identified potentially distinct roles for NLRP3 in both professional and non-professional immune cells. These functions parallel involvement in both systolic heart disease, with increased inflammatory infiltrate, and diastolic disease, with myocardial fibrosis and myofibroblast differentiation. Further experiments were required though, in order to better delineate how these different roles proceed and which pathways are involved.

Chapter Four: **NLRP3 is a Regulator of TGFβ Induced Fibrotic Signalling in Cardiac Fibroblasts**

Overview

Our prior results indicate a potential role for NLRP3 in the development of fibrosis. Inflammation and fibrosis are temporally linked following acute injurious stimuli, and many soluble factors released by innate immune cells initiate fibrotic signalling in resident cells[5]. We therefore next looked to identify the specific cellular and molecular mediators involved in NLRP3-driven fibrotic processes. ASC has previously been shown to induce "inflammasome" assembly in isolated cardiac fibroblasts, regulating caspase-1 and IL-1β production[186]. Despite this, the expression of all inflammasome components has not been adequately demonstrated in cardiac fibroblasts, and their ability to secrete physiologically relevant levels of cytokine is not universally accepted. Moreover, the specific role of these pathways in fibrosis during chronic injury has not been evaluated.

We characterized the expression and role of NLRP3 in both murine and human cardiac fibroblasts using primary cell culture, molecular approaches, microscopy and signalling assays. Interestingly, we found that NLRP3-deficient cardiac fibroblasts did not fully differentiate into myofibroblasts in response to profibrotic stimuli such as AngII or TGFβ. We went on to characterize the signalling pathways that were defective in *Nlrp3*$^{-/-}$cells, and identified impaired receptor associated Smad (R-Smad) activation in response to TGFβ. NLRP3 regulated R-Smad signalling in response to TGFβ through its NACHT domain in an ATP binding-dependent mechanism, similar to what has been reported for its involvement in inflammasome assembly and cytokine processing. To further understand how NLRP3 could regulate such processes, we went on to characterize its subcellular localization in human cardiac fibroblasts. NLRP3 localized dominantly to mitochondrial structures, where it promoted the production of reactive oxygen species (ROS). Importantly, the regulation of mitochondrial ROS, R-Smad signalling and

myofibroblast differentiation in cardiac fibroblasts occurred independently from IL-1β, IL-18 and caspase-1, supporting a novel "inflammasome" independent mechanism. Our results show, for the first time, a complete mechanistic insight into NLRP3 function in cardiac fibroblasts and fibrosis. They support our *in vivo* and histological findings in both mouse and human tissue, and further elaborate on novel aspects of NLRP3 signalling in non-professional immune cells to regulate cellular physiology.

4.1 NLRP3 Regulates Cardiac Myofibroblast Differentiation

Since our histological data in human left ventricular tissue showed NLRP3 expression predominantly within interstitial cardiac fibroblasts, and NLRP3-deficient mice were protected against AngII-induced hypertensive cardiac fibrosis, we looked to explore the general expression and biology of NLRP3 in primary isolated cardiac fibroblasts (CFs) during activation to myofibroblasts. We began by validating our primary cell culture technique. Isolated cells responded to early treatment with TGFβ, as indicated by the phosphorylation and nuclear accumulation of Smad2/3 after 15 minutes. Following 24 hours of treatment with TGFβ, primary cells increased αSMA in cytoplasmic stress fibers, again consistent with fibroblast to myofibroblast transdifferentiation (Figure 12A-C)[59].

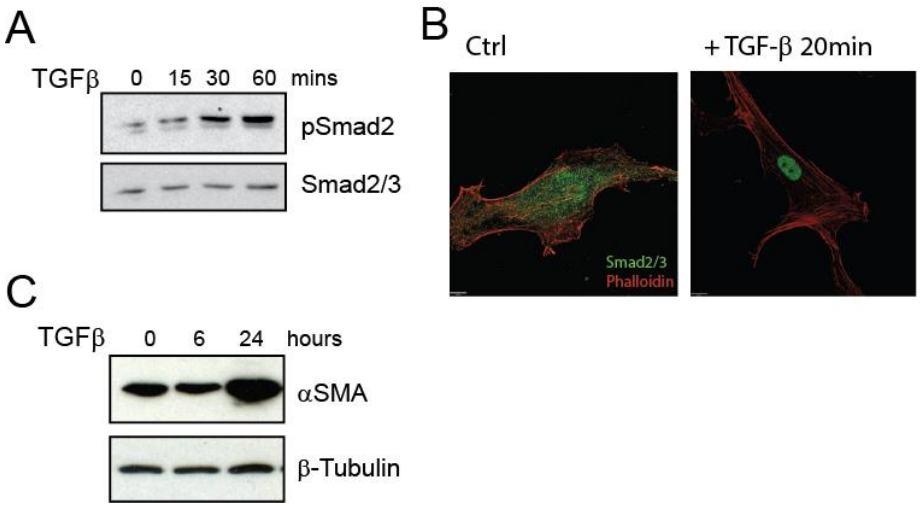

Figure 12. Effects of TGFβ on murine cardiac fibroblasts.

A. Immunoblot for pSmad2 in response to TGFβ stimulation for indicated time points. **B.** Immunocytochemistry for Smad2 nuclear accumulation in response to TGFβ. **C.** Immunoblot for αSMA in murine CFs treated with TGFβ for indicated time points.

Consistent with previous reports, primary murine ventricular CFs expressed the general "inflammasome" components pro-caspase-1, ASC and NLRP3 at the protein level (Figure 13A). Interestingly, the treatment of CFs with TGFβ for 24 hours resulted in a significant increase in NLRP3. This occurred in association with elevated αSMA, indicating that NLRP3 may directly participate in myofibroblast differentiation (Figure 13B). To establish its function and relevance, primary murine ventricular CFs were isolated from WT and *Nlrp3*$^{-/-}$mice and differentiated into myofibroblasts by TGFβ or AngII. While WT CFs responded to both TGF and AngII stimulation by upregulating αSMA, *Nlrp3*$^{-/-}$cells displayed impaired differentiation. We detected significantly reduced levels of αSMA both under baseline unstimulated conditions and following treatment with TGFβ or AngII (Figure 13C). The upregulation of αSMA and activation of cardiac fibroblasts is also associated with increased matrix proteins and soluble growth factors[208]. We also detected reduced levels of CTGF and MMP2 following stimulation of *Nlrp3*$^{-/-}$ CFs for 24 and 48 hours by TGFβ, again consistent with impaired myofibroblast differentiation. To directly visualize changes in myofibroblasts differentiation in WT and *Nlrp3*$^{-/-}$cells, we performed fluorescent immunocytochemistry to detect αSMA in response to TGFβ (Figure 13D). Under unstimulated conditions, both WT and *Nlrp3*$^{-/-}$ CFs displayed little αSMA. In WT cells, TGF stimulation resulted in increased αSMA within cytoplasmic radiating stress fibers. These were again largely absent from *Nlrp3*$^{-/-}$CFs, which showed little change compared to unstimulated conditions. Together, these data show that NLRP3 regulates the differentiation of cardiac fibroblasts into myfibroblast phenotype in response to soluble fibrotic factors TGFβ and AngII.

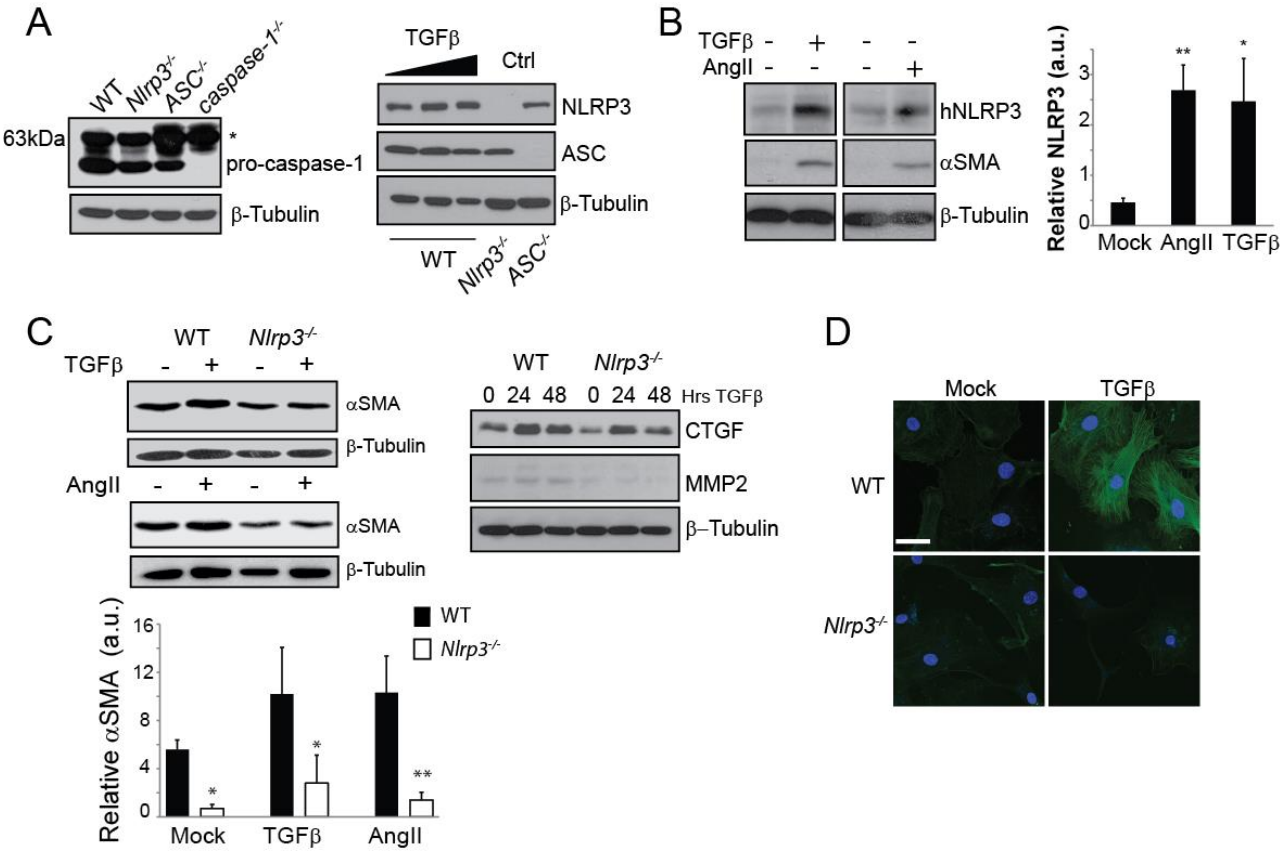

Figure 13. NLRP3 regulates cardiac myofibroblast differentiation

A. Immunoblotting for NLRP3, ASC and procaspase-1 in murine CFs. Asterisk denotes a nonspecific band present at 63 kDa. **B.** Immunoblotting and semiquantitative analysis for NLRP3 and αSMA in human CFs stimulated for 24 hours with TGFβ (10 ng/ml) or AngII (1 μM). **C.** Immunoblotting for CTGF, MMP2 and αSMA and semiquantitative analysis in WT and *Nlrp3*$^{-/-}$ CFs following 24-hour stimulation with TGFβ or AngII *p<0.05, **p<0.01, n=3. **D.** Confocal fluorescent immunocytochemistry of αSMA in WT and *Nlrp3*$^{-/-}$ CFs following 24-hour stimulation with TGFβ. Scale=10 μm.

4.2 NLRP3 Regulates TGFβ-Induced R-Smad Activation

The observation that NLRP3 regulates cardiac myofibroblasts differentiation in response to both TGFβ and AngII implies a potential role in modulating a shared signalling element. TGFβ signalling proceeds following binding of soluble TGFβ to the type II TGF receptor (TBRII)[67]. This results in the dimerization and phosphorylation of the type I TGF receptor (TGFRI), which results in the recruitment of R-Smads 2 and 3 to the receptor I/II complex[209]. R-Smad phosphorylation on conserved serine/threonine residues by the TGF receptors leads to their association with co-Smad Smad4, forming the activated transcription factor complex that translocates to the nucleus and regulates pro-fibrotic gene expression. While many of the effects of AngII in the vasculature involve signalling through protein kinase C and IP3/calcium, it has also been shown to regulate the activation of R-Smads during fibrosis[74,77,210]. The Smad pathway represents a critical point in cardiac fibroblast differentiation to myofibroblasts in both isolated cell culture and animal models of cardiac injury. Therefore we looked to explore the role of the R-Smads in the early signalling response in WT and *Nlrp3*$^{-/-}$CFs. WT cells displayed an early activation time course for Smad2 phosphorylation, with increased levels present at 15 minutes and persisting to later time points (Figure 14A). In comparison, *Nlrp3*$^{-/-}$CFs displayed significantly reduced phosphorylation of Smad2 after TGFβ stimulation. We also observed reduced R-Smad nuclear accumulation in *Nlrp3*$^{-/-}$CFs using fluorescent immunocytochemistry (Figure 14B). While *Nlrp3*$^{-/-}$cells still showed relative Smad activation after stimulation with TGFβ, there was consistently less phosphorylation and nuclear accumulation in comparison to WT cells. These data point to a potential role in fine-tuning the early pro-fibrotic signalling pathways in cardiac fibroblasts. For validation of the knock-out phenotype, we next looked to determine whether these apparent responses were also present in human CFs, given the

compensatory mechanisms that arise in genetically altered animal strains. In the context of NLRP3-mediated IL-1β signalling in macrophages, the sulfonylurea anti-diabetic agent glibenclamide has been reported to antagonize NLRP3 function[211]. We established that in human PMA-differentiated THP-1 macrophages, glibenclamide inhibits IL-1β secretion with an apparent IC50 of approximately 20 μM (Figure 14C). To determine whether glibenclamide treatment was able to recapitulate the *Nlrp3*$^{-/-}$ phenotype on R-Smad signalling, human CFs were pretreated for 15 minutes in 20 μM glibenclamide and stimulated with TGFβ. Similar to the phenotype observed in *Nlrp3*$^{-/-}$ CFs, glibenclamide also reduced the phosphorylation of Smad2 in response to TGFβ in human cells. Collectively these data support the premise that NLRP3 contributes to TGFβ-induced R-Smad activation and myofibroblast differentiation in both human and mouse CFs.

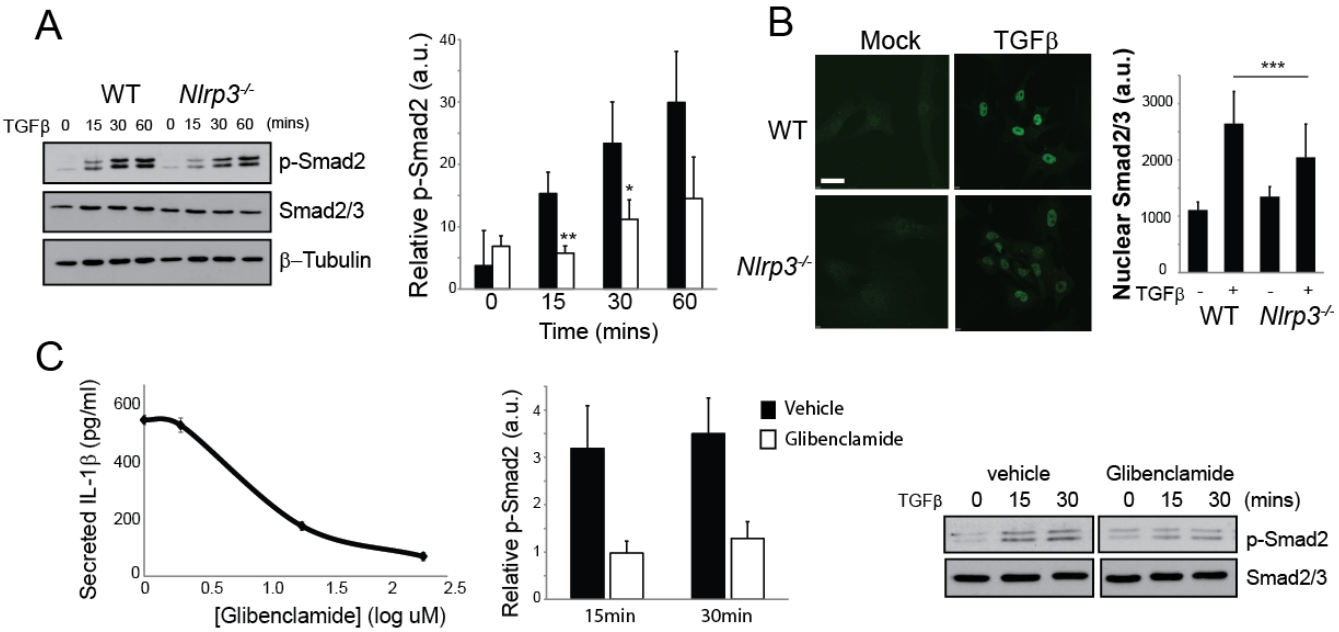

Figure 14. NLRP3 regulates R-Smad signalling.

A. Immunoblotting and semiquantitative analysis for p-Smad2 in TGFβ stimulated WT and *Nlrp3*$^{-/-}$ CFs. **B.** Confocal immunofluorescence of Smad2/3 in WT and *Nlrp3*$^{-/-}$ CFs with and without TGFβ for 15 minutes (n>30 cells in at least 4 fields of view). Scale=40 μm. **C.** Dose-response curve for glibenclamide inhibition of IL-1β secretion in PMA differentiated THP1 cells (left). Immunoblot of p-Smad2 in TGFβ stimulated human CFs pretreated 15 minutes in 20 μM glibenclamide. (*p<0.05, ***p<0.001).

A role for NLRP3 in R-Smad activation could involve multiple diverse signalling pathways. In addition to Smads, TGFβ induces the early signalling through a number of non-canonical kinase cascades[212]. The facilitation of R-Smads to the TGFRI/II receptor complex also involves a growing list of regulators that mediate degradation, apoptosis, translocation and endocytosis processes[68]. Therefore we looked to understand whether NLRP3 participated in these additional aspects of TGFβ signalling within cardiac fibroblasts. We found that TGFβ stimulation resulted in comparable levels of AKT phosphorylation in both WT and *Nlrp3*$^{-/-}$CFs (Figure 15). Erk1/2, in contrast, was not activated in WT or *Nlrp3*$^{-/-}$CFs during TGFβ treatment, implying that it does not significantly participate in CF differentiation in this system. We also observed a similar increase in cytoplasmic levels of TGFRII and Smad4 in WT and *Nlrp3*$^{-/-}$ CFs, further ruling out a role for NLRP3 in differential regulation of TGF receptors or coSmad recruitment.

NLRP3 has been proposed to interact with a wide variety of signalling proteins to facilitate cellular processes in macrophages. We looked to explore this possibility in the TGFβ machinery using co-immunoprecipitation studies. We did not detect consistently significant or meaningful protein-protein interactions using overexpression systems between NLRP3 and Smad2, Smad3, Smad4, Smad7, TGFRI ,TGFRII or the anchoring protein SARA (Figure 16A, B). NLRP3 has previously been shown to undergo regulation via de-ubiquitination in macrophages, which may play a role in priming cells before stimulation[213]. We assessed whether NLRP3 and its separate domains underwent differential ubiquitination during the course of TGFβ stimulation. Flag tagged NLRP3 or its separate domain plasmid constructs were co-transfected in 293T cells with TGFRII. Cells were then treated with TGFβ for 6 hours, immunoprecipitated and immunoblotted for ubiquitin (Figure 16C). We did not detect any

changes in NLRP3 ubiquitination in any of the constructs tested. Together, these results indicate that the involvement of NLRP3 in TGFβ-induced R-Smad signalling may secondarily relate to other intracellular components and organelles, operating in a nonspecific manner.

