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Mitochondrial DNA is a danger to the heart

PhD thesis

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To my mother, who took me to the library.

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Oslo, February 2015

Lars Henrik Mariero

Lars Henrik Mariero

List of papers

Paper I: Increased circulating mitochondrial DNA after myocardial infarction
Marte Bliksøen[1], **Lars Henrik Mariero**[1], Ingrid Kristine Ohm[1], Fred Haugen, Arne Yndestad, Svein Solheim, Ingebjørg Seljeflot, Trine Ranheim, Geir Øystein Andersen, Pål Aukrust, Guro Valen[2], Leif Erik Vinge[2]. *Int J Cardiol*. 2012 Jun 28;158(1):132-4

Paper II: Extracellular mtDNA activates NF-κB via TLR9 and induces cell death in cardiomyocytes
Marte Bliksøen, **Lars Henrik Mariero**, Kirsti Ytrehus, Anton Baysa, Fred Haugen, Ingebjørg Seljeflot, Jarle Vaage, Guro Valen[2], Kåre-Olav Stensløkken[2]
To be submitted

Paper III: Blocking cell surface nucleolin in heart cells prevents uptake of immunogenic DNA
Lars Henrik Mariero, Anton Baysa, Yuchuan Li, May-Kristin Torp, Guro Valen, Jarle Vaage, Kåre-Olav Stensløkken
To be submitted

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[1]These authors contributed equally to the paper
[2]These authors have a shared last authorship

Abbreviations

CF	Cardiac fibroblast
CM	Cardiomyocyte
CpG	Cytosine-phosphate-Guanine
DAMP	Damage-associated molecular pattern
IFNα1	Interferon alpha 1
IFNβ1	Interferon beta 1
IHD	Ischemic heart disease
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
i.p.	Intraperitoneal
I/R	Ischemia/reperfusion
H/R	Hypoxia/reoxygenation
IRF	Interferon regulatory factor
MAPK	Mitogen-activated protein kinase
mtDNA	Mitochondrial DNA
MyD88	Myeloid differentiation primary response gene 88
nDNA	Nuclear DNA
NF-κB	Nuclear factor kappa-light chain enhancer of activated B-cells
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
SAP	Stable angina pectoris
STEMI	ST elevation myocardial infarction
TLR	Toll-like receptor
TNFα	Tumor-necrosis factor alpha

Introduction

This thesis investigates how cellular debris can cause inflammation which may damage heart cells after acute myocardial infarction. The purpose of my introduction is to contextualize our findings in the clinical settings that we model and to provide the reader with a theoretical rationale for the research questions we have posed and our experimental approaches.

Clinical perspectives

Although the mortality of acute myocardial infarction is declining in Norway, survivors of large AMIs are at high risk for heart failure, a major cause of mortality and morbidity. Despite advanced treatment, heart failure remains a deadly disease[1]. Although there is evidence that the post-infarction inflammatory response is maladaptive[2], it is currently not a target for treatment. Improved fundamental understanding of the innate immune response that acute myocardial infarction holds the promise of better therapy.

Ischemic heart disease is a global health issue

In the last two decades there has been a dramatic improvement in prevention strategies, treatment options and subsequently short- and long-term prognosis for acute myocardial infarction and other ischemic heart disease. However, ischemic heart disease remains a leading cause of death in the industrialized world, and each year 3.8 million men and 3.4 million women die from the disease world wide[3]. In Norway, ischemic heart disease (ICD I20-25) was the cause of 4852 deaths in 2012, accounting for 12 per cent of all deaths[4]. On a global scale, low income countries are disproportionately affected, where over 40 % of deaths in persons under the age of 60 are attributable to cardiovascular disease, versus 4 % in high income countries[5].

Current diagnostics and treatment

Early recognition of possible acute myocardial infarction followed by early and successful reperfusion therapy by thrombolysis or percutaneous coronary intervention (PCI) is crucial to a favorable outcome[6]. A reduction in symptom-to-reperfusion time and the implementation of a number of treatment strategies has dramatically reduced morbidity and mortality. Current strategies are focused on primary and secondary preventative measures including weight, glucose, cholesterol and blood pressure management, public anti-smoking campaigns and dietary guidelines advising reduced intake of saturated fatty acids. Acute management focuses on reperfusion therapy by thrombolysis and increasingly, primary PCI.

Post-infarction medical care including β1 adrenergic antagonists, inhibition of the renin-angiotensin-aldosterone system by angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, cholesterol management with statins, as well as a number of other antihypertensive and -lipidemic agents represent important advances in treatment. In addition to pharmaceuticals, treatment such as cardiac resynchronization therapy and valvular or revascularization surgery in selected patients have contributed to reduced mortality and morbidity from ischemic heart disease. In addition to prompt pharmacological or mechanic reperfusion therapy, appropriate prehospital care is vital. Current treatment comprises analgesia (usually opioids), vasodilatation (nitric oxide donors, such as glyceryl trinitrate), platelet inhibition, β1 adrenergic antagonists, each of which has clinically proven risk-reward benefits[7,8] and high-flow oxygen. Although it is difficult to identify the most important causes of reduced mortality from ischemic heart disease, the mortality from acute myocardial infarction in Norway has fallen dramatically in the last three decades (figure 1). However, despite all these advances, ischemic heart disease remains a major killer in industrialized nations and it is even on the rise in many developing countries[9].

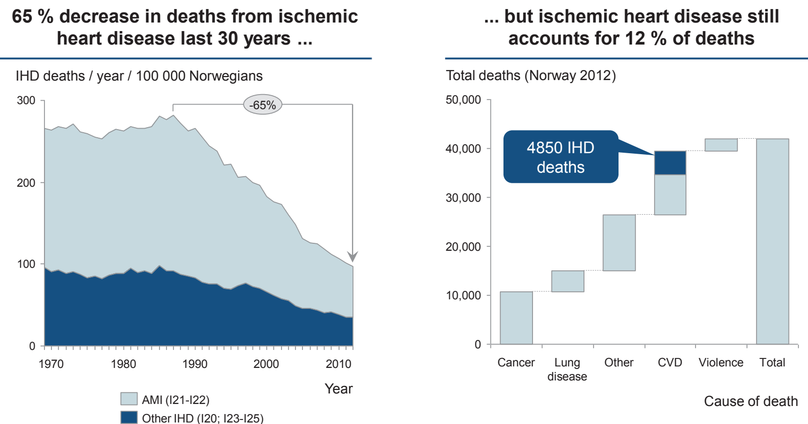

Figure 1. Historical development of population-adjusted mortality from ischemic heart disease and ischemic heart disease deaths in 2012. Modified from data from Statistics Norway[4].