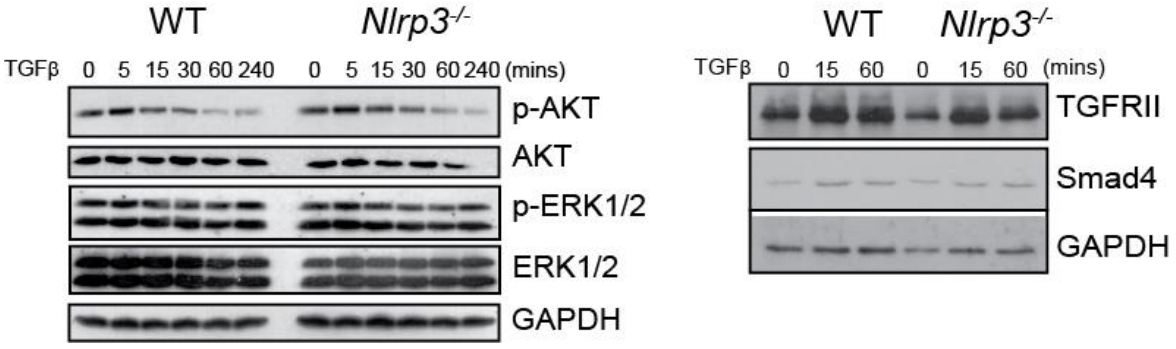

Figure 15. TGFβ signalling in *Nlrp3*$^{-/-}$ cells.

Immunoblotting for p-AKT, p-ERK1/2, TGFRII and Smad4 in WT and *Nlrp3*$^{-/-}$ CFs stimulated with TGFβ.

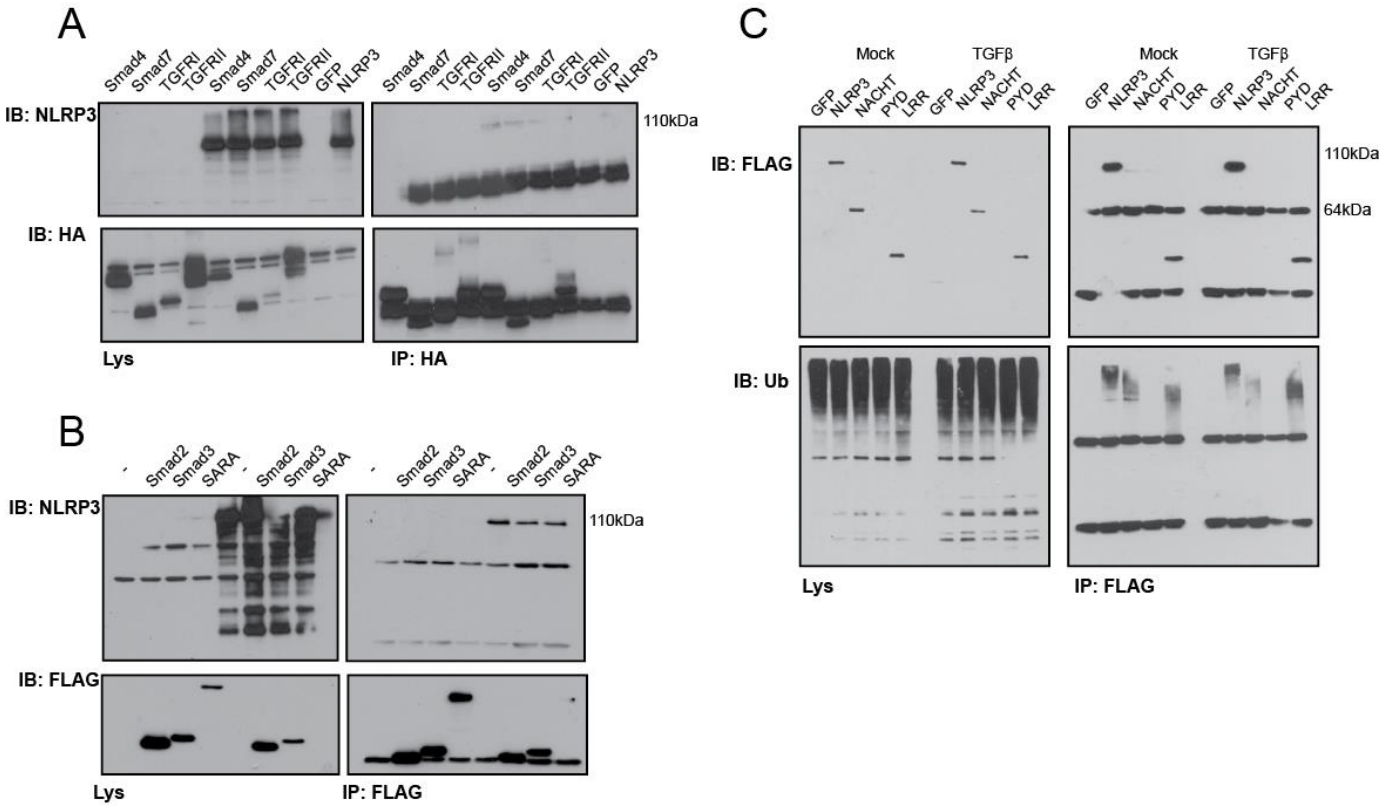

Figure 16. NLRP3 does not interact with members of the TGFβ machinery.

A. Immunoblot for NLRP3 and HA in co-immunoprecipitates from 293T cells transfected with WT NLRP3 and indicated HA-tagged constructs. **B.** Immunoblot for NLRP3 and FLAG in co-immunoprecipitates from 293T cells transfected with WT NLRP3 and indicated FLAG-tagged constructs. 110 kDa marks the expected location for NLRP3, which is either absent or present non-specifically in immunoprecipitated samples. **C.** Immunoblot for FLAG and ubiquitin in control and TGFβ-stimulated immunoprecipitates from 293T cells transfected with NLRP3 FLAG-tagged domain constructs.

4.3 The NLRP3 NACHT Domain is Required for NLRP3-Induced R-Smad Signalling

To further explore the general mechanism by which NLRP3 might modulate R-Smad activation, experiments were performed to identify the essential NLRP3 structural elements underlying the observed response. Using an R-Smad-responsive luciferase reporter assay, 293T cells were transfected with a variety of NLR mutant constructs and activation of the Smad-responsive SBE4-luciferase plasmid was determined after TGFβ treatment. A significant increase in R-Smad activity was observed in NLRP3-overexpressing cells compared to GFP-transfected controls, consistent with our previous observations in the primary murine and human ventricular CFs (Figure 17A). Interestingly, augmented Smad signalling was not observed with expression of NLRP9 or NLRP10 constructs, suggesting a unique and specific role for NLRP3 amongst the NLRs in the TGFβ pathway. To determine which specific structural elements were required, NLRP3 domain mutants including NACHT, PYD-NACHT, LRR or PYD constructs were employed (Figure 17B). Compared to control GFP transfected cells, only plasmids carrying the NACHT domain enhanced Smad activity. PYD and LRR-only constructs did not increase Smad-dependent luciferase activity, whereas the NACHT and NACHT-PYD both increased Smad activity similar to full length NLRP3, identifying a critical function for the NACHT domain.

As previously indicated, all NLR proteins contain a conserved NBD responsible for nucleotide coordination and hydrolysis[87]. The consensus Walker A motif, characterized by GXXXXGK(T/S), has been shown to be critical in preferential ATP binding, formation of the inflammasome and IL-1β processing by NLRP3 in macrophages[150]. To determine if the NLRP3 NACHT Walker A site was also necessary to modulate Smad activation, we created ATP-binding defective NLRP3 mutants (Figure 17C). Mutation of G231, K232 and T233 to alanine

residues resulted in significantly reduced Smad activation in both full length NLRP3 and NLRP3-NACHT constructs, indicating that the NLRP3-NACHT domain is required for NLRP3-dependent Smad activation in response to TGFβ. These molecular findings indicate that NLRP3 maintains shared structural requirements in regulating canonical cytokine processing in macrophages and non-canonical pathways in structural cell types.

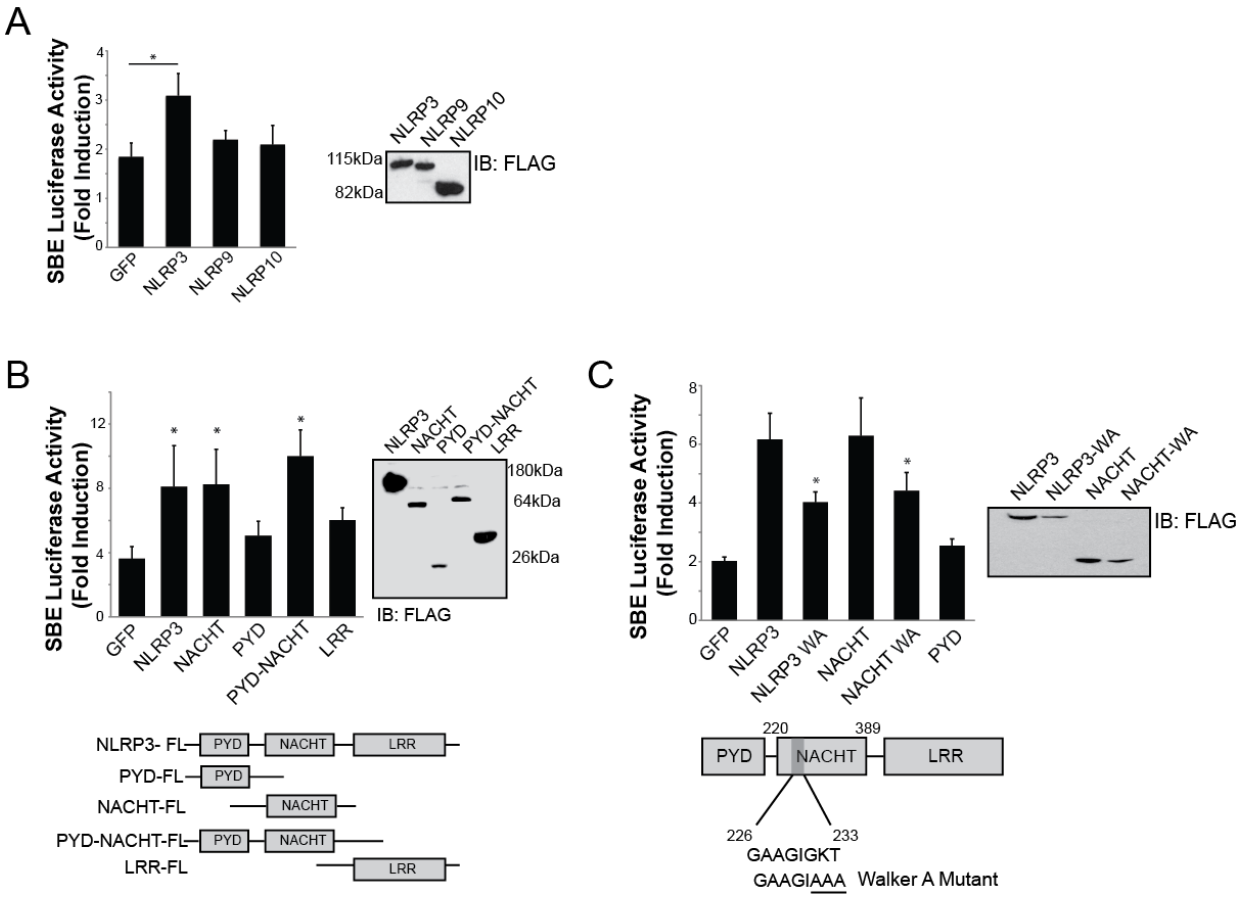

Figure 17. NLRP3 regulates R-Smad signalling through the NACHT domain.

A. Luciferase assay and expression of FLAG-tagged NLRP3, NLRP9 and NLRP10 constructs with SBE4-luciferase reporter in 293T cells transfected with control GFP or NLRs. **B.** Luciferase assay in 293T cells transfected with control GFP, NLRP3, NACHT, PYD, PYD-NACHT or LRR constructs. Data are expressed as fold induction of luciferase activity following TGFβ stimulation (10 ng/ml) compared to mock-treated cells (n=3, *p<0.05 versus GFP). **C.** Structure and expression of the NLRP3 Walker A (WA) mutation in full-length NLRP3 and NACHT constructs. SBE4 luciferase assay with control GFP, NLRP3, NLRP3 WA, NACHT, NACHT WA or PYD constructs. (n=3, *p<0.05).

4.4 Mitochondrial NLRP3 Regulates R-Smad Signalling Through the Production of ROS

Since we failed to identify specific molecular interactions governing a role for NLRP3 in R-Smad signalling, we looked to gain clues for its mechanistic involvement in cardiac fibroblasts from subcellular localization studies. NLRP3 has been previously proposed to localize to mitochondria after activation in macrophages using tagged overexpression constructs[156,191]. We employed conventional fluorescent immunocytochemistry using endogenous proteins to determine its presence in primary human CFs. Under unstimulated conditions, we detected NLRP3 predominantly at the mitochondria, with some cytoplasmic staining (Figure 18A). NLRP3 substantially co-localized with Mitotracker Red, but not other organelles like the Golgi or nucleus. Interestingly, stimulating human CFs with TGFβ for 6 hours did not result in significant translocation, as NLRP3 still maintained a predominantly mitochondrial localization (Figure 18B). Moreover, the destabilization of mitochondria by treating CFs with the electron transport complex I inhibitor rotenone (10 μM) resulted in fragmented mitochondrial morphology. Despite altered organelle structure, NLRP3 was still detected at mitochondria, indicating a potentially intrinsic association.

Since NLRP3 localized predominantly to mitochondria both in unstimulated and TGFβ-stimulated human CFs, we went on to explore how mitochondrial function could regulate R-Smad signalling towards myofibroblast differentiation. The mitochondria are energetically important organelles involved in diverse processes. Their production of reactive oxygen species (ROS) is of particular importance in cell signalling. For example, fibroblasts and epithelial cells have been well documented to utilize mitochondrial ROS (mROS) as second messengers to facilitate diverse signal transduction pathways[214,215]. TGFβ has recently been reported to increase mROS, which is required for optimal differentiation of myofibroblasts[215]. Since mROS have also

been extensively implicated in activating NLRP3-dependent caspase-1 and IL-1β processing in macrophages, we looked to explore the role of mROS in R-Smad signalling. Consistent with previous findings, treatment of TGFRII-expressing 293T cells with TGFβ resulted in an early induction of mROS (Figure 19A). The stimulation of human CFs with Rotenone (10 μM) also resulted in the early phosphorylation of Smad2, confirming the importance of ROS in TGFβ signalling.

A function for NLRP3 in mROS and TGFβ signalling could operate similar to previous reports documenting ROS-mediated activation of the NLRP3 inflammasome[159,216]. Alternatively, NLRP3 could have uncharacterized roles in mitochondrial function. As previously mentioned, the role of ROS in activating the "inflammasome" remains correlational, with putative activating stimuli merely increasing ROS levels in association with NLRP3-dependent cytokine production[159]. Nobody to date has explored the possibility that NLRP3 could regulate the degree of ROS induction. To resolve this, we looked to explore the relative degree of mROS production in control GFP and NLRP3-expressing 293T cells by flow cytometry using MitoSOX. Surprisingly, the overexpression of NLRP3 resulted in modest, but significantly increased mROS compared to GFP controls (Figure 19B). To determine the biological relevance of these observations, we went on to explore whether antagonizing ROS could impact NLRP3-dependent R-Smad signalling in our over-expression luciferase reporter system. 293T cells were transfected with NLRP3 and the SBE4 luciferase construct as before, and the TGFβ-induced R-Smad response was measured in the presence of the ROS inhibitor aminopyrrolidine-2, 4-dicarboxylate (APDC), which has previously been shown to inhibit NLRP3 inflammasome activation[167]. We observed a dose-dependent reduction in NLRP3-mediated Smad activity with increasing concentrations of APDC (Figure 19C). A similar response was seen in the presence of

N-Acetyl Cysteine (NAC, Figure 19D). While the blockade of ROS also reduced Smad activity in control GFP cells, the effect was significantly more pronounced in NLRP3-expressing cells, demonstrating that NLRP3 likely exerts its effects on TGFβ signalling through modulation of ROS.

To clarify the importance of ROS in primary CFs and determine its relevance, we next looked to directly visualize any potential differences in mROS production. We performed live cell imaging using isolated WT and *Nlrp3*$^{-/-}$ ventricular CFs loaded with Mitotracker Green to label mitochondria and MitoSOX to visualize mROS (Figure 20). WT CFs showed a time dependent increase in MitoSOX staining. This signal was inhibited by pretreatment with the mROS inhibitor MitoTEMPO, validating the specificity of our microscopy system and the treatment conditions. In contrast, *Nlrp3*$^{-/-}$ CFs displayed reduced mROS compared to WT controls, consistent with our previous findings and supporting its role in the regulation of mitochondrial function. These results further indicate that NLRP3 operates intrinsically at the mitochondria in cardiac fibroblasts to regulate the degree of mROS, which acts upstream of R-Smads to modulate TGFβ signalling.

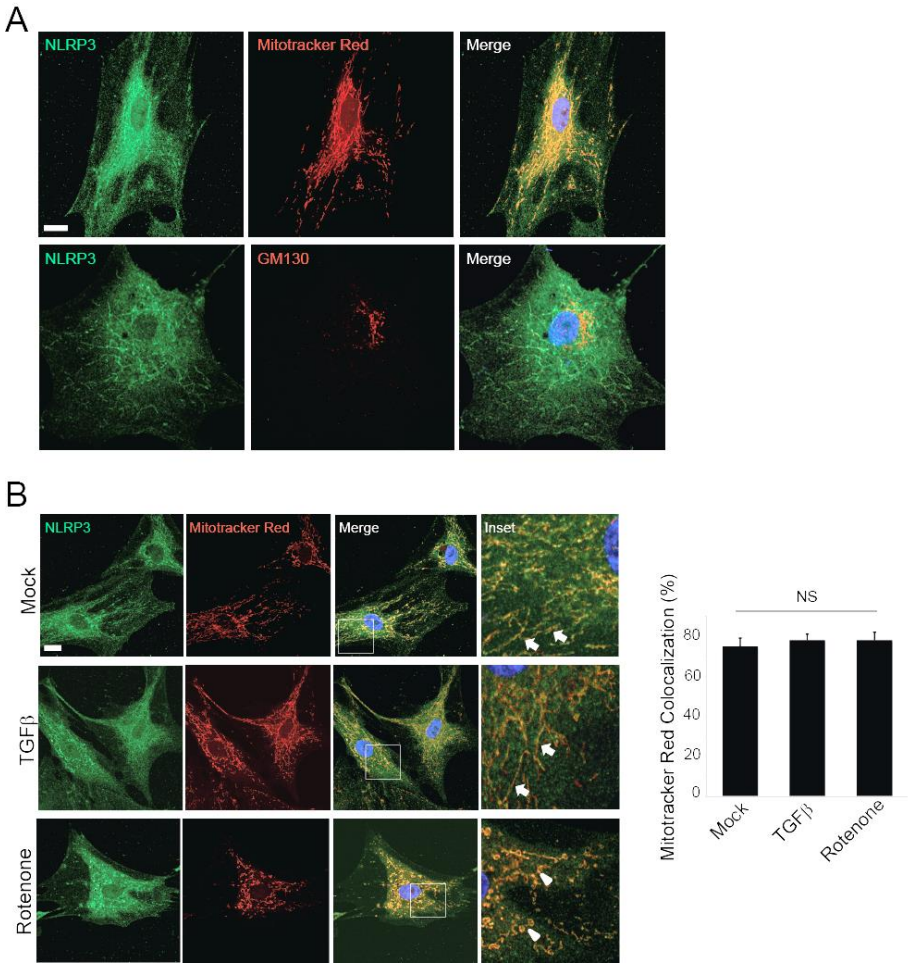

Figure 18. NLRP3 localization in human cardiac fibroblasts.

A. Confocal fluorescent immunocytochemistry for endogenous NLRP3 and Mitotracker Red (top) or the cis-Golgi marker GM130 (bottom) in unstimulated human CFs. **B.** Confocal fluorescent immunocytochemistry for endogenous NLRP3 and Mitotracker Red in human CFs stimulated with TGFβ for 24 hours or Rotenone (10 μM) for 6 hours. Solid arrows are directed at mitochondria with linear morphology, triangles are directed at mitochondria with fragmented morphology. **C.** Semiquantitative analysis of endogenous NLRP3/Mitotracker red colocalization in mock, TGFβ or Rotenone treated human CFs. Scales=20 μm.

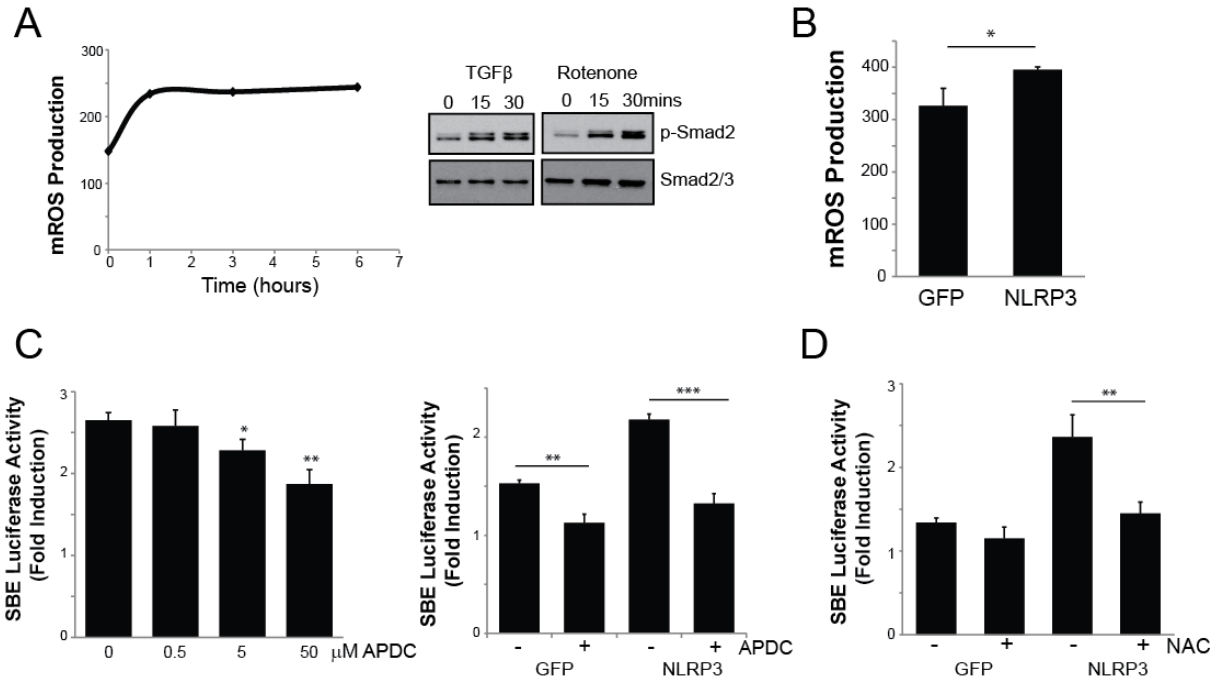

Figure 19. Role of ROS in NLRP3-mediated Smad signalling.

A. (Left) Flow cytometry on MitoSOX loaded (5 μM) 293T cells transfected with control GFP and the TGF type II receptor, and stimulated with TGFβ. Data are expressed as mean fluorescence intensity units. (Right) Immunoblot for phosphorylated Smad2 in human CFs treated with either TGFβ or Rotenone (10 μM). **B.** Flow cytometry mean fluorescence of MitoSOX loaded (5 μM) 293T cells transfected with either control GFP or NLRP3. **C.** Luciferase assay with SBE4-luciferase reporter in 293T cells transfected with NLRP3 treated with increasing concentrations of APDC (0-50 μM). Effects of APDC (25 μM) on SBE4-luciferase in transfected 293T cells. **D.** Luciferase assay comparing GFP vs NLRP3 transfected 293T cells treated with N-Acetyl Cysteine (NAC, 10 mM) n=3, *p<0.05, **p<0.01, ***p<0.001.

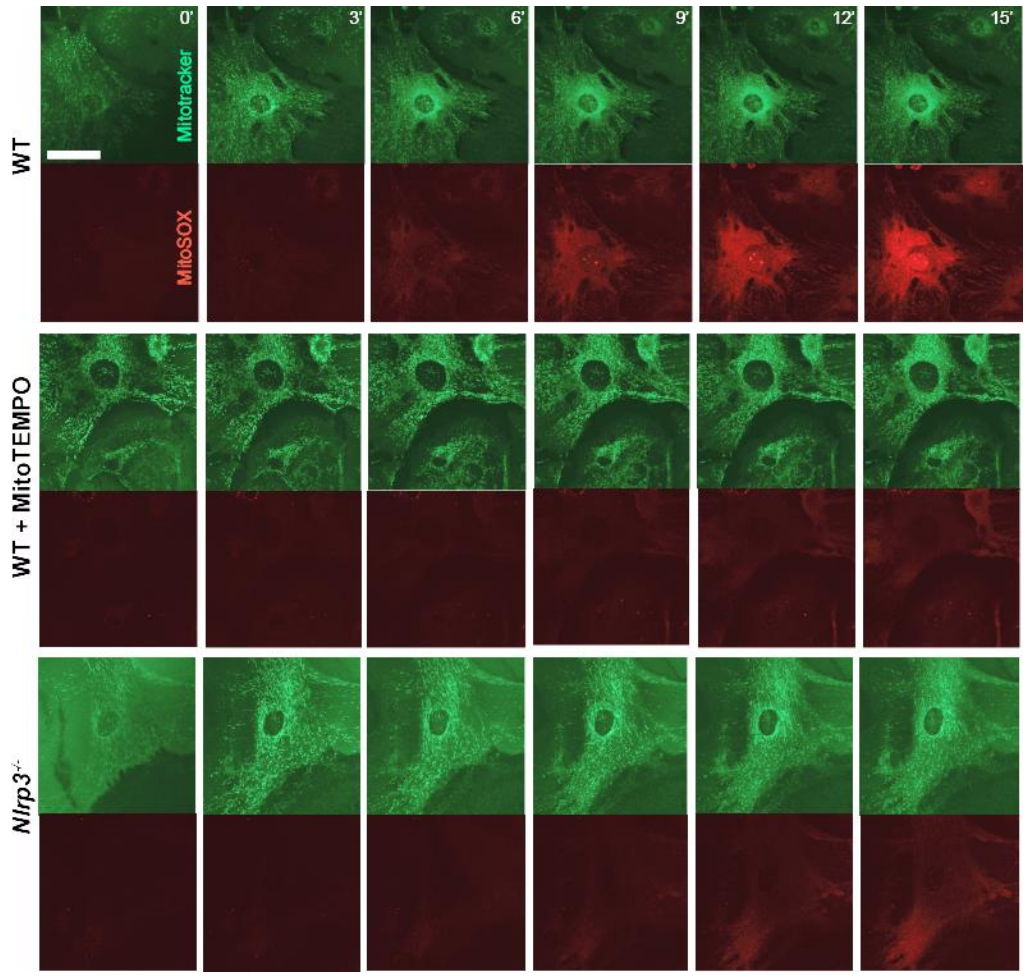

Figure 20. Live Cell Imaging of WT and *Nlrp3*$^{-/-}$ CF mROS

WT and *Nlrp3*$^{-/-}$ CFs were pre-treated with Mitotracker Green (400nM) and loaded with MitoSOX (5uM) for mROS visualization prior to confocal microscopy for the indicated time points. WT cells were pretreated for 8 hours in MitoTEMPO (400mM) prior to live cell imaging.

4.5 NLRP3 Regulates R-Smad Signalling, mROS Production and Myofibroblast Differentiation Independent from the "Inflammasome"

The previous experiments identify roles for NLRP3 in the regulation of mROS production, R-Smad signalling and differentiation of cardiac fibroblasts. To date, most established functions of NLRP3 have been presumed to relate to its regulation of inflammatory cytokine production via caspase-1. However the mechanistic data in support of these conclusions remains speculative, as endogenous molecular complexes have not been observed with endogenous proteins. Moreover, the relevance of NLRP3 with respect to cytokine processing within structural cells like cardiac fibroblasts has not yet been established. Our *in vivo* experiments with AngII indicated that NLRP3 might regulate homeostasis within the heart distinct from overt inflammatory processes. Therefore any role for NLRP3 cannot be assumed to intrinsically result from caspase-1, IL-1β or IL-18.

We initially assessed the ability of isolated cardiac fibroblasts in culture to secrete IL-1β following diverse stimuli. In LPS-primed primary peritoneal macrophages, ATP induces the robust release of IL-1β into supernatant (Figure 21A). This occurs in association with the intracellular cleavage of procaspase-1 detected by immunoblotting (Figure 21B). In contrast, stimulation of WT murine CFs with LPS and ATP did not result in significant IL-1β release. Moreover, stimulation of CFs with AngII or TGFβ occurred in the absence of IL-1β secretion or caspase-1 intracellular processing. These data suggest that alternative mechanisms may operate in macrophages compared to resident cardiac cells, and raise the possibility of "inflammasome-independent" pathways.

While unlikely, local cytokine secretion could theoretically still impact cardiac fibroblasts via low-level effects below the threshold of detection limits. To further rule out

inflammasome-derived cytokine products as mediators of cardiac myofibroblast transformation, fibroblasts were treated with IL-1β (1 ng/ml), TGFβ (10 ng/ml) or both cytokines (Figure 21C). As anticipated, both TGFβ and IL-1β activated NFκB signalling. However, only TGFβ robustly increased αSMA following 24 hours of stimulation. To directly address the relevance of the inflammasome in NLRP3 regulation of TGFβ signalling, we stimulated WT and *Nlrp3*$^{-/-}$ CFs with TGFβ alone or TGFβ plus IL-1β and IL-18 and measured Smad2 phosphorylation (Figure 21D). The addition of inflammasome-related cytokines to *Nlrp3*$^{-/-}$ cells still induced IκBα phosphorylation, but did not rescue the defective Smad2 response, further indicating that NLRP3 regulation of TGFβ signalling occurs independently from the inflammasome and its downstream elements.

Since CFs do not secrete IL-1β or activate caspase-1 in response to TGFβ or AngII, we next looked to explore the relevance of other inflammasome components[122]. We isolated ventricular CFs from WT, *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$, and *caspase-1*$^{-/-}$ mice and stimulated them with TGFβ for early time points to examine R-Smad activation profiles. Whereas *Nlrp3*$^{-/-}$ cells continued to show reduced Smad2 responsiveness to TGFβ, *caspase-1*$^{-/-}$ CFs responded in a similar manner to WT (Figure 22A). *ASC*$^{-/-}$ cells responded as an intermediary phenotype, consistent with previous descriptions in kidney epithelium[20]. To further assess these other proteins, we performed immunocytochemistry to examine Smad2/3 localization. Similar to our phosphorylation results, we also observed a comparable degree of Smad2/3 nuclear accumulation following 15 minutes of TGFβ in WT, ASC$^{-/-}$and *caspase-1*$^{-/-}$ CFs, whereas *Nlrp3*$^{-/-}$ cells again displayed a significantly impaired R-Smad response (Figure 22B). Since these observations indicate distinct NLRP3 function, we finally looked to determine whether its role in mROS production was reliant on the inflammasome. We isolated primary ventricular CFs from WT, *Nlrp3*$^{-/-}$and

caspase-1$^{-/-}$ mice, loaded them with MitoSOX and analyzed the signal by flow cytometry. Consistent with our previous live cell imaging experiments, NLRP3-deficient CFs showed substantially reduced levels of mROS compared to WT. Interestingly, *caspase-1*$^{-/-}$ CFs behaved similar to WT, with comparable profiles of MitoSOX staining (Figure 22C). Taken together, these results strongly support an inflammasome-independent mechanism. They show that NLRP3 participates in TGFβ-induced R-Smad activation, mROS production and myofibroblast differentiation independently from caspase-1 or inflammasome-dependent cytokines IL-1β or IL-18.

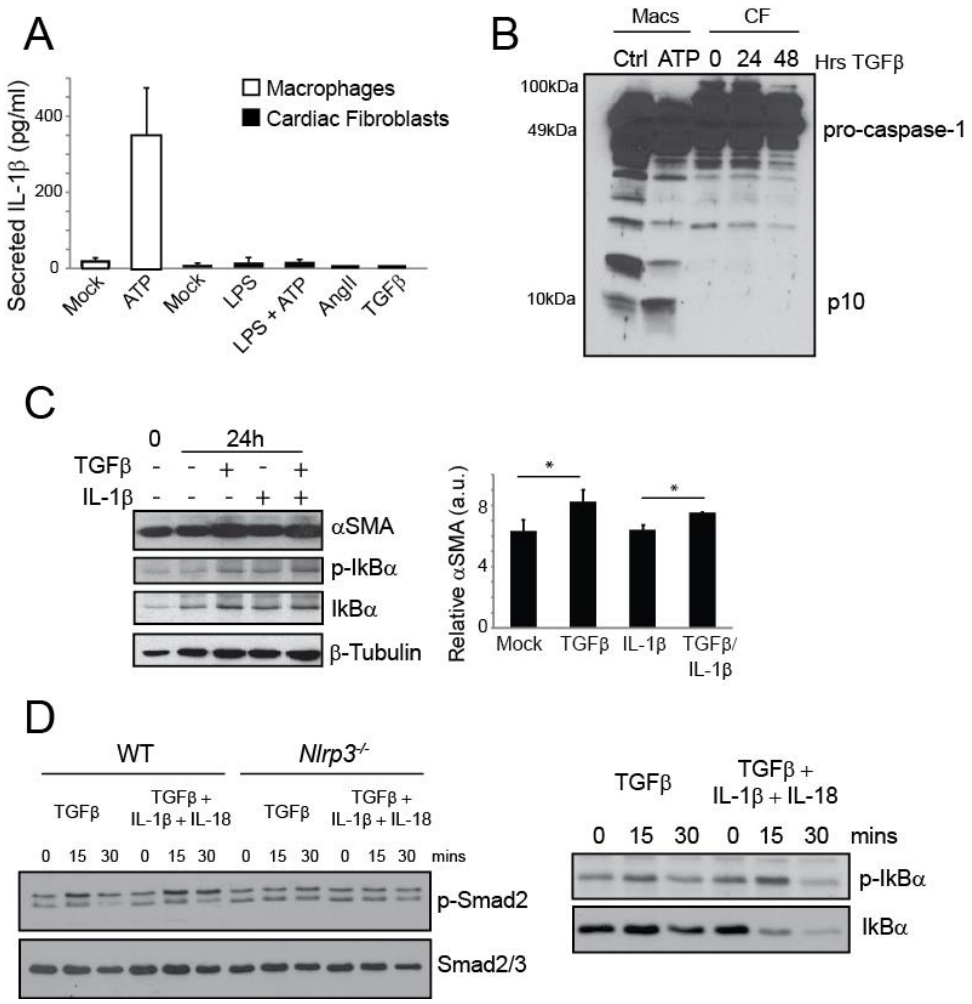

Figure 21. Inflammasome-independence of NLRP3 in TGFβ signalling.

A. IL-1β quantification in supernatant from WT CFs primed with LPS (10 ng/ml) followed by ATP (5 mM), or stimulated with AngII or TGFβ for 6 hours. **B.** Immunoblotting for caspase-1 cleavage in WT CFs stimulated for 20 mins or 24 hours with TGFβ or AngII. **C.** Immunoblotting for αSMA and p-IκBα in fibroblasts stimulated with TGFβ, IL-1β (1 ng/ml) or both (n=3, *p<0.05). **D.** Immunoblotting for p-IκBα in *Nlrp3*$^{-/-}$ CFs and p-Smad2 in WT and *Nlrp3*$^{-/-}$ CFs stimulated with TGFβ and IL-1β/IL-18 (1 ng/ml/10 ng/ml).

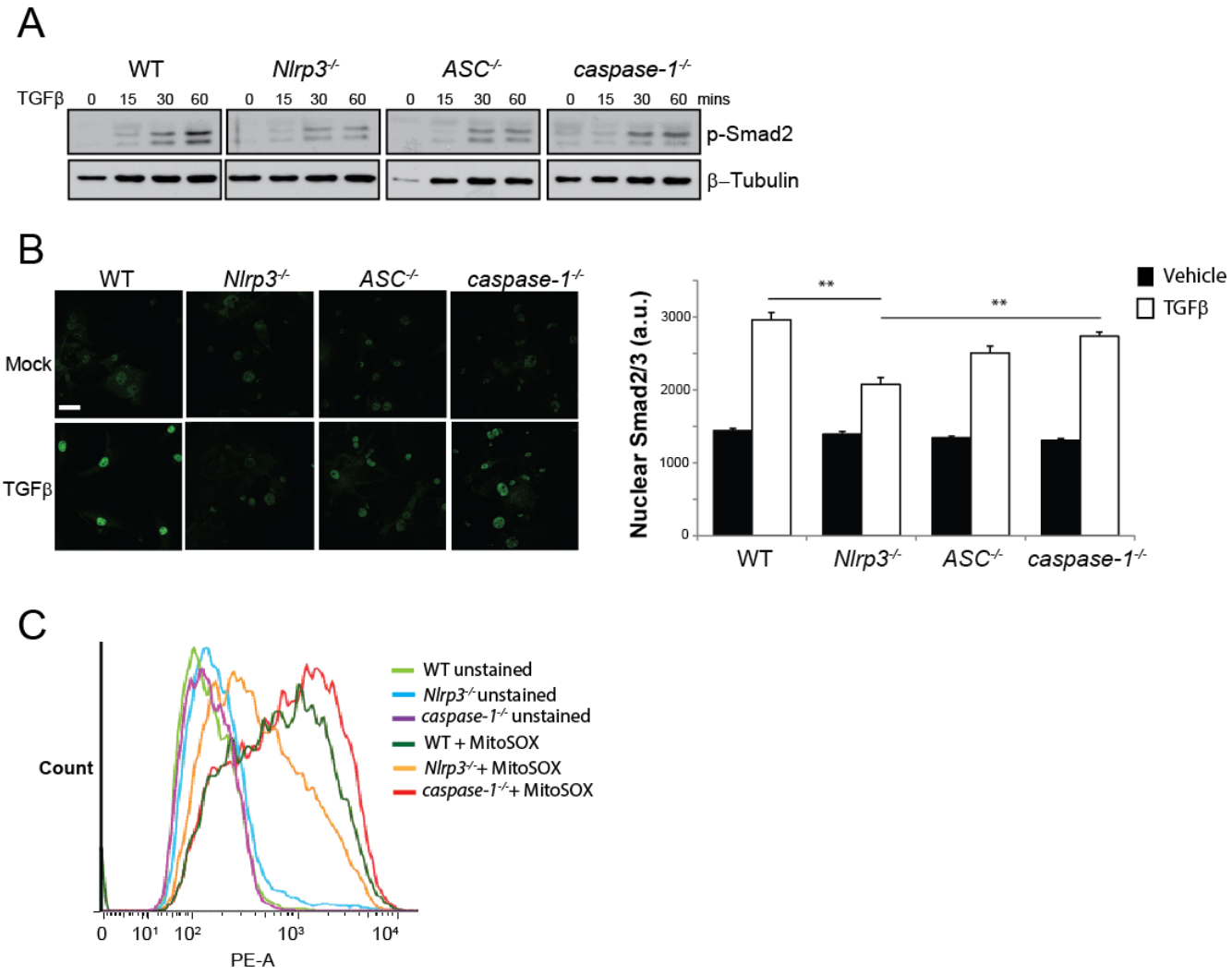

Figure 22. Role of caspase-1, ASC and IL-1r in TGFβ signalling.

A. Immunoblot for p-Smad2 in WT, *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$ and *IL-1R*$^{-/-}$ CFs stimulated with TGFβ. **B.** Confocal immunofluorescence and quantification of nuclear Smad2/3 in WT, *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$, and *caspase-1*$^{-/-}$ CFs stimulated with TGFβ for 15 minutes (** $p<0.01$, error bars indicate standard error). Scale=40 μm. **C.** Flow cytometry on control and MitoSox loaded WT, *Nlrp3*$^{-/-}$ and *caspase-1*$^{-/-}$ CFs.

4.6 Discussion

4.6.1 Inflammasome-Independent Mechanisms

The initial concept that inflammasome components NLRP3 and ASC could be capable of exerting distinct roles has been implicated in a number of different systems. Initial studies focusing on kidney injury models revealed that the abrogation of IL-1β and ASC did not completely protect animals against ischemia reperfusion to the same degree as NLRP3 deletion[176]. Our group has shown that in renal unilateral ureteric obstruction, deletion of ASC does not provide a protective phenotype, whereas NLRP3 deletion improves fibrosis and inflammation markers (unpublished). In the heart, similar results have been proposed with distinctly different conclusions by others. The deletion of NLRP3 in isolated mouse hearts resulted in protection against ischemia reperfusion injury, which occurred in the absence of IL-1β[188]. Interestingly, there was reduced IL-6 in *Nlrp3*$^{-/-}$ hearts, suggesting that NLRP3 could secondarily exert differential regulation of inflammatory processes. However the report that *ASC*$^{-/-}$ mice are also protected from cardiac ischemic injury is inconsistent with models in the kidney[186]. Additionally, this group reported protection in ASC-deficient mice via reduction in already low levels of IL-1β, raising the question of whether the "inflammasome" was truly present.

While these studies represent indirect *in vivo* evidence, others have already reported distinct roles for ASC independent from NLRP3. ASC was shown to regulate the expression of Dock2, with subsequently reduced actin-polymerization and impaired adaptive immunity in *ASC*$^{-/-}$mice[217]. However these results were not universally observed in other ASC knockout strains generated by different labs, suggesting that the effects could be a result of the gene targeting

method used to generate the animals[218]. Indeed, these results raise the difficulties in conducting studies on primary cells and *in vivo* work using only one strain of knockout mouse. We cannot comment on the potential for confounding genetic abrogation in our strain of *Nlrp3*$^{-/-}$animals, despite their extensive prior use in other laboratories[152]. However we note that our findings in the luciferase reporter assay are consistent, and we additionally observed that glibenclamide was able to recapitulate the NLRP3 knockout phenotype in primary human cells. These results are also internally consistent with our group's observations in kidney epithelium, again supporting that NLRP3 does exert distinct effects independent from cytokine products or caspase-1.

4.6.2 Fibroblasts and Cytokine Secretion

While the concept of inflammasome-independent mechanisms represents a point of controversy, our observation that fibroblasts do not make IL-1β should not be surprising.

A growing body of evidence has implicated that NLRP3 and ASC exert protective effects both in the bone marrow and solid organ system compartments. Some groups attribute beneficial effects of NLRP3 and ASC deletion to functional regulation of cytokine processing, as in fibroblasts or macrophages[186,189]. These conclusions raise a number of issues based on both scientific methodology and plausible rationale. IL-1β is synthesized as an inactive precursor protein within the cell, and is regulated post-translationally[118]. Therefore the transcriptional measurement of cytokine mRNA is invalid as a metric of so called "activation". Moreover, the currently available commercial ELISA kits are unable to distinguish between pro- IL-1β and the cleaved, active and secreted forms. Therefore any process that causes unregulated cell death will induce the release of pro- IL-1β, giving rise to false positive results.

Perhaps more important is the issue of plausible rationale. As mentioned earlier, higher orders of organisms have developed complex mechanisms of wound healing. For the processes of avoidance, resistance and tolerance to function, different cellular players must exert distinctly specialized roles based on thresholds of activation. Not all cells are capable of all processes, and when overwhelming injury proves too much for one cell type to handle, additional compartments are recruited. From a teleological standpoint then, it makes little sense for resident cells to be capable of secreting highly potent cytokines like IL-1β. Rather, fibroblasts are more likely to participate in tissue tolerance to injury, attempting to limit its burden on whole organ integrity and function. When an injury becomes too severe, recruitment of professional cytokine-secreting innate immune cells proceeds. Our results support this model, and identify NLRP3 as a critical mediator of both processes. Accordingly, the inflammasome may not necessarily be synonymous with NLRP3.

4.6.3 The NACHT Domain

While few others have proposed inflammasome-independent pathways, nobody has proposed experimentally validated molecular determinants that govern their effects. Our finding that the NLRP3-NACHT domain is required for augmentation of R-Smad signal transduction is consistent with its role in cytokine regulation in macrophages[150]. While these studies must be interpreted in the context of *in vitro* structural assays and may not necessarily reflect NLRP3 biology *in vivo*, consistency with previous reports continues to support a critical role for the NACHT domain and nucleotide hydrolysis in various signalling paradigms. The NACHT domain is a complex structure containing at least seven conserved distinct motifs[97]. Its conservation in the NLR family implies structural relevance. However we cannot discount the

possibility based on our own analyses that mutagenesis of the Walker A motif results in a misfolded protein or altered cellular distribution. Indeed, the generation of the Walker A mutant did not completely abolish NLRP3-induced R-Smad signalling, indicative of other important regulatory elements within the NACHT domain. These same patterns are observed in previous work by others for NLRP3 in macrophages[150]. Clearly the protein structure is more complex than is currently accepted. Moreover, NLRP9 and NLRP10 failed to increase R-Smad activation. The NACHT domains of these NLRs are very similar, though non-identical. Clearly other regions aside from the Walker A and Walker B motifs are important, though we could not identify specific patterns based on secondary amino-acid structure. While we examined the only plasmid constructs available to us at the time, the role of the other NLRs in R-Smad signalling remains to be determined.

4.6.4 NLRP3 Mitochondrial Localization and ROS Signalling

The subcellular localization of NLRP3 has recently been shown to target mitochondria on activation of innate immune cells, however all currently published reports propose that NLRP3 is purely cytosolic under unstimulated conditions[156]. These studies have employed overexpression systems with tagged NLRP3 constructs, and were expressed using unconventional methodologies. Our results in primary human CFs indicate that endogenous NLRP3 is constitutively associated with the mitochondria, with a smaller pool localizing to peri-mitochondrial cytoplasm. While the specific roles of these two pools must be further addressed, our data is somewhat consistent with recent reports suggesting the presence of a putative N-terminal mitochondrial localization signal, targeting NLRP3 to mitochondrial membranes[191]. Our results do not necessarily support a specific targeting sequence, and further experiments are

required to determine specifically where in the mitochondria endogenous NLRP3 is found. Since the expression of NLRP3 in CFs is significantly lower than innate immune cells, differential localization patterns could also be a result of altered levels of gene expression.

Our findings of reduced mROS production in NLRP3-deficient cells represent a potentially novel explanation for its function at the mitochondria. While the inflammasome has been suggested to "activate" in response to ROS production, and caspase-1/IL-1β cleavage both require ROS for NLRP3-dependent inflammatory signalling, nobody has explored the possibility that ROS could be downstream of NLRP3. The fact that many NLRP3 activators induce ROS towards cytokine secretion further supports that this mechanism may also operate in macrophages. There are many ways in which NLRP3 could exert such effects. While unlikely, it is possible that NLRP3 directly regulates electron transfer at the inner mitochondrial membrane to produce hydroxyl radical, or elsewhere to facilitate the conversion of peroxide to superoxide. However we believe it more likely that NLRP3 could interact with proteins involved in the secondary quenching and scavenging of ROS within the cell. The regulation of ROS and redox status of proteins is a growing topic that has come to be known as the maintenance of the cysteine proteome[219]. In principle, NLRP3 could act at the mitochondria as one member of this complex regulatory network involved in the fine-tuning of redox status via the thioredoxins, glutaredoxins or peroxiredoxins. While NLRP3 has already been proposed to interact with one such member, TXNIP, the functional relevance of this interaction has not been validated by other groups[216]. The principle stands, however, that NLRP3 could exert dual roles in both the production and response to ROS within diverse cell types in inflammasome-dependent and independent pathways.

Chapter Five: **Resolving NLRP3 in Professional and Non-Professional Immune Cells**

Overview

NLRP3 was initially characterized by its ability to regulate processing of IL-1β and caspase-1[149]. These processes were linked to paradigms first developed in apoptosis relating to homotypic protein interactions, and the molecular players determined through biochemical overexpression systems[103]. Since then, the field has erupted with the use of diverse knockout mouse strains employing various disease models that may or may not reflect true function of the proteins studied[220-222]. The verification for NLRP3 mechanisms, however, remains to be demonstrated using endogenous proteins in cell culture. Moreover, the mounting evidence to support distinct functions for NLRP3 independent from cytokines in non-professional immune cells raises the question of whether there are truly dual roles, or whether there is more to be learned regarding how the NLRs operate in general.

Given our previous work in cardiac fibroblasts, we looked to identify any observed consistencies for NLRP3 in diverse cell types. Our initial experiments led to the repeated finding that, in contrast to others, endogenous NLRP3 localized constitutively with mitochondria regardless of stimulation. While we maintain that NLRP3 is critical for caspase-1 and IL-1β processing and secretion in immune cells, we were unable to discern experimental evidence of its incorporation into a large quaternary complex. Given the robust mitochondrial localization profile and consistent results across diverse cell types, we further utilized *in silico* modeling software to predict an inner mitochondrial membrane profile for NLRP3, which was supported biochemically by fractionation experiments. We conclude with a model for NLRP3 function, localization and topology in both professional and non-professional immune cells, and offer insight into future directions to better understand how the NLRs regulate homeostasis.

5.1 NLRP3 Tightly Associates with Mitochondrial Membranes in Diverse Cell Types

NLRP3 and the other NLRs are colloquially referred to as cytosolic pattern recognition receptors[223]. While this terminology differentiates them from the plasma membrane-bound TLR family, it provides potentially misleading information on their structure and ultimately their function. In macrophages, NLRP3 was proposed to localize predominantly in the cytosol in unstimulated conditions[156]. In response to activating stimuli like ATP, MSU or ROS, NLRP3 reportedly translocates to the mitochondria, where it is said to facilitate inflammasome formation in binding to ASC and caspase-1, generating cleaved IL-1β. In some cases, elaborate models have been drawn to include microtubules "pulling" NLRP3 to mitochondria and the release of mitochondrial DNA activating NLRP3 through direct binding[224,225]. Despite the extensive literature, our results in cardiac fibroblasts showed distinctly mitochondrial localization in unstimulated cells. We co-localized NLRP3 with both Mitotracker Red and cytochrome C, supporting a mitochondrial pattern (Figure 23A). Additionally, we observed NLRP3 present at mitochondria in HeLa cells, reflecting potentially conserved localization amongst parenchymal fibroblast and epithelial cell types (Figure 23B). 293T cells do not express NLRP3, and displayed no signal with our endogenous NLRP3 staining protocol in comparison (Figure 23C). Since mitochondrial localization for NLRP3 in resting cells is inconsistent with recent reports in the literature, we looked to explore the localization of endogenous NLRP3 in professional immune cells using our own imaging protocols. Surprisingly, PMA-differentiated THP-1 cells showed similar results to fibroblast and epithelial cell types, with distinctly mitochondrial NLRP3 even under un-stimulated conditions (Figure 24A). Treatment of THP-1 cells with ATP resulted in the release of IL-1β, but did not induce meaningful translocation of NLRP3 from mitochondria.

Since we were unable to determine any movement of NLRP3 during stimuli that activate IL-1β processing, we next to looked to explore the downstream consequences of NLRP3 "activation" with respects to secretion. ATP induces the processing and release of both caspase-1 and IL-1β. While these components are both secreted during pyroptosis following DAMP stimulation of macrophages, the fate of the other "inflammasome" components has not been described[158]. If NLRP3 does in fact complex with ASC and caspase-1 to form a quaternary inflammasome structure, ASC and NLRP3 should also be secreted following stimulation of macrophages. To address this, we stimulated PMA-differentiated THP-1 cells with ATP in the presence and absence of glibenclamide to inhibit "inflammasome" formation and determine the specificity of our detection methods. ATP induced both the processing of intracellular IL-1β and its release into the supernatant, which were all inhibited by glibenclamide as previously reported (Figure 24B)[211]. We did not detect β-tubulin in secretions from ATP-treated THP-1 cells, suggesting that any protein present reflects an active secretory mechanism and not passive release of intracellular contents during cell death. While ASC was robustly present in supernatants of activated cells, NLRP3 was not detected, indicating that it is either not released or is present at levels below our limits of detection. This was surprising, because activation of IL-1β in response to ATP requires both proteins to be present[149,153].

To further determine the intracellular localization of all inflammasome components during activating stimuli, PMA-differentiated THP-1 cells were crudely separated into cytosolic (C) and heavy membrane (HM) fractions, enriched in β-tubulin and cytochrome c respectively (Figure 24C). Under baseline conditions, ASC, pro-caspase-1 and pro-IL-1β localized predominantly to the cytosol. NLRP3 appeared in both cytosolic and membrane fractions, again suggesting the presence of two pools within the cell. Stimulation with 5mM ATP for 1 hour

resulted in the intracellular maturation of caspase-1 and IL-1β, which were still found in the cytosolic fractions together with ASC. Consistent with our microscopy results, however, NLRP3 still maintained a similar localization profile upon ATP stimulation. We did not observe translocation of NLRP3 to heavy membrane compartments, in contrast to the reports of others. Accordingly, these data collectively support a different model, implicating intrinsic localization of NLRP3 to mitochondria and potentially other cytoplasmic vesicular membranes.

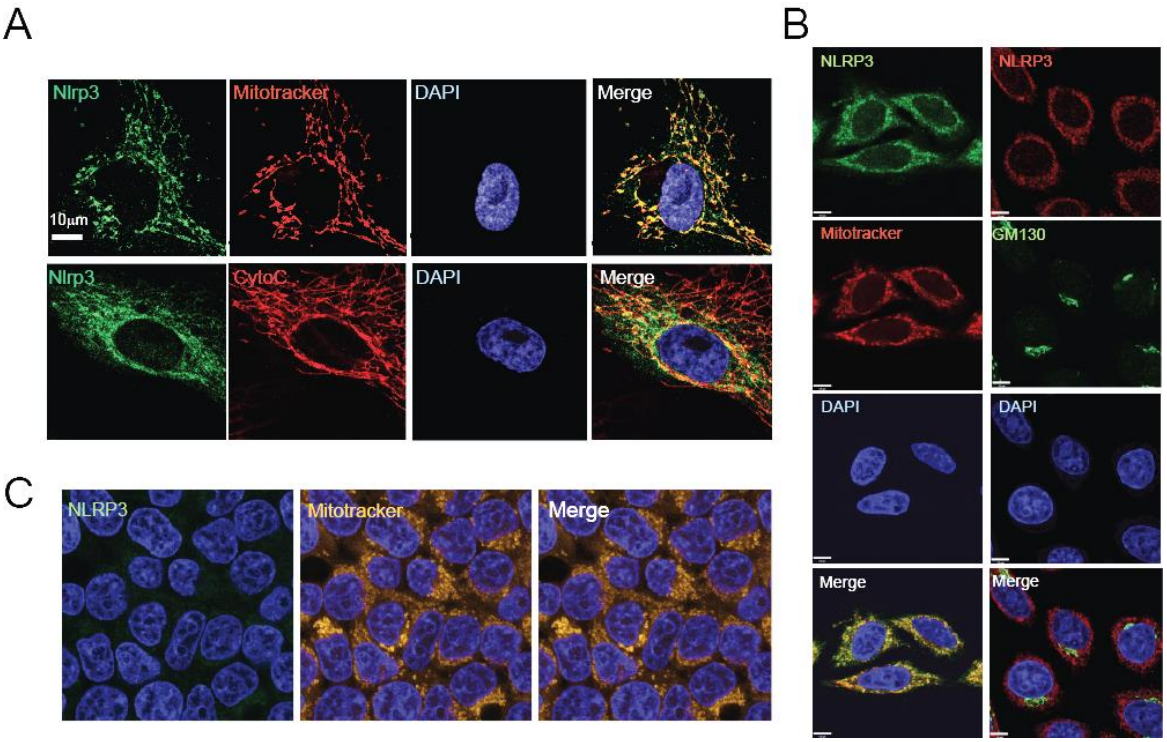

Figure 23. Mitochondrial localization of NLRP3.

A. Florescent confocal immunocytochemistry of endogenous NLRP3 and Mitotracker Red or cytochrome C in primary cardiac fibroblasts. **B.** Confocal imaging of endogenous NLRP3 and Mitotracker Red or Golgi (GM130) in HeLa cells. **C.** Confocal imaging of endogenous NLRP3 and Mitotracker Red in un-transfected 293T cells as a negative control. Scale is 10 μm.

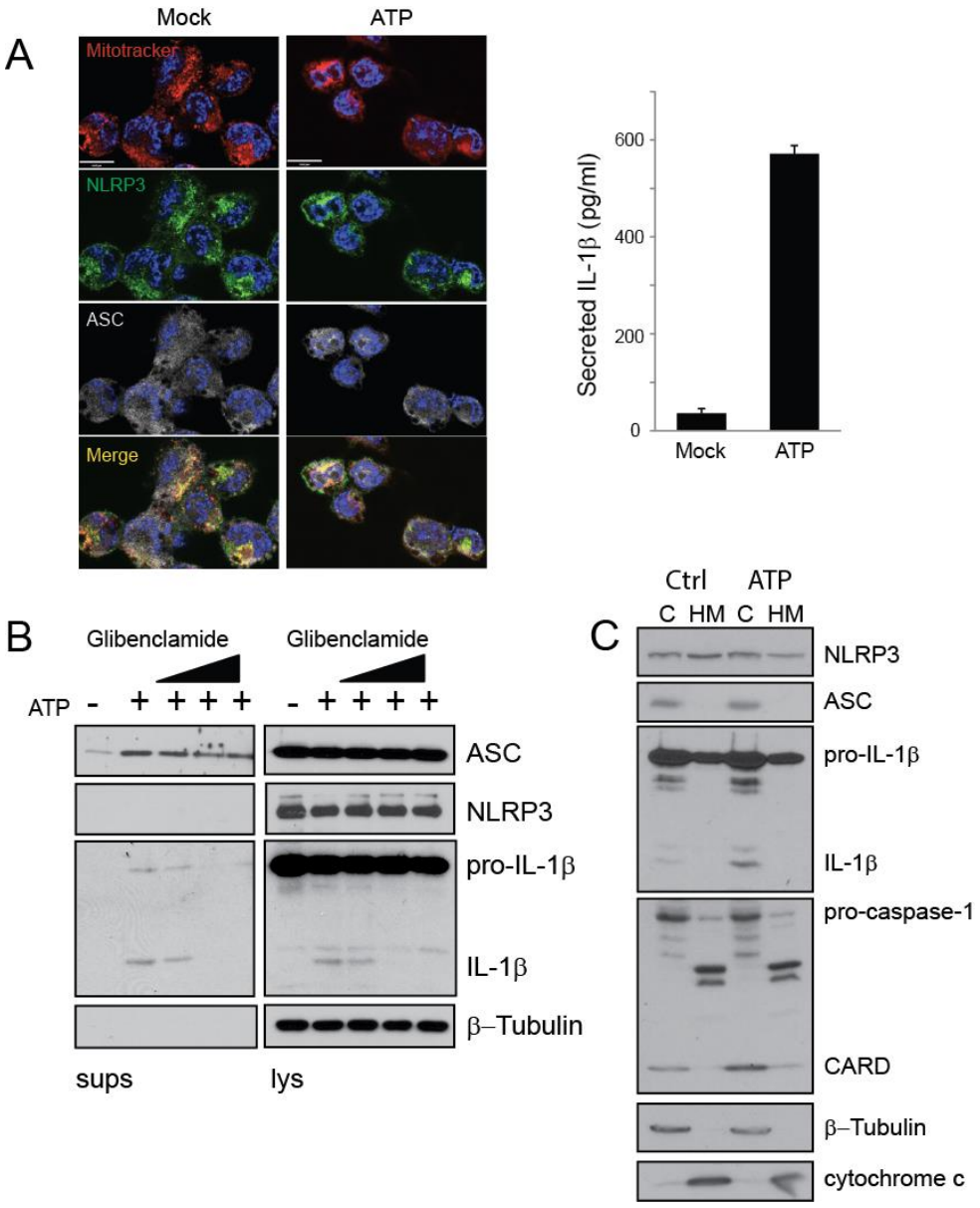

Figure 24. Mitochondrial Localization of NLRP3 in THP-1 cells.

A. Confocal imaging for endogenous NLRP3, ASC and Mitotracker Red in PMA-differentiated THP-1 cells stimulated with 5mM ATP for 60 minutes. Scale is 10 µm. ELISA measurement of IL-1β from THP-1 supernatants during ATP stimulation. **B.** Immunoblot for ASC, NLRP3 and IL-1β in PMA-differentiated THP-1 cells stimulated with 5mM ATP for 60 minutes. Cells were

pretreated in glibenclamide (0-200μM) for 15minutes. **C.** Immunoblot for NLRP3, caspase-1, ASC and IL-1β in THP-1 cells separated into cytosolic (C) and heavy membrane (HM) fractions.

5.2 Unifying Models of NLRP3 Function

The previous findings were largely inconsistent with the current paradigm for NLRP3 in regulating cytokine production, though internally consistent with our prior work in cardiac fibroblasts and renal tubular epithelial cells. Moreover, to our knowledge this represents the only attempts at imaging endogenous NLRP3 in the absence of overexpression systems with tagged constructs. Accordingly, our results may better reflect the biology of wild type NLRP3 under its natural promoter. To resolve these issues, we looked to better understand the structural protein biochemistry of NLRP3 using *in silico* analysis. Since others have reported the presence of an N-terminal mitochondrial targeting sequence that resulted in putative translocation and binding to mitochondrial MAVS, we looked to employ similar software[191]. While our analysis correctly predicted the localization of other proteins, such as Bcl-2 and MAVS to the outer mitochondrial membrane and ASC to the cytosol, the highest probability for NLRP3 was assigned to the inner mitochondrial membrane (Figure 25A).

In contrast to previous reports, we did not detect highly probabilistic predictions for a mitochondrial targeting sequence at the N-terminus. Instead, we identified putative transmembrane regions within the NLRP3 secondary structure. Of three potential membrane-spanning regions, one in particular was consistently identified. This highest probability score corresponded to a membrane spanning S1 segment ranging from amino acids 406 to 421 (Figure 25B). Analysis of the sequence alignments for all 14 NLRPs showed extensive conservation of NLRP3 L406, W417, C420 and L423 residues within this region, indicating that it may represent an important general role (Figure 26). Such domain architecture thus predicted NLRP3 as a type 1b transmembrane protein in the inner mitochondrial membrane, given the large degree of predicted hydrophobicity. Theoretically, this topology fits for a single pass through biological

membranes, as one 20-amino acid stretch of hydrophobic residues is consistently within the limits previously reported for integral membrane proteins[226]. Since the identified S1 segment localizes to a theoretical linker region between the NACHT and LRR domains, this would place the N-terminal PYD and NACHT domains in the intermembrane space, and the LRR in the mitochondrial matrix. Alternatively, if NLRP3 localized to the outer membrane, the PYD and CARD domains would face the cytosol and the LRR at the intermembrane space.

The *in silico* analysis of secondary protein structure is imperfect, as motifs are often missed and signal sequences can be mistaken for transmembrane helices[226]. To begin to experimentally validate our predictions and test whether NLRP3 was present in mitochondrial membranes, we performed initial biochemical experiments on isolated mitochondria. The disruption of mitochondrial membranes with increasing concentrations of digitonin has reportedly been used to preferentially dissociate outer membrane proteins at low concentrations, and inner membrane protein at higher amounts[227]. We isolated crude mitochondria from PMA-differentiated THP-1 cells and exposed them to increasing amounts of digitonin, then analyzed the mitochondrial pellet for remaining protein (Figure 25C). We found that at concentrations below 0.125%, digitonin resulted in the early dissociation of the outer mitochondrial protein Bcl-2, whereas higher concentrations were required for the disruption of inner mitochondrial protein cytochrome c oxidase IV (COXIV). Consistent with our imaging, NLRP3 was again present in mitochondria under baseline conditions, but required digitonin concentrations above 0.2% for its slow disruption from the membranes. These results again support an intrinsic role for NLRP3 at the mitochondria, and are consistent with our *in silico* analysis of a potentially inner membrane or matrix-associated protein with a transmembrane domain.

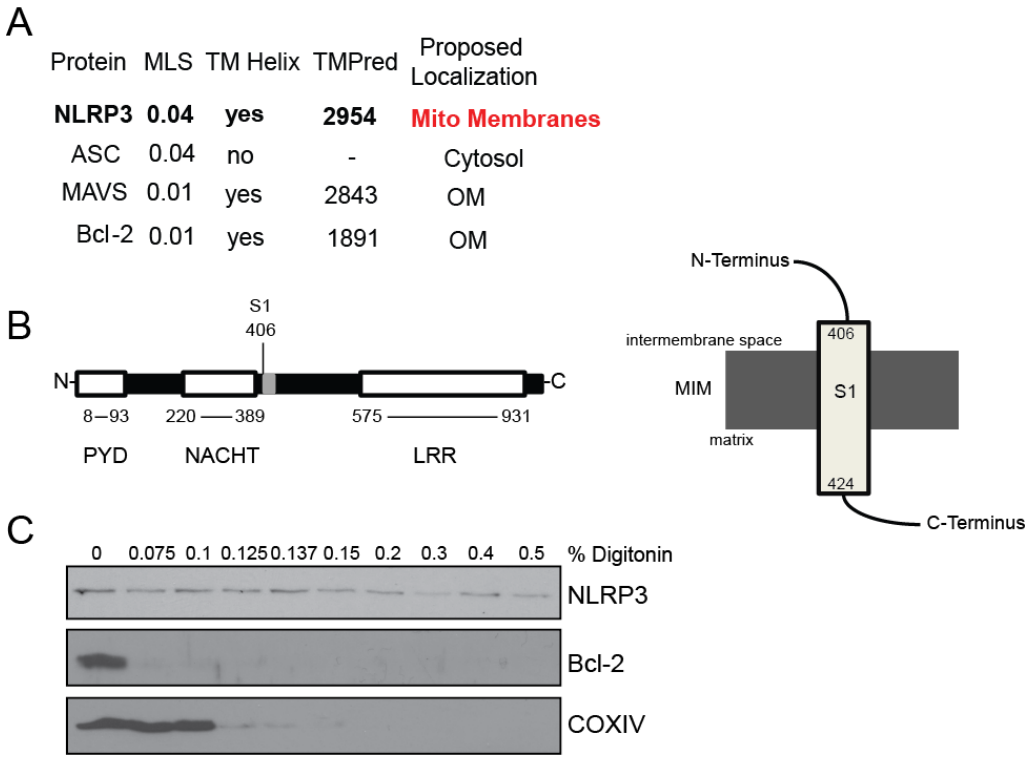

Figure 25. Mitochondrial membrane localization of NLRP3 in THP-1 cells.

A. *in silico* modeling for NLRP3 based on secondary protein structure. ASC, MAVS and Bcl-2, with previous experimentally determined localization profiles, were used as controls for validation. Algorithms were used for mitochondrial localization sequences (MitoProt II) and transmembrane helices (TMPred). MitoProt reports probability scores for mitochondrial targeting sequences ranging from 0-1. TMPred predicts mitochondrial localization, with scores greater than 1750 considered significant. **B.** Predicted membrane topology for S1 transmembrane region with highest probability returned from TMPred, and organization with respect to NLRP3 structure. **C.** Immunoblot for NLRP3, Bcl-2 and COXIV in digitonin-treated isolated mitochondrial pellets.

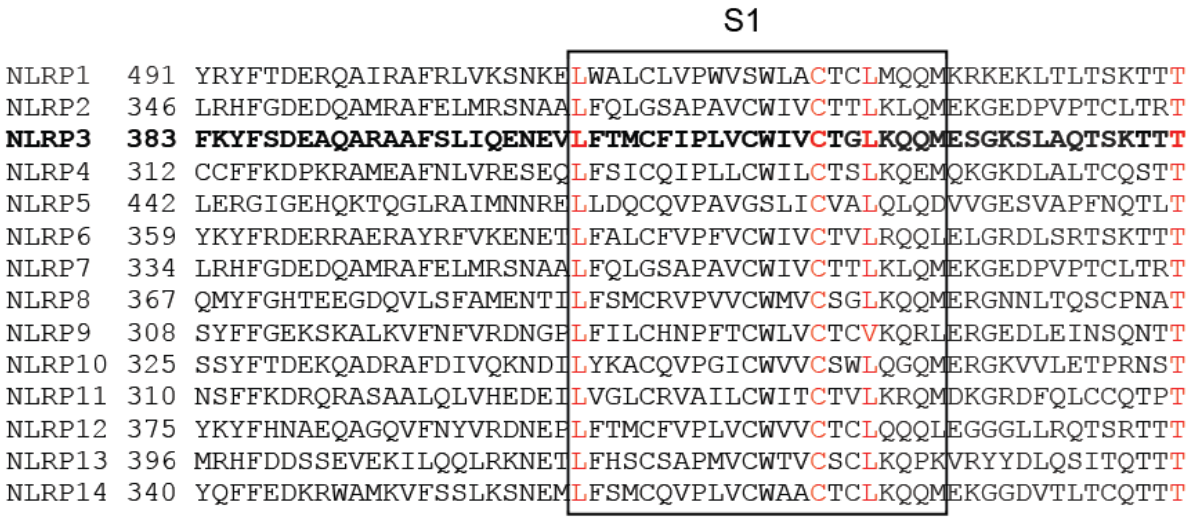

Figure 26. Conservation of hydrophobic residues within the putative S1 transmembrane region of NLRP3 in other NLRPs.

5.3 Discussion

5.3.1 The Need for a New Model

The biology of NLRP3 and the NLRs in general is becoming increasingly complex. Recently, experimental methodologies have turned from predominantly cell culture and biochemistry-based to animal models. Many are moving to develop therapeutic strategies aimed at "blocking" NLRP3[228]. However we still do not have a fundamental understanding of its function, localization or how it is regulated in different cell types, despite seemingly ubiquitous expression profiles. Therefore there is a distinct need to understand these principles so that ongoing work can explore potential for subsequent therapeutic utility.

Homotypic protein-protein interactions represent an appealing concept to biologists, because they imply that secondary structural analysis alone can give rise to meaningful identification of domains that operate and signal together. However this simplification has drawbacks. Given the terminology of the PYD and CARD as death fold domains, many groups have proposed NLR interaction with various proteins involved in inflammation, apoptosis and redox signalling, including Bcl-2, MAVS, TXNIP and caspase-8[216,229-231]. Moreover, a growing number of stimuli have now been reported to activate NLRP3-dependent cytokine processing to fit the initial paradigm first proposed. The result is a large list of activators, interacting proteins and responses that are not further explored following initial discovery. Accordingly, one cannot fit the various pieces of the inflammasome puzzle together to construct any coherent picture. Here we attempted to explore the fundamental aspects of NLRP3 cellular biology in the absence of *a priori* assumptions. Our results continue to support NLRP3 as a critical regulator of IL-1β and caspase-1 processing, but differ in the complete mechanistic overview. Despite these radical

differences, our models are based on experimental evidence that is internally consistent within our working group, rather than previously reported findings and speculation by others.

5.3.2 NLRP3 as a Membrane Protein

The prospect of NLRP3 as a membrane-bound protein is actually indirectly supported by the results of others. NLRP3 has previously been reported to be very difficult to express in bacterial systems, and ultimately was only accomplished in one report using insect cells[150]. Even then, little soluble protein was present in preparations, suggesting that these technical issues could be related to its underlying structure. In addition, membrane-bound proteins are notoriously difficult to crystalize. While the crystal structure of the PYD is known, the NACHT domain and full-length protein have not been successful[232]. Given the proximity of the NACHT domain to the putative S1 segment, these challenges could also be taken as indirect evidence in support of its membrane-bound localization.

While others report on an N-terminal mitochondrial localization signal, we were unable to validate these findings using similar open source modeling software packages. This does not necessarily refute a mitochondrial role. The mitochondria contain a distinct genome, though the vast majority of mitochondrial proteins are transcribed in the nucleus and translated in the cytoplasm[233]. Protein targeting to mitochondrial membranes then proceeds via two broad mechanisms. Many proteins contain localizing signals that target them for insertion directly through the membranes, with subsequent cleavage sites and transport recognition motifs determining the ultimate location. One pertinent example is the atypical NLR family members NLRX1, which contains an N-terminal signal sequence instead of a PYD or CARD domain[111]. NLRX1 is potentially targeted to the mitochondria through the TOM/TIM complex, where it is

likely cleaved into the matrix to associate with the inner membrane. In contrast, other proteins that lack N-terminal addressing sequences can still localize to mitochondria. For example, BLC-2 does not contain a signal, and instead stably inserts itself into biological membranes relatively non-specifically. We are currently unable to fully distinguish where NLRP3 precisely localizes in mitochondria, though it is evident that it stably incorporates in membranes. It is possible that, similar to Bcl-2, NLRP3 also non-specifically inserts in various biological membranes to perform different functions[234]. We still observe a small pool of NLRP3 in peri-mitochondrial cytoplasm on imaging. This could reflect membrane-bound NLRP3 in small cytoplasmic organelles, and requires further investigation.

Lastly, we also note that various residues within the S1 segment are highly conserved across all other NLRPs. While some appear to have diverged with the presence of charged resides (NLRP5), this region may represent an important feature of the NLRPs that has gone unnoticed. Alternatively, it may simply reflect similar backbone architecture in the general structure of this protein subfamily, without any functional relevance. Former classification of the NLRs placed them as cytosolic in comparison to the TLRs. It may be possible that the NLRs are homologous to the TLRs in that they are both potentially membrane-associated, but differ in their surveillance of extracellular versus intracellular environments. For this reason, TLRs would have adapted extracellular LRR domains capable of sensing DAMPs and PAMPs directly, while the NLRs maintained within the cytoplasm in membranous organelles to survey the intracellular milieu. Further investigation into all of the NLRs is necessary to broadly begin to categorize how these proteins act within diverse cell types to maintain cellular homeostasis.

Chapter Six: **General Discussion and Future Considerations**

6.1 Synopsis

The work presented in this thesis aimed to characterize the biology of NLRP3 in the heart during cardiac injury. Using animal models of chronic heart disease, we identified distinct roles for NLRP3 in IL-1β production through its regulation of caspase-1, and fibrosis through non-cytokine mediated effects. Our observations that tissue from human patients with heart disease showed highly expressing NLRP3 positive interstitial cells within fibrotic regions confirmed this, and further implicated an important role in fibrosis and myofibroblast differentiation. We went on to explore NLRP3 function in isolated cardiac fibroblasts, where it appeared to regulate TGFβ and AngII-induced myofibroblast differentiation through the R-Smad pathway. Using reporter systems and overexpression constructs we found that only the central NACHT domain was required for NLRP3-induced R-Smad activation, consistent with its importance in cytokine processing through caspase-1. However despite these similarities, the role of NLRP3 in myofibroblast activation was found to be independent of caspase-1, IL-1β or IL-18, supporting an inflammasome-independent mechanism. Since NLRP3 was localized to mitochondria in cardiac fibroblasts, we further explored the role of reactive oxygen species in R-Smad signalling. NLRP3 overexpression increased the production of ROS from mitochondria, and NLRP3-deficient cells displayed reduced ROS even under unstimulated conditions. These results pointed to an intrinsic role for NLRP3 at the mitochondria. To understand how NLRP3 localized within the cell and rationalize potential mechanisms differentiating inflammasome-independent and dependent pathways, we explored the similarities between professional and non-professional immune cells. In contrast to others, we showed consistent mitochondrial localization in both cell types regardless of stimulation using microscopy and fractionation methodologies. These studies were supported by preliminary biochemical work that showed tight-association with

mitochondrial membranes. We moved on to model NLRP3 based on secondary structure analysis. These results further supported a role for NLRP3 at mitochondria, and predicted a transmembrane site present within a putative linker region between the NACHT and LRR domains. Consistent with these analyses, stimulation of macrophages by ATP results in the secretion of all inflammasome components with the exception of NLRP3, which is likely retained within the cell as a membrane bound protein.

We realized that our progress in both cardiac fibroblasts/epithelial cells and monocytes/macrophages reached a challenging point at the same mechanistic question- how is NLRP3 regulating function from mitochondria? Contrary to a cytosolic quaternary complex, we suggest that NLRP3 instead acts as a membrane bound sensor of cellular stress to fine-tune diverse processes based on underlying regulation of mitochondrial and/or vesicular function (Figure 27). We offer two theoretical models for NLRP3 biology. These represent our attempts to rationalize seemingly divergent roles in professional and non-professional immune cells using our own internally consistent results from both paradigms. Importantly, these models rely on testable underlying hypotheses that must be further explored experimentally for validation.

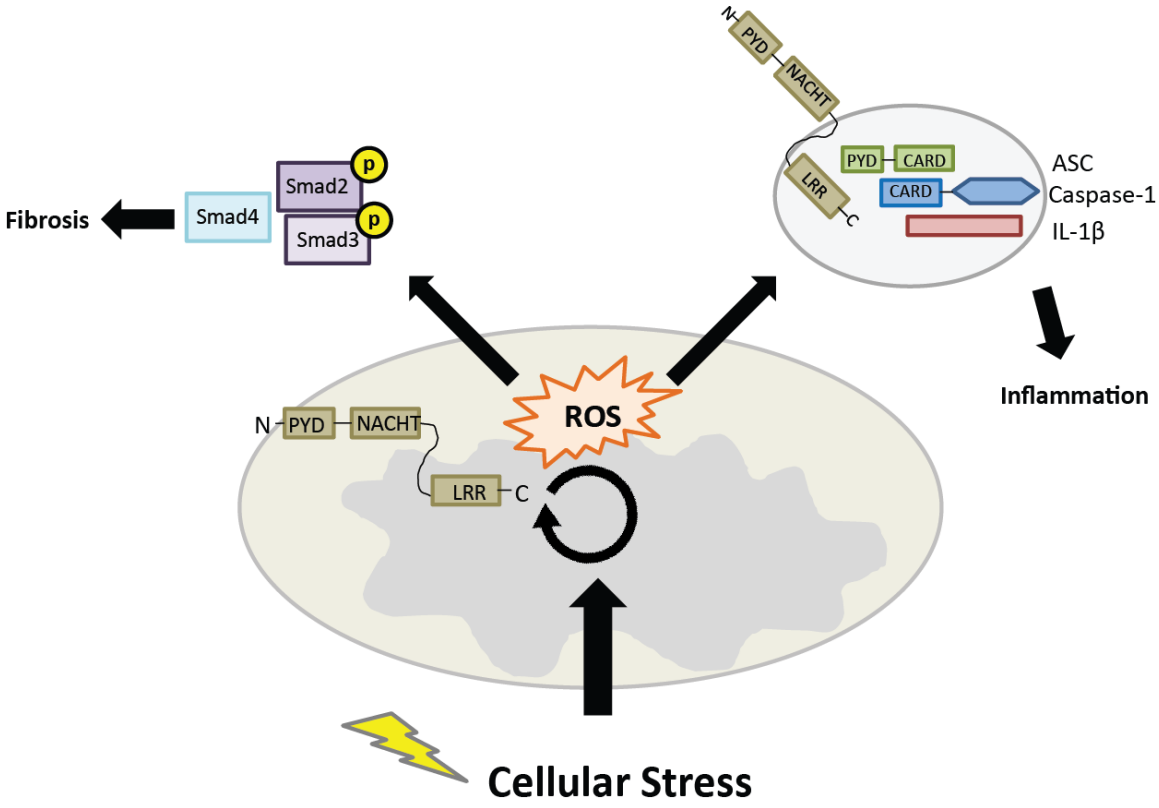

Figure 27. Unifying model of NLRP3 function in diverse cell types.

NLRP3 localizes to both mitochondrial and vesicular membranous structures within the cytoplasm. In mitochondria, NLRP3 both augments and responds to ROS in response to diverse injurious activating stimuli. While the NLRP3 response to ROS facilitates inflammasome formation and caspase-1-dependent cytokine processing, the induction of ROS additionally promotes fibrotic signal transduction in structural cells.

6.2 Testable Models for NLRP3 in Cellular Biology

6.2.1 NLRP3 Regulates Post-Translational Endomembrane Packaging of pro- IL-1β, ASC and pro-caspase-1

It is interesting that attention has turned to study NLRP3 in the biological regulation of the inflammasome, since so little is still known regarding IL-1β itself within innate immune cells. As mentioned, IL-1β is an atypical cytokine that lacks an N-terminal addressing sequence. How the pro-form is trafficked remains to be answered, despite observations that it accumulates in peri-membranous vesicles prior to a secondary stimulus causing its release[118]. Since it is well established that caspase-1 is also secreted, and we observed the co-release of ASC during ATP stimulation in THP-1 cells, it is possible that NLRP3 acts to package these components into vesicles prior to their joint release. Of note, nobody has attempted to differentiate between caspase-1/IL-1β activation versus secretion as separate entities. We hypothesize that it is possible to uniquely stimulate processing/activation while retaining caspase-1 and IL-1β within the cell, with NLRP3 regulating only their release. Additionally, NLRP3 has been proposed to reside at ER/Golgi/mitochondrial contact sites, and the mitochondria are diverse organelles that interact with various components of the endomembrane system[224,235]. We therefore suggest the possibility that NLRP3 could reside in these theoretical contact sites to regulate the packaging of pro-caspase-1, ASC and pro-IL-1β into vesicles that are secreted on secondary activation by danger signals.

This model is consistent with reports that various heterogeneous signals are capable of activating NLRP3-dependent IL-1β secretion, and link the trafficking of all proteins involved in the so-called inflammasome formation. However they do not explain the findings that in our

system, NLRP3 increased production of reactive oxygen species from mitochondria. Additionally, we cannot confirm the presence of ASC aggregates/specks at these mitochondrial sites using imaging so far, though they may occur in relatively low abundance[191]. Such an explanation is consistent with the severity of the NLRP3-mediated response, as IL-1β is a very potent cytokine capable of significant responses when released. In that way, very low levels of NLRP3 and ASC may accumulate below our detection limits. Thus we are currently unable to link all aspects of our findings into this model, and further exploration of the relevance of the NACHT domain and requirements for ATP hydrolysis must also be incorporated.

6.2.2 NLRP3 Regulates Vesicular Transport of pro-IL-1β, ASC and pro-Caspase-1

Perhaps more interesting is the potential for NLRP3 to act as a membrane bound regulator of diverse aspects in cellular behaviour. We have speculated on the ability for NLRP3 to non-specifically insert itself stably into biological membranes. In association with our findings for mROS potentiation, NLRP3 could act in diverse roles due to differential pools exerting different behaviour. We believe that NLRP3, in this way, could regulate both R-Smad signalling and myofibroblast differentiation in cardiac fibroblasts, and caspase-1 and IL-1β processing in innate immune cells depending on whether on not the substrates of the "inflammasome" are being actively translated. In addition, its role in vesicles could regulate the redox system indirectly, giving rise to the increased levels present on NLRP3 induction.

In an entirely speculative model, it is also possible that NLRP3 resides within membranes present on vesicles containing caspase-1, IL-1β and ASC and facilitates their recruitment and/or incorporation for downstream activation. Once activated, these vesicles would travel to the

membrane and fusion would result in secretion of ASC, IL-1β and caspase-1 with retention of the membrane bound NLRP3. In unicellular organisms, the incorporation of proteins without leader sequences occurs in part through a family of ATP-binding cassette transporters (ABC)[236]. This large family of proteins are involved in various cellular functions, and are conserved across diverse organisms back to prokaryotes. The general structure of ABC proteins contains an N-terminal nucleotide binding domain (with a Walker A motif) and a C-terminal transmembrane region[237]. In ATP-dependent mechanisms these proteins facilitate transport of many proteins across vesicles and endomembranous organelles including the plasma membrane. Given the predicted architecture for NLRP3, there is a modest similarity that could reflect underlying functions. While the ABC protein family typically contains multiple repeating units of transmembrane domains, they also oligomerize in a similar fashion to that predicted for NLRP3[238]. Although there is no experimental evidence reporting increasing amounts of NLRP3 present at the plasma membrane after stimulation of innate immune cells, attention has been focussed almost exclusively on mitochondria in overexpression studies, and such results may have been overlooked. Again it is possible that very low levels of NLRP3 are required for its function, and very high resolution with actively temporal imaging are required in order to truly exclude these hypothetical possibilities.

6.3 Future Methods of Experimentation for NLRP3

As previously alluded to, the field of NLRP3 and the inflammasome have erupted as of late towards extensive *in vivo* models of study. While *in vivo* work is paramount in establishing so called clinically relevant research, such little is still known regarding the cellular and

molecular biology of NLRP3 that any additional work is built on an incomplete story. We have established that overexpression systems pose problems when studying the NLRs, since their localization profiles are not consistent with endogenous protein. Given the previously described theoretical models for NLRP3, further analysis must proceed in the context of endogenous protein under its natural promoter.

Since both of our models rely on the membranous localization of NLRP3 and differ in its potential translocation, live cell imaging with fluorescently labelled NLRP3, ASC and caspase-1 would identify where these proteins are located under un-stimulated and stimulated conditions and whether they actually associate in response to activating stimuli. Furthermore, imaging NLRP3 during differentiation of structural cells like cardiac fibroblasts and renal tubular cells in comparison to innate immune cells would highlight potential areas of distinction between inflammasome-dependent and independent effects currently reported in the literature. The endogenous tagging of constructs *in situ* within the genome using novel genomic editing technologies would also enable the use of co-immunoprecipitation probing for NLRP3 interacting partners[239]. It is established that the NLRP3 inflammasome cannot simply be reconstituted by transfection with NLRP3, ASC and caspase-1 in reporter cell lines (unpublished reports). Clearly additional proteins are involved, and their identification will only come through large-scale "discovery" methods like mass spectrometry or yeast two-hybrids.

Lastly, the specific roles of the pattern recognition receptor superfamily and the innate immune system in general rely on conserved domains. These domains were described to fully form an immune system when rearranged, and clearly they impart functions dependent on such permutations and combinations[85,86]. There have been reports on some PRRs that support various

duplication events and genomic rearrangements forming functional receptors throughout evolution[240]. In this way, it may not necessarily be the PRRs that are of importance, but rather the actual domains themselves. In conventional biological and medical sciences, research paradigms dictate that to understand the functions of proteins, single genetic deletions are the gold standard (one protein=few functions). However given the likelihood for functional redundancy, especially within immunology, a novel approach may be to genetically delete whole sets of domains. For example, deletion of all PYD domains from the genome may produce a more significant *in vivo* phenotype during injurious stimuli. Such a "domain centric" view of biology seems more consistent with evolution, considering the shared architecture of many proteins that appear to have evolved separately from one another by convergence[241].

6.4 Therapeutic Implications for IL-1β and NLRP3 Antagonism

The therapeutic strategies aimed at managing patients with heart failure have not changed substantially in the last 20 years[242]. Since the understanding that many chronic diseases like heart failure and kidney disease carry a systemic inflammatory component, novel therapeutic interventions have been proposed on many fronts. These approaches should proceed with caution, however, as a complete understanding of their relevance must predate targeted strategies. For example, observations that serum from patients with longstanding heart disease contained elevated levels of TNFα led to clinical trials aimed at its neutralization with monoclonal antibodies. Paradoxically, some heart failure patients treated with anti-TNFα agents displayed increased mortality, indicating that we do yet have a complete understanding of inflammation in chronic disease[243].

Nevertheless, early clinical trials have been underway for IL-1β blockade in many areas, including post-myocardial infarction in ischemic heart disease[183,184]. These trials support both the safety and therapeutic efficacy for IL-1β antagonism in the setting of ST-elevation MI. The clinical end-points used for Anakinra treatment following myocardial infarction have been measurement of left ventricular end systolic volume index. This appears to be an independent risk factor for HF hospitalization and is a suitable measure of disease severity[244]. However the number of patients remained small, and further large scale double blinded placebo controlled trials are necessary. In addition, our data in the calcineurin model would elaborate the use of Anakinra to some patients with nonischemic heart disease. Regardless of etiology, it appears that IL-1β production in end-stage HF is a common finding that likely reflects extensive injury and on-going inflammatory responses. Since our observations that protective effects take place in the absence of changes to myocardial wall thickness or hypertrophy, patients may additionally benefit from a dual therapeutic approach using IL-1β neutralizing agents in addition to ACE-inhibitors and beta-blockers. These combination-based therapeutic strategies have not yet been addressed; though surely will in the next few years.

Given the protective benefits already shown for IL-1β, NLRP3 antagonism has already been proposed and attempted in animal models[228]. This raises a number of issues related to drug design and general pharmacological approaches. In principle, therapeutic strategies using drugs should be aimed at key players in pathological processes. For rational drug design, compounds should be targeted to well-described regions to facilitate high affinity binding with minimal side effects. Since NLRP3 has not been crystalized, and little is known regarding its true biology, it serves as a poor candidate. Moreover, currently adopted chemical strategies have utilized

glibenclamide as a model compound, attempting to modify its structure in hopes of better targeting. Glibenclamide is a sulfonylurea agent formerly used in managing type II diabetes that exerts various actions in diverse systems[245]. Its ability to antagonize NLRP3 function is more of a research tool than hopeful clinical strategy. Finally, NLRP3 is only one of many regulators of inflammatory cytokine production. The knockout phenotypes observed indicates an important- but not critical role in health and disease. Instead of NLRP3 directly regulating one process, it is more likely that it finely regulates a number of cellular functions, participating in complex maintenance of homeostasis. In drug design, one generally looks to target critical regulators. Thus, it is of this author's opinion that it is still too soon for NLRP3-guided therapeutic intervention, despite the various studies implicating potential distinct roles from IL-1β or caspase-1.

6.5 Hierarchical Immune Mechanisms

In summary, it is apparent that NLRP3 exerts wound-healing effects in both professional and non-professional immune cells during the course of various injurious stimuli. As such, these two pools of NLRP3-expressing cells exemplify how vertebrates utilize hierarchical pathways in the maintenance of homeostasis. As previously mentioned, three potential types of injury occur: pathogenic, acute non-microbial and chronic non-microbial[17]. While pathogenic infection and acute non-microbial injuries result in the early recruitment of innate immune cells and overt inflammation, chronic injury proceeds through more subtle long-term changes in tissue structure and function, with only mild inflammatory cytokine involvement and infiltrating leukocytes. Clearly the resident cells comprising tissue are also capable of their own wound healing and

defence pathways, as we have observed the differentiation of myofibroblasts and remodelling of extracellular matrix elements even in the absence of inflammatory cytokine involvement. However when injuries progress beyond some predetermined threshold, damage becomes overwhelming and requires the recruitment of additional highly specialized pools of immune cells. In this way, tissue homeostasis is analogous to home repair. In response to a theft or home invasion, highly specialized law enforcement personnel are immediately recruited for assistance. When the toilet breaks, a plumber is contacted for help as soon as possible. However the various day-to-day issues, like a leaky faucet, are typically managed by the homeowner directly. While these patch jobs are initially helpful, over time they accumulate and ultimately decompensate. In wound healing, the patch jobs that accompany chronic injury represent tolerance pathways, rendering the host less susceptible to ongoing damage via processes like fibrosis and remodelling.

In this thesis, we have found that NLRP3 jointly participates in resistance and tolerance pathways in leukocytes and fibroblasts, respectively. Since there are only 30-40,000 genes encoded in the human genome, it is not unexpected to have seemingly distinct roles for a single protein in different tissue types. Such a model is consistent with the need to respond to diverse signals and injurious stimuli through the hierarchical recruitment of different wound healing pathways. It makes sense, then, to have similar proteins function in different levels, with the ultimate difference resting in the presence or absence of downstream effector molecules like cytokines. Thus we propose that NLRP3, and perhaps other members of the NLR family, represent general proteins involved in the maintenance of homeostasis.

6.6 Concluding Remarks

The identification of diverse sets of germline-encoded pattern recognition receptors has uncovered complicated mechanisms by which organisms have evolved to regulate homeostasis. While the TLRs have been well characterized, the NLRs are now being recognized to regulate the intracellular environment in response to injurious stimuli, participating in chronic injuries and cellular stress. Technological advances in molecular biology, biochemistry and cell biology with imaging modalities will surely give rise to further information on how these proteins operate. Our results are only beginning to reveal novel roles for the NLRs in both professional and non-professional immune cells, suggesting that they have broad functions. These new findings will lead to a greater understanding of general signalling mechanisms for NLRs in all cell types, ultimately shedding new light on the complicated nature of the innate immune system.

References

1. Morris SC. The Cambrian 'explosion': Slow-fuse or megatonnage ? Proc Natl Acad Sci 97, 4426-4429 (2000).

2. Medzhitov R. Toll-Like Receptors and Innate Immunity. Nat Rev Immunol 1, 135-145 (2001).

3. Akira S, Takeda K. Toll-Like Receptor signaling. Nat Rev Immunol 4, 499-511 (2004).

4. Janeway CA, Medzhitov R. Innate Immune Recognition. Ann Rev Immunol 20, 197-216 (2002).

5. Nathan C, Ding A. Nonresolving Inflammation. Cell 140, 871-882 (2010).

6. Janeway CA. Approaching the Asymptote? Evolution and Revolution in Immunology. Cold Spring Harb Symp Quant Biol. 54, 1-13 (1989).

7. Matzinger P. Friendly and dangerous signals: is the tissue in control? Nat Immunol 8, 11-3 (2007).

8. Matzinger P. Tolerance, Danger and the Extended Family. Ann Rev Immunol 12, 991-1045 (1994).

9. Matzinger P. The Danger Model : A Renewed Sense of Self. Science 301, (2008).

10. Hogquist KA, Netr MA, Unanuet ER, Chaplin DD. Interleukin-1 is processed and released during apoptosis. Proc Natl Acad Sci USA 88, 8485-8489 (1991).

11. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 425, 516-521 (2003).

12. Timmers L, Pasterkamp G, de Hoog VC, Arslan F, Appelman Y, de Kleijn DP. The innate immune response in reperfused myocardium. Cardiovasc Res 94, 276-283 (2012).

13. Hedayat M, Mahmoudi MJ, Rose NR, Rezaei N. Proinflammatory cytokines in heart failure: double-edged swords. Heart Fail Rev 15, 543-562 (2010).

14. Chen W, Frangogiannis NG. The role of inflammatory and fibrogenic pathways in heart failure associated with aging. Heart Fail Rev 415-422 (2010).

15. Jessup M, Brozena S. Heart failure. N Engl J Med 348, 2007-2018 (2003).

16. Gajarsa JJ, Kloner RA. Left ventricular remodeling in the post-infarction heart: a review of cellular, molecular mechanisms, and therapeutic modalities Trandolapril Cardiac Evaluation trial. Heart Fail Rev 16, 13-21 (2011).

17. Medzhitov R, Schneider DS, Soares MP. Disease Tolerance as a Defense Strategy. Science 335, 936-941 (2012).

18. Kavaliers M, Choleris E, Anders A, Pfaff DW. Olfactory-mediated parasite recognition and avoidance: linking genes to behavior. Hormones and Behavior 46, 272 - 283 (2004).

19. Casadevall A, Pirofski L. Host-Pathogen Interactions: Redefining the Basic Concepts of Virulence and Pathogenicity. Infection Immun 67, 3703-3713 (1999).

20. Råberg L, Sim D, Read A. Disentangling Genetic Variation for Resistance and Tolerance to Infectious Diseases in Animals. Science 318, 812-814 (2007).

21. Ferreira A, Marguti I, Bechmann I, Jeney V, Chora A, Palha NR, Rebelo S, Henri A, Beuzard Y, Soares MP. Sickle Hemoglobin Confers Tolerance to Plasmodium Infection. Cell. Apr 29;145(3):398-409 (2011)

22. Schneider DS, Ayres JS. Two ways to survive infection: What resistance and tolerance can teach us about treating infectious diseases. Nat Rev Immunol 8, 889-895 (2008).

23. Råberg L, Graham AL, Read AF. Decomposing health : tolerance and resistance to parasites in animals. Philos Trans R Soc Lond B Biol Sci. 364, 37-49 (2009).

24. Foreman R. Mechanisms of Cardiac Pain. Ann Rev Physiol 61, 143-167 (1999).

25. Pinckard RN, Olson MS, Giclas PC, Terry R, Boyer JT, O'Rourke RA. Consumption of Classical Complement Components by Heart Subcellular Membranes In Vitro and in Patients after Acute Myocardial Infarction. J Clin Invest 56, 740-750 (1975).

26. Rossen RD, Michael LH, Hawkins HK, Youker K, Dreyer WJ, Baughn RE, Entman ML. Cardiolipin-Protein Complexes and Initiation of Complement Activation After Coronary Artery Occlusion. Circulation Research 75, 546-555 (1994).

27. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 204, 3037-47 (2007).

28. Bournazou I, Pound JD, Duffin R, Bournazos S, Melville LA, Brown SB, Rossi AG, Gregory CD. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. J Clin Invest 119, (2009).

29. Frangogiannis NG. Regulation of the Inflammatory Response in Cardiac Repair. Circ Res 110, 159-173 (2012).

30. Frangogiannis NG, Smith CW, Entman, ML. The inflammatory response in myocardial infarction. Cardiovasc Res 53, 31-47 (2002).

31. Beltrami CA, Finato N, Rocco M, Feruglio GA, Puricelli C, Cigola E, Quaini F, Sonnenblick EH, Olivetti G, Anversa P. Structural Basis of End-Stage Failure in Ischemic Cardiomyopathy in Humans. Circulation 89, 151-163 (1994).

32. St John Sutton MG, Sharpe N. Left Ventricular Remodeling After Myocardial Infarction: Pathophysiology and Therapy. Circulation 101, 2981-2988 (2000).

33. Cleutjens JPM, Blankesteijn WM, Daemen MJAP, Smits JFM. The infarcted myocardium : Simply dead tissue, or a lively target for therapeutic interventions. Cardiovasc Res 44, 232-241 (1999).

34. Weber KT. Cardiac Interstitium in Health and Disease : The Fibrillar Collagen Network. J Am Cardiol 13, (1989).

35. Kramann R, Dirocco DP, Humphreys BD. Understanding the origin , activation and regulation of matrix-producing myofibroblasts for treatment of fibrotic disease. J Pathol 231, 273-289 (2013).

36. Ikeuchi M, Tsutsui H, Shiomi T, Matsusaka H, Matsushima S, Wen J, Kubota T, Takeshita A. Inhibition of TGF signaling exacerbates early cardiac dysfunction but prevents late remodeling after infarction. Cardiovasc Res 64, 526 - 535 (2004).

37. Davis J, Burr AR, Davis GF, Birnbaumer L, Molkentin JD. A TRPC6-Dependent Pathway for Myofibroblast Transdifferentiation and Wound Healing In Vivo. Deve Cell 23, 705-715 (2012).

38. Assomull RG, Prasad SK, Lyne J, Smith G, Burman ED, Khan M, Sheppard MN, Poole-Wilson PA, Pennell DJ. Cardiovascular Magnetic Resonance, Fibrosis, and Prognosis in Dilated Cardiomyopathy. J Am Col Cardiol 48, 1977-1985 (2006).

39. Hunt SA, Abraham WT, Chin MH, Feldman AM, Francis GS, Ganiats TG, Jessup M, Konstam MA, Mancini DM, Michl K, Oates JA, Rahko PS, Silver MA,Stevenson LW, Yancy CW. 2009 focused update incorporated into the ACC/AHA 2005 Guidelines for the Diagnosis and Management of Heart Failure in Adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines: develope. Circulation 119, e391-479 (2009).

40. Ho K, Anderson K, Kannel W, Grossman W, Levy D. Survival After the Onset of Congestive Heart Failure in Framingham Heart Study Subjects. Circulation 88, 107-115 (1993).

41. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Stafford R, Thom T, Wasserthiel-Smoller S, Wong ND, Wylie-Rosett J; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart Disease and Stroke Satistics-2010 Update: A Report from the American Heart Association. Circulation 121, 410-528 (2010).

42. Curtis LH, Greiner MA, Hammill BG, Kramer JM, Whellan DJ, Schulman KA, Hernandez AF. Early and long-term outcomes of heart failure in elderly persons, 2001-2005. Arch Int Med 168, 2481-8 (2008).

43. Beyar R, Dong SJ, Smith ER, Belenkie I, Tyberg JV. Ventricular interaction and septal deformation : a model compared with experimental data. Am J Physiol 256, H2044-2056 (1993).

44. Redfield MM, Jacobsen SJ, Burnett JC Jr, Mahoney DW, Bailey KR, Rodeheffer RJ. Burden of systolic and diastolic ventricular dysfunction in the community: appreciating the scope of the heart failure epidemic. JAMA 289, 194-202 (2003).

45. Willenheimer R, van Veldhuisen DJ, Silke B, Erdmann E, Follath F, Krum H, Ponikowski P, Skene A, van de Ven L, Verkenne P, Lechat P; CIBIS III Investigators. Effect on survival and hospitalization of initiating treatment for chronic heart failure with bisoprolol followed by enalapril, as compared with the opposite sequence: results of the randomized Cardiac Insufficiency Bisoprolol Study (CIBIS) III. Circulation 112, 2426-2435 (2005).

46. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med 341, 709-17 (1999).

47. Pitt B, Segal R, Martinez FA, Meurers G, Cowley AJ, Thomas I, Deedwania PC, Ney DE, Snavely DB, Chang PI. Randomised trial of losartan versus captopril in patients over 65 with heart failure (Evaluation of Losartan in the Elderly Study, ELITE). Lancet 349, 747-52 (1997).

48. Aurigemma GP, Zile MR, Gaasch WH. Contractile Behavior of the Left Ventricle in Diastolic Heart Failure : With Emphasis on Regional Systolic Function. Circulation 113, 296-304 (2006).

49. Zile R, Carolina S, Simsic JM. Diastolic Heart Failure : Diagnosis and Treatment. Cong Heart Fail 3, 13-24 (2003).

50. Mandinov L, Eberli FR, Seiler C, Hess OM. Diastolic heart failure. Cardiovasc Res 45, 813-25 (2000).

51. Hein S, Arnon E, Kostin S, Schönburg M, Elsässer A, Polyakova V, Bauer EP, Klövekorn WP, Schaper J. Progression from Compensated Hypertrophy to Failure in the Pressure-Overloaded Human Heart. Circulation 107, 984-991 (2003).

52. Fedak PW, Verma S, Weisel RD, Li R. Cardiac remodeling and failure From molecules to man (Part II). Cardiovasc Pathol 14, 49 - 60 (2005).

53. Aplin AE, Howe AK, Juliano RL. Cell adhesion molecules, signal transduction and cell growth. Curr Opin Cell Biol 11, 737-744 (1999).

54. Gonzalez A, Lopez B, Diez J. Myocardial fibrosis in arterial hypertension. Eur Heart J 4, 18-22 (2002).

55. Weber KT, Jalil JE, Janicki JS, Pick R. Myocardial Collagen Remodeling in Pressure. Am J Hypertens 2, 931-940 (1989).

56. Snider P, Standley KN, Wang J, Azhar M, Doetschman T, Conway SJ. Origin of Cardiac Fibroblasts and the Role of Periostin. Circ Res 2009 Nov 6;105(10):934-47

57. Gabbiani G. The myofibroblast in wound healing and fibrocontractive disease. J Pathol 200, 500-503 (2003).

58. Dugina V, Fontao L, Chaponnier C, Vasiliev J, Gabbiani G. Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. J Cell Sci 114, 3285-3296 (2001).

59. Borne SWMVD, Diez J, Blankesteijn WM, Verjans J, Hofstra L. Myocardial remodeling after infarction : the role of myofibroblasts. Nat Rev Cardiol 7, 30-37 (2009).

60. Porter KE, Turner NA. Cardiac fibroblasts : At the heart of myocardial remodeling. Pharmacol Ther 123, 255-278 (2009).

61. Rohr S. Cardiac Fibroblasts in Cell Culture Systems : Myofibroblasts All Along ? J Cardiovasc Pharmacol 57, 389-399 (2011).

62. Lim H, Zhu YZ. Role of transforming growth factor-β in the progression of heart failure. Cell Mol Life Sci 63, 2584-2596 (2006).

63. Schultz-Cherry S, Murphy-Ullrich J. Thrombospondin Causes Activation of Latent Transforming Growth Factor- Secreted by Endothelial Cells by a Novel Mechanism. J Cell Biol 122, 923-932 (1993).

64. Koitabashi N, Danner T, Zaiman AL, Pinto YM, Rowell J, Mankowski J, Zhang D, Nakamura T, Takimoto E, Kass DA. Pivotal role of cardiomyocyte TGF-β signaling in the murine pathological response to sustained pressure overload. J Clin Invest 121, 2301-2312 (2011).

65. Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. Cardiovasc Res 63, 423-32 (2004).

66. Yang YC, Piek E, Zavadil J, Liang D, Xie D, Heyer J, Pavlidis P, Kucherlapati R, Roberts AB, Böttinger EP. Hierarchical model of gene regulation by transforming growth factor beta. Proc Natl Acad Sci USA 100, 10269-1074 (2003).

67. Massagué J. How Cells Read TGF-b Signals. Nature reviews. Molecular Cell Biology 1, 169-178 (2000).

68. Runyan CE, Poncelet AC, Schnaper HW. TGF-beta receptor-binding proteins: complex interactions. Cell Signal 18, 2077-88 (2006).

69. Gordon KJ, Blobe, GC Role of transforming growth factor-β superfamily signaling pathways in human disease. Biochim Biophys Acta 1782, 197 - 228 (2008).

70. Akhurst RJ, Hata A. Targeting the TGFβ signalling pathway in disease. Nat Rev Drug Disc 11, 790-811 (2012).

71. Flavell R, Sanjabi S, Wrzesinski S, Limón P. The polarization of immune cells in the tumour environment by TGFβ. Nat Rev Immunol 10, 554-567 (2010).

72. Effects of Enalapril on Mortality in Severe Congestive Heart Failure. N Engl J Med 316, 1429-1435 (1987).

73. Yang F, Chung A, Huang X, Lan H. Angiotensin II Induces Connective Tissue Groth Factor and Collagen I Expression vi TRansforming Growth Factor-b-Dependent and-Independent Smad Pathways: The Role of Smad3. Hypertension 54, 877-884 (2009).

74. Wang W, Huang XR, Canlas E, Oka K, Truong LD, Deng C, Bhowmick NA, Ju W, Bottinger EP, Lan HY. Essential Role of Smad3 in Angiotensin II – Induced Vascular Fibrosis. Circulation 98, 1032-1039 (2006).

75. Zhuo JL, Li XC. New insights and perspectives on intrarenal renin-angiotensin system: Focus on intracrine/intracellular angiotensin II. Peptides 32, 1551-65 (2011).

76. Yang F, Huang XR, Chung AC, Hou CC, Lai KN, Lan HY. Essential role for Smad3 in angiotensin II-induced tubular epithelial – mesenchymal transition. J Pathol 221, 390-401 (2010).

77. Carvajal G, Rodríguez-Vita J, Rodrigues-Díez R, Sánchez-López E, Rupérez M, Cartier C, Esteban V, Ortiz A, Egido J, Mezzano SA, Ruiz-Ortega M. Angiotensin II activates the Smad pathway during epithelial mesenchymal transdifferentiation. Kidney International 74, 585-595 (2008).

78. Rodríguez-Vita J, Sánchez-López E, Esteban V, Rupérez M, Egido J, Ruiz-Ortega M. Angiotensin II Activates the Smad Pathway in Vascular Smooth Muscle Cells by a Transforming Growth. Circulation 111, 2509-2517 (2005).

79. Leask A. Potential Therapeutic Targets for Cardiac Fibrosis. Circ Res 105, 1675-1680 (2010).

80. Schultz Jel J, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, Kimball TR, Doetschman T. TGF-β1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. J Clin Invest 109, 715-716 (2002).

81. Lee MS, Kim Y. Pattern-Recognition Receptor Signaling Initiated From Extracellular, Membrane, and Cytoplasmic Space. Molecules and Cells 23, 1-10 (2007).

82. Hoffmann J, Kafatos F, Janeway C, Ezekowitz R. Phylogenetic Perspectives in Innate Immunity. Science 284, 1313-1318 (1999).

83. Medzhitov R, Janeway C. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 9, 4-9 (1997).

84. Janeway CA. The Immune System Evolved to Discriminate Infectious Nonself from Noninfectious Self. Immunology Today 13, 11-16 (1992).

85. Palsson-McDermott EM, O'Neill LAJ. Pattern-Recognition Receptors in Human Disease Building an immune system from nine domains. Biochem Soc 35, 8-10 (2007).

86. Werts C, Girardin SE, Philpott DJ. TIR, CARD and PYRIN: three domains for an antimicrobial triad. Cell Death Diff 13, 798-815 (2006).

87. Martinon F, Tschopp J. NLRs join TLRs as innate sensors of pathogens. Trends Immunol 26, 447-454 (2005).

88. Vallejo JG. Role of Toll-like receptors in cardiovascular diseases. Clinical science (London, England: 1979) 121, 1-10 (2011).

89. Chao W. Toll-like receptor signaling : a critical modulator of cell survival and ischemic injury in the heart. Am J Physiol Heart Circ Physiol 296, 1-12 (2009).

90. Neill LAJO, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7, 353-364 (2007).

91. Xu Y, Tao X, Shen B, Horng T, Medzhitov R, Manley JL, Tong L. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature 408, 111-115 (2000).

92. Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA Jr. MyD88 Is an Adaptor Protein in the hToll/ IL-1 Receptor Family Signaling Pathways. Mol Cell 2, 253-258 (1998).

93. Ozinsky A, Underhill DM, Fontenot JD, Hajjar AM, Smith KD, Wilson CB, Schroeder L, Aderem A. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. Proc Natl Acad Sci USA 57, 13766-13771 (2000).

94. Kobe B, Kajava AV. The leucine-rich repeat as a protein recognition motif. Curr Opin Struc Biol 7-10

95. Kim HM, Oh SC, Lim KJ, Kasamatsu J, Heo JY, Park BS, Lee H, Yoo OJ, Kasahara M, Lee JO. Structural Diversity of the Hagfish Variable Lymphocyte Receptors. J Biol Chem 282, 6726-6732 (2006).

96. Ehrhardt RA, Ceitlin J, Gartland GL, Cooper MD. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. Nature 430, 174-180 (2004).

97. Tschopp, J., Martinon, F. & Burns, K. NALPs: a novel protein family involved in inflammation. Nat Rev Mol Cell Biol 4, 95-104 (2003).

98. Koonin E, Aravind L. The NACHT family – a new group of predicted NTPases implicated in apoptosis and MHC transcription activation. Trends Biomed Sci 25, 223-224 (2000).

99. Aravind L, Dixit VM, Koonin EV. The domains of death : evolution of the apoptosis machinery. Trends Biomed Sci 24, 47-53 (1999).

100. Walker JE, Saraste M, Runswick MJ, Gay, NJ. Distantly related sequences in the a and b subunits of ATP synthase, myosin and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO I, 945 - 951 (1982).

101. Lamkanfi M, Dixit VM. Inflammasomes: guardians of cytosolic sanctity. Immunol Rev 227, 95-105 (2009).

102. Leipe DD, Koonin EV, Aravind L. STAND , a Class of P-Loop NTPases Including Animal and Plant Regulators of Programmed Cell Death : Multiple , Complex Domain Architectures , Unusual Phyletic Patterns , and Evolution by Horizontal Gene Transfer. J Mol Biol 343, 1-28 (2004).

103. Martinon F, Hofmann K, Tschopp J. The pyrin domain : a possible member of the death domain-fold family implicated in apoptosis and inflammation. Curr Biol 10, 118-120

104. Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS. A Unified Model for Apical Caspase Activation. Mol Cell 11, 529-541 (2003).

105. Hofmann K, Bucher P, Tschopp J. The CARD domain: a new apoptotic signalling motif. Trends Biomed Sci 22, 155-156 (1997).

106. Fairbrother WJ, Gordon NC, Humke EW, O'Rourke KM, Starovasnik MA, Yin JP, Dixit VM. The PYRIN domain: A member of the death domain-fold superfamily. Protein Science 2, 1911-1918 (2001).

107. Hille R. Folding Pyrin into the Family. Structure 11, 1190-1191 (2003).

108. Kufer TA, Fritz H, Philpott DJ. NACHT-LRR proteins (NLRs) in bacterial infection and immunity. Trends Microbiol 13, 381-388 (2005).

109. Ting JP, Lovering RC, Alnemri ES, Bertin J, Boss JM, Davis BK, Flavell RA, Girardin SE, Godzik A, Harton JA, Hoffman HM, Hugot JP, Inohara N, Mackenzie A,Maltais LJ, Nunez G, Ogura Y, Otten LA, Philpott D, Reed JC, Reith W, Schreiber S, Steimle V, Ward PA. The NLR gene family: a standard nomenclature. Immunity 28, 285-7 (2008).

110. Proell M, Riedl SJ, Fritz H, Rojas AM, Schwarzenbacher R. The Nod-Like Receptor (NLR) Family : A Tale of Similarities and Differences. PLoS One 3, 1-11 (2008).

111. Arnoult D, Soares F, Tattoli I, Castanier C, Philpott DJ, Girardin SE. An N-terminal addressing sequence targets NLRX1 to the mitochondrial matrix. J Cell Sci 122, 3161-3168 (2009).

112. Moore CB, Bergstralh DT, Duncan JA, Lei Y, Morrison TE, Zimmermann AG, Accavitti-Loper MA, Madden VJ, Sun L, Ye Z, Lich JD, Heise MT, Chen Z, Ting JP. NLRX1 is a regulator of mitochondrial antiviral immunity. Nature 451, 573-579 (2008).

113. Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E, Bailly-Maitre B, Volkmann N, Hanein D, Rouiller I, Reed JC. Reconstituted NALP1 Inflammasome Reveals Two-Step Mechanism of Caspase-1 Activation. Mol Cell 25, 713-724 (2007).

114. Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller SI, Aderem A. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1b via Ipaf. Nat Immunol 7, 569-575 (2006).

115. Dinarello CA. Immunological and Inflammatory Functions of the Interleukin-1 Family. Ann Rev Immunol 27, 519-550 (2009).

116. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 87, 2095-2147 (1996).

117. Schindler R, Ghezzi P, Dinarello CA. IL-1 Induces IL-1 IV . IFN-y Suppresses IL-1 but Not Lipopolysaccharide-Induced Transcription of IL-ll. J Immunol 144, 2216-2222 (1990).

118. Andrei C, Dazzi C, Lotti L, Torrisi MR, Chimini G, Rubartelli A. The Secretory Route of the Leaderless Protein Interleukin 1b Involves Exocytosis of Endolysosome-related Vesicles. Mol Biol Cell 10, 1463-1475 (1999).

119. Perregaux D, Gabels A. Interleukin-lP Maturation and Release in Response to ATP and Nigericin. The Journal of Biological Chemistry 21, 15195-15203 (1994).

120. Webe, A, Wasiliew P, Kracht M. Interleukin-1 (IL-1) Pathway. Sci Signal 3, 1-6 (2010).

121. Eisenberg SP, Evans RJ, Arend WP, Verderber E, Brewer MT, Hannum CH, Thompson RC. Primary structure and functional expression form complementary DNA of a human interleukin-1 receptor antagonist. Nature 343, 341-346 (1990).

122. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 117, 3720-3732 (2011).

123. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS,Hanauer SB, Nuñez G, Cho JH. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411, 603-606 (2001).

124. Dodé C, Le Dû N, Cuisset L, Letourneur F, Berthelot JM, Vaudour G, Meyrier A, Watts RA, Scott DG, Nicholls A, Granel B, Frances C, Garcier F, Edery P,Boulinguez S, Domergues JP, Delpech M, Grateau G. New Mutations of CIAS1 That Are Responsible for Muckle-Wells Syndrome and Familial Cold Urticaria : A Novel Mutation Underlies Both Syndromes. Am J Human Genet 70, 1498-1506 (2002).

125. Hoffman HM., Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle–Wells syndrome. Nat Genet 29, 301-305 (2001).

126. Dinarello CA, Meer JWMVD. Treating inflammation by blocking interleukin-1 in humans. Seminar Immunol 1-16 (2013).

127. Tian X, Pascal G, Monget P. Evolution and functional divergence of NLRP genes in mammalian reproductive systems. BMC Evol Biol 13, 1-13 (2009).

128. Chu ZL, Pio F, Xie Z, Welsh K, Krajewska M, Krajewski S, Godzik A, Reed JC. A Novel Enhancer of the Apaf1 Apoptosome Involved in Cytochrome c-dependent Caspase Activation and Apoptosis. J Biol Chem 276, 9239 -9245 (2001).

129. Hlaing T, Guo RF, Dilley KA, Loussia JM, Morrish TA, Shi MM, Vincenz C, Ward PA. Molecular Cloning and Characterization of DEFCAP-L and -S , Two Isoforms of a Novel Member of the Mammalian Ced-4 Family of Apoptosis Proteins. J Biol Chem

130. Lahm A, Paradisi A, Green DR, Melino G. Death fold domain interaction in apoptosis. Cell Death Diff 10, 10-12 (2003).

131. Salvesen GS, Dixit VM. Caspases : Intracellular Signaling by Proteolysis. Cell 91, 443-446 (1997).

132. Martinon F, Tschopp J. Inflammatory Caspases : Linking an Intracellular Innate Immune System to Autoinflammatory Diseases. Cell 117, 561-574 (2004).

133. Wajant H. The Fas Signaling Pathway : More Than a Paradigm. Science 1635, 1635-1636 (2002).

134. Boldin MP, Goncharov TM, Goltsev YV. Wallach D. Involvement of MACH , a Novel MORT1 / FADD-Interacting Protease , in Fas/APO-1 and TNF Receptor – Induced Cell Death. Cell 85, 803-815 (1996).

135. Micheau O, Tschopp J. Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes. Cell 114, 181-190 (2003).

136. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. FLICE , A Novel FADD-Homologous ICE/CED-3-like Protease, Is Recruited to the CD95 (Fas/APO-1) Death-Inducing Signaling Complex. Cell 85, 817-827 (1996).

137. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO 14, 5579-5588 (1995).

138. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-Dependent Formation of Apaf-1 / Caspase-9 Complex Initiates an Apoptotic Protease Cascade. Cell 91, 479-489 (1997).

139. Pan G, O'Rourke K, Dixit VM. Caspase-9 , Bcl-X L , and Apaf-1 Form a Ternary Complex. J Biol Chem 273, 5841-5845 (1998).

140. Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes to Cells 3, 697-707 (1998).

141. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 Interacting Protein , Mediates Cytochrome c Release from Mitochondria in Response to Activation of Cell Surface Death Receptors. Cell 94, 481-490 (1998).

142. Cerretti DP, Kozlosky CJ, Mosley B, Nelson N, Van Ness K, Greenstreet TA, March CJ, Kronheim SR, Druck T, Cannizzaro LA. Molecular Cloning of the Interleukin- 1 IB Converting Enzyme. Science 256, 97-100 (1992).

143. Kuida K Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA Altered Cytokine Export and Apoptosis in Mice Deficient in Interleukin-1 , Converting Enzyme. Science 267, (1995).

144. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J et al. A novel heterodimeric cysteine protease is required for interleukin-1b processing in monocytes. Nature 356, 768-774 (1992).

145. Masumoto J, Taniguchi S, Ayukawa K, Sarvotham H, Kishino T, Niikawa N, Hidaka E, Katsuyama T, Higuchi T, Sagara J. ASC, a Novel 22-kDa Protein , Aggregates during Apoptosis of. J Biol Chem 274, 33835-33838 (1999).

146. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10, 417-26 (2002).

147. Kovarova M, Hesker PR, Jania L, Nguyen M, Snouwaert JN, Xiang Z, Lommatzsch SE, Huang MT, Ting JP, Koller BH. NLRP1 dependent pyroptosis leads to acute ung injury and morbidity in mice. J Immunol 189, 2006-2016 (2012).

148. Albrecht M, Domingues FS, Schreiber S, Lengauer T. Structural localization of disease-associated sequence variations in the NACHT and LRR domains of PYPAF1 and NOD2. FEBS Letters 554, 520-528 (2003).

149. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 Forms an IL-1 beta -Processing Inflammasome with Increased Activity in Muckle-Wells Autoinflammatory Disorder. Immunity 20, 319-325 (2004).

150. Duncan JA, Bergstralh DT, Wang Y, Willingham SB, Ye Z, Zimmermann AG, Ting JP. Cryopyrin/NALP3 binds ATP/dATP, is and ATPase, and requires ATP binding to mediate inflammatory signaling. Proc Natl Acad Sci USA 104, 8041-8046 (2007).

151. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol 9, 847-856 (2008).

152. Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature 440, 237-41 (2006).

153. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, Lee WP, Weinrauch Y, Monack DM, Dixit VM. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440, 228-32 (2006).

154. Luheshi NM, Giles J, Lopez-Castejon G, Brough D. Sphingosine regulates the NLRP3-inflammasome and IL-1β release from macrophages. Eur J Immunol 42, 1-10 (2011).

155. Muruve DA, Pétrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ, Tschopp J. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. Nature 452, 103-7 (2008).

156. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221-226 (2010).

157. Takeuchi O, Kawai T, Mühlradt PF, Morr M, Radolf JD, Zychlinsky A, Takeda K, Akira S. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 13, 933-940 (2001).

158. Fernandes-Alnemri T, Wu J, Yu JW, Datta P, Miller B, Jankowski W, Rosenberg S, Zhang J, Alnemri ES. The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Diff 14, 1590-1604 (2007).

159. Stutz A, Golenbock DT, Latz E. Inflammasomes: too big to miss. J Clin Invest 119, 3502-3511 (2009).

160. Walev I, Reske K, Palmer M, Valeva A, Bhakdi S. Potassium-inhibited processing of IL-1b in human monocytes. EMBO 14, 1607-1614 (1995).

161. Colomar A, Marty V, Médina C, Combe C, Parnet P, Amédée T. Maturation and Release of Interleukin-1β by Lipopolysaccharide-primed Mouse Schwann Cells Require the Stimulation of P2X7 Receptors. J Biol Chem 278, 30732-30740 (2003).

162. Petrilli V, Dostert C, Mayor A, Martinon,F, Tschopp J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. Cell Death Diff 14, 1583-1589 (2007).

163. Vu C, Bortner C, Cidlowski J. Differential Involvement of Initiator Caspases in Apoptotic Volume Decrease and Potassium Efflux during Fas- and UV-induced Cell Death. J Biol Chem 276, 37602-37611 (2001).

164. Koo IC, Wang C, Raghavan S, Morisaki JH, Cox JS, Brown EJ. ESX-1-dependent cytolysis in lysosome secretion and inflammasome activation during mycobacterial infection. Cell Microbiol 10, 1866-1878 (2008).

165. Duncan JA, Gao X, Huang MT, O'Connor BP, Thomas CE, Willingham SB, Bergstralh DT, Jarvis GA, Sparling PF, Ting JP. Neisseria gonorrhoeae Activates the Proteinase Cathepsin B to Mediate the Signaling Activities of the NLRP3 and ASC-containing inflammasome. J Immunol 12, 6460-6469 (2009).

166. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca2+ and cAMP. Nature 492, 123-127 (2012).

167. Dostert C, Pétrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through the Nalp3 inflammasome sensing of asbestos and silica. Science 674, 674-677 (2013).

168. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, Carter AB, Rothman PB, Flavell RA, Sutterwala FS. The Nalp3 inflammasome is essential for the development of silicosis. Proc Natl Acad Sci 105, 9035-9040 (2008).

169. Nathan C. Points of control in inflammation. Nature 420, 846-52 (2002).

170. Hofmann U, Frantz S. How can we cure a heart “‘ in flame ””? A translational view on inflammation in heart failure. Basic Res Cardiol 108, 1-19 (2013).

171. Bujak M, Frangogiannis NG. The role of IL-1 in the pathogenesis of heart disease. Arch Immunol Ther Exp (Warsz) 57, 165-76 (2009).

172. Topkara VK, Evans S, Zhang W, Epelman S, Staloch L, Barger PM, Mann DL. Therapeutic targeting of innate immunity in the failing heart. J Mol Cell Cardiol 51, 594-599 (2011).

173. Xiao L, Liu Y. Chronic kidney disease: Fibrosis and anaemia in CKD—two beasts, one ancestor. Nat Rev Nephrol 9, 563-565 (2013).

174. Frangogiannis NG The immune system and cardiac repair. Pharmacol Res 58, 88-111 (2008).

175. Mann DL. The emerging role of innate immunity in the heart and vascular system: for whom the cell tolls. Circ Res 108, 1133-45 (2011).

176. Shigeoka AA, Mueller JL, Kambo A, Mathison JC, King AJ, Hall WF, Correia Jda S, Ulevitch RJ, Hoffman HM, McKay DB. An Inflammasome-Independent Role for Epithelial-Expressed Nlrp3 in Renal Ischemia-Reperfusion Injury. J Immunol 185, 6277-6285 (2010).

177. Vilaysane A, Chun J, Seamone ME, Wang W, Chin R, Hirota S, Li Y, Clark SA, Tschopp J, Trpkov K, Hemmelgarn BR, Beck PL, Muruve DA. The NLRP3 inflammasome promotes renal inflammation and contributes to CKD. J Am Soc Nephrol 21, 1732-44 (2010).

178. Thaik CM, Calderone A, Takahashi N, Colucci WS Interleukin-1b Modulates the Growth and Phenotype of Neonatal Rat Cardiac Myocytes. J Clin Invest 96, 1093-1099 (1995).

179. Nishikawa K, Yoshida M, Kusuhara M, Ishigami N, Isoda K, Miyazaki K, Ohsuzu F. Left ventricular hypertrophy in mice with a cardiac-specific overexpression of interleukin-1. Am J Physiol Heart Circ Physiol 291, 176-183 (2006).

180. Frantz S. Targeted deletion of caspase-1 reduces early mortality and left ventricular dilatation following myocardial infarction. J Mol Cell Cardiol 35, 685-694 (2003).

181. Pomerantz BJ, Reznikov LL, Harken AH, Dinarello CA. Inhibition of caspase 1 reduces human myocardial ischemic dysfunction via inhibition of IL-18 and IL-1b. Proc Natl Acad Sci USA 98, 2871-2876 (2001).

182. Merkle S, Frantz S, Schön MP, Bauersachs J, Buitrago M, Frost RJ, Schmitteckert EM, Lohse MJ, Engelhardt S. A role for caspase-1 in heart failure. Circ Res 100, 645-53 (2007).

183. Abbate A, Kontos MC, Grizzard JD, Biondi-Zoccai GG, Van Tassell BW, Robati R, Roach LM, Arena RA, Roberts CS, Varma A, Gelwix CC, Salloum FN, Hastillo A, Dinarello CA, Vetrovec GW; VCU-ART Investigators. Interleukin-1 blockade with anakinra to prevent adverse cardiac remodeling after acute myocardial infarction (Virginia Commonwealth University Anakinra Remodeling Trial [VCU-ART] Pilot study). Am J Cardiol 105, 1371-1377.(2010).

184. Abbate A, Van Tassell BW, Biondi-Zoccai G, Kontos MC, Grizzard JD, Spillman DW, Oddi C, Roberts CS, Melchior RD, Mueller GH, Abouzaki NA, Rengel LR, Varma A, Gambill ML, Falcao RA, Voelkel NF, Dinarello CA, Vetrovec GW. Effects of Interleukin-1 Blockade With Anakinra on Adverse Cardiac Remodeling and Heart Failure After Acute Myocardial Infarction [from the Virginia Commonwealth University-Anakinra Remodeling Trial (2) (VCU-ART2) Pilot Study]. Am J Cardiol 111, 1394-1400 (2013).

185. Abbate A, Salloum FN, Vecile E, Das A, Hoke NN, Straino S, Biondi-Zoccai GG, Houser JE, Qureshi IZ, Ownby ED, Gustini E, Biasucci LM, Severino A, Capogrossi MC,

Vetrovec GW, Crea F, Baldi A, Kukreja RC, Dobrina A. Anakinra, a recombinant human interleukin-1 receptor antagonist, inhibits apoptosis in experimental acute myocardial infarction. Circulation 117, 2670-83 (2008).

186. Kawaguchi M, Takahashi M, Hata T, Kashima Y, Usui F, Morimoto H, Izawa A, Takahashi Y, Masumoto J, Koyama J, Hongo M, Noda T, Nakayama J, Sagara J, Taniguchi S, Ikeda U. Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. Circulation 123, 594-604 (2011).

187. Mezzaroma E, Toldo S, Farkas D, Seropian IM, Van Tassell BW, Salloum FN, Kannan HR, Menna AC, Voelkel NF, Abbate A. The inflammasome promotes adverse cardiac remodeling following acute myocardial infarction in the mouse. Proc Natl Acad Sci USA 108, 2-7 (2011).

188. Zuurbier CJ, Jong WM, Eerbeek O, Koeman A, Pulskens WP, Butter LM, Leemans JC, Hollmann MW. Deletion of the Innate Immune NLRP3 Receptor Abolishes Cardiac Ischemic Preconditioning and Is Associated with Decreased Il-6/ STAT3 Signaling. PLoS One 7, 1-8 (2012).

189. Sandanger Ø, Ranheim T, Vinge LE, Bliksøen M, Alfsnes K, Finsen AV, Dahl CP, Askevold ET, Florholmen G, Christensen G, Fitzgerald KA, Lien E, Valen G, Espevik T, Aukrust P, Yndestad A. The NLRP3 inflammasome is up-regulated in cardiac fibroblasts and mediates myocardial ischaemia – reperfusion injury. Cardiovasc Res 99, 164-174 (2013).

190. Green D.R, Reed JC. Mitochondria and Apoptosis. Science 1309, (2013).

191. Subramanian N, Natarajan K, Clatworthy MR, Wang Z, Germain RN. The Adaptor MAVS Promotes NLRP3 Mitochondrial Localization and Inflammasome Activation. Cell 153, 348-361 (2013).

192. Somers JR, Beck PL, Lees-Miller JP, Roach D, Li Y, Guo J, Loken S, Zhan S, Semeniuk L, Duff HJ. iNOS in cardiac myocytes plays a critical role in death in a murine model of hypertrophy induced by calcineurin. Am J Physiol Heart Circ Physiol 295, H1122-H1131 (2008).

193. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN. A Calcineurin-Dependent Transcriptional Pathway for Cardiac Hypertrophy. Cell 93, 215-228 (1998).

194. Dong D, Duan Y, Guo J, Roach DE, Swirp SL, Wang L, Lees-Miller JP, Sheldon RS, Molkentin JD, Duff HJ. Overexpression of calcineurin in mouse causes sudden cardiac death associated with decreased density of K-1 channels. Cardiovasc Res 57, 320-332 (2003).

195. Chu G, Carr AN, Young KB, Lester JW, Yatani A, Sanbe A, Colbert MC, Schwartz SM, Frank KF, Lampe PD, Robbins J, Molkentin JD, Kranias EG. Enhanced myocyte contractility and Calcium handling in a calcineurin transgenic model of heart failure. Cardiovasc Res 54, 105-116 (2002).

196. Zhong J, Basu R, Guo D, Chow FL, Byrns S, Schuster M, Loibner H, Wang XH, Penninger JM, Kassiri Z, Oudit GY. Angiotensin-converting enzyme 2 suppresses pathological hypertrophy, myocardial fibrosis, and cardiac dysfunction. Circulation 122, 717-28, 18 p following 728 (2010).

197. Borja J, Arnáiz E, Izquierdo I, Souto, M. Controversies in ventricular remodelling. Lancet 367, 1315-6 (2006).

198. Zile MR, Baicu CF, Gaasch WH. Diastolic heart failure--abnormalities in active relaxation and passive stiffness of the left ventricle. N Engl J Med 350, 1953-9 (2004).

199. Zhang H, Gharaee-Kermani G, Phan S. Regulation of Lung Fibroblast a-Smooth Muscle Actin Expression, Contractile Phenotype, and Apoptosis by IL-1B. J Immunol 158, 1393-1399 (1997).

200. Weber K, Brilla C. Pathological Hypertrophy and Cardiac Interstitium. Circulation 83, 1849-1865 (1991).

201. Waller TA, Hiser WL, Capehart JE, Roberts WC. Comparison of Clinical and Morphologic Cardiac Findings in Patients Having Cardiac Transplantation for Ischemic Cardiomyopathy , Idiopathic Dilated Cardiomyopathy , and Dilated Hypertrophic Cardiomyopathy. Am J Cardiol 9149, 884-894 (1998).

202. Weber KT, Sun Y, Bhattacharya SK, Ahokas RA, Gerling IC. Myofibroblast-mediated mechanisms of pathological remodelling of the heart. Nat Rev Cardiol 10, 15-26 (2012).

203. Milano CA, Simeone AA. Mechanical circulatory support: devices, outcomes and complications. Heart Fail Rev 18, 35-53 (2013).

204. Kostin S, Hein S, Arnon E, Scholz D, Schaper J. The Cytoskeleton and Related Proteins in the Human Failing Heart. Heart Fail Rev 5, 271-280 (2000).

205. Guillfin I, Castell V. Cytokine signaling during myocardial infarction : sequential appearance of IL-1beta and IL-6. Cytokine 269, R229-35 (1995).

206. Munkvad S, Gram J, Jespersen J. Interleukin-1 and tumor necrosis factor-alpha in plasma of patients with acute ischemic heart disease who undergo thrombolytic therapy: a randomized, placebo-controlled study. Lymphokine Cytokine Res 10, 325–327 (1991).

207. Artlett CM, Sassi-Gaha S, Rieger JL, Boesteanu AC, Feghali-Bostwick CA, Katsikis PD. The Inflammasome Activating Caspase 1 Mediates Fibrosis and Myofibroblast Differentiation in Systemic Sclerosis. Arthritis Rheum 63, 3563-3574 (2011).

208. González A, Ravassa S, Beaumont, J, López B, Díez J. New Targets to Treat the Structural Remodeling of the Myocardium. J Am Col Cardiol 58, 1833-1843 (2011).

209. Massague J. TGFβ Signal Transduction. Ann Rev Biochem 67, 753-791 (1998).

210. Hao J, Wang B, Jones SC, Jassal DS, Dixon IMC. Interaction between angiotensin II and Smad proteins in fibroblasts in failing heart and in vitro. Am J Physiol Heart Circ Physiol 279, H3020-H3030 (2012).

211. Lamkanfi M, Mueller JL, Vitari AC, Misaghi S, Fedorova A, Deshayes K, Lee WP, Hoffman HM, Dixit VM. Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. J Cell Biol 187, 61-70 (2009).

212. Derynck R, Zhang YE. Smad-dependent and Smad-Independent pathways in TGF-B Family Signaling. Nature 4, 577-584 (2003).

213. Juliana C, Fernandes-Alnemri T, Kang S, Farias A, Qin F, Alnemri ES. Non-transcriptional Priming and Deubiquitination Regulate NLRP3 Inflammasome Activation. J Biol Chem 287, 36617-36622 (2012).

214. Sena LA, Chandel NS. Physiological Roles of Mitochondrial Reactive Oxygen Species. Molecular Cell 48, 158-167 (2012).

215. Jain M, Rivera S, Monclus EA, Synenki L, Zirk A, Eisenbart J, Feghali-Bostwick C, Mutlu GM, Budinger GR, Chandel NS. Mitochondrial Reactive Oxygen Species Regulate Transforming Growth Factor-b Signaling. J Biol Chem 288, 770 -777 (2013).

216. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. Nat Immunol 11, 136-140 (2009).

217. Ippagunta SK, Malireddi RK, Shaw PJ, Neale GA, Walle LV, Green DR, Fukui Y, Lamkanfi M, Kanneganti TD. The inflammasome adaptor ASC regulates the function of adaptive immune cells by controlling Dock2-mediated Rac activation and actin polymerization. Nat Immunol 12, 1010-1017 (2012).

218. Ippagunta SK, Malireddi RK, Shaw PJ, Neale GA, Walle LV, Fukui Y, Green DR, Lamkanfi M, Kanneganti TD. Addendum: Defective Dock2 expression in a subset of ASC-deficient mouse lines. Nat Immunol 13, 701-702 (2012).

219. Jones DP, Go Y. Mapping the cysteine proteome: analysis of redox-sensing thiols. Curr Opin Chem Biol 15, 103-112 (2011).

220. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nuñez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 464, 1357-1362 (2010).

221. Wen H, Gris D, Lei Y, Jha S, Zhang L, Huang MT, Brickey WJ, Ting JP. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. Nat Immunol 12, 408-415 (2011).

222. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482, 179-185 (2012).

223. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. Ann Rev Immunol 27, 229-65 (2009).

224. Misawa T, Takahama M, Kozaki T, Lee H, Zou J, Saitoh T, Akira S. Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. Nat Immunol 14, 454-461 (2013).

225. Shimada K, Crother TR, Karlin J, Dagvadorj J, Chiba N, Chen S, Ramanujan VK, Wolf AJ, Vergnes L, Ojcius DM, Rentsendorj A, Vargas M, Guerrero C, Wang Y, Fitzgerald KA, Underhill DM, Town T, Arditi M. Oxidized Mitochondrial DNA Activates the NLRP3 Inflammasome during Apoptosis. Immunity 36, 401-414 (2012).

226. Krogh A, Larsson È, Heijne GV, Sonnhammer ELL Predicting Transmembrane Protein Topology with a Hidden Markov Model : Application to Complete Genomes. J Mol Biol 305, 567-580 (2001).

227. Schmidt B, Wachter E, Sebald W, Neupert W. Processing peptidase of Neurospora mitochondria. Eur J Biochem 588, 581 -588 (1984).

228. Marchetti C, Chojnacki J, Toldo S, Mezzaroma E, Tranchida N, Rose SW, Federici M, Van Tassell BW, Zhang S, Abbate A. A novel pharmacologic inhibitor of the NLRP3 inflammasome limits myocardial injury following ischemia-reperfusion in the mouse. J Cardiovasc Pharmacol 1-28 (2013).

229. Faustin B, Chen Y, Zhai D, Le Negrate G, Lartigue L, Satterthwait A, Reed JC. Mechanism of Bcl-2 and Bcl-X L inhibition of NLRP1 inflammasome : Loop domain-dependent suppression of ATP binding and oligomerization. Proc Natl Acad Sci USA. 106, 1-6 (2009).

230. Park S, Juliana C, Hong S, Datta P, Hwang I, Fernandes-Alnemri T, Yu JW, Alnemri ES. The Mitochondrial Antiviral Protein MAVS Associates with NLRP3 and Regulates Its Inflammasome Activity. J Immunol 191, 4358-4366 (2013).

231. Masumoto J, Dowds TA, Schaner P, Chen FF, Ogura Y, Li M, Zhu L, Katsuyama T, Sagara J, Taniguchi S, Gumucio DL, Núñez G, Inohara N. ASC is an activating adaptor for NF-kB and caspase-8-dependent apoptosis. Biochem Biophys Res Commun 303, 69-73 (2003).

232. Bae J, Park H. Crystal Structure of NALP3 Protein Pyrin Domain (PYD) and Its Implications in Inflammasome Assembly. J Biol Chem 286, 39528-39536 (2011).

233. Neupert W. Protein Import into Mitochondria. Ann Rev Biochem 66, 863-917 (1997).

234. Kaufmann T, Schlipf S, Sanz J, Neubert K, Stein R, Borner C. Characterization of the signal that directs Bcl-xl, but not Bcl-2, to the mitochondrial outer membrane. J Cell Biol 60, 53-64 (2002).

235. Heid ME, Keyel PA, Kamga C, Watkins SS, Salter RD. Mitochondrial Reactive Oxygen Species Induces NLRP3-Dependent Lysosomal Damage and Inflammasome Activation. J Immunol 191, 1-10 (2013).

236. KK, AR, BH. Unusual secretory pathways: from bacteria to man. (Austin: R.G. Landes: 1997).

237. Rees DC, Johnson E, Lewinson O. ABC transporters: the power to change. Nat Rev Mol Cell Biol 10, 218-227 (2009).

238. Dawson RJP, Locher KP. Structure of a bacterial multidrug ABC transporter. Nature 443, 180-186 (2006).

239. Gaj T, Gersbach CA, Barbas CF. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31, 397-405 (2013).

240. Brunette RL, Young JM, Whitley DG, Brodsky IE, Malik HS, Stetson DB. Extensive evolutionary and functional diversity among mammalian AIM2-like receptors. J Exp Med 209, 1969-1983 (2012).

241. de Lima Morais DA, Fang H, Rackham OJ, Wilson D, Pethica R, Chothia C, Gough J. SUPERFAMILY 1.75 including a comain-centric gene ontology method. Nucleic Acids Res 39, 427-434 (2011).

242. Mudd JO, Kass D. Tackling heart failure in the twenty-first century. Nature 451, 919-28 (2008).

243. Chung,ES, Packer M, Lo KH, Fasanmade AA, James T. Randomized double blind, placebo controlled, pilot trial of infiximad, a chimeric monoclonal antibody to TNFa, in patients with moderate to severe heart failure: results of the anti-TNF therapy against congestive heart failure (ATTACH) trial. Circulation 107, 3133-3140 (2003).

244. Carabello B, Spann J. The uses and limitations of end-systolic indexes of left ventricular function. Circulation 69, 1058-1064 (1984).

245. Coppack SW, Lant AF, Mcintosh CS, Rodgers AV. Pharmacokinetic and pharmacodynamic studies of glibenclamide in non-insulin dependent diabetes mellitus. B J Clin Pharmacol 29, 673-684 (1990).

Chapter Seven: **Appendix**

7.1 Appendix A

During the course of work, material from this thesis has been published in peer reviewed journals. This appendix contains accepted and published manuscripts.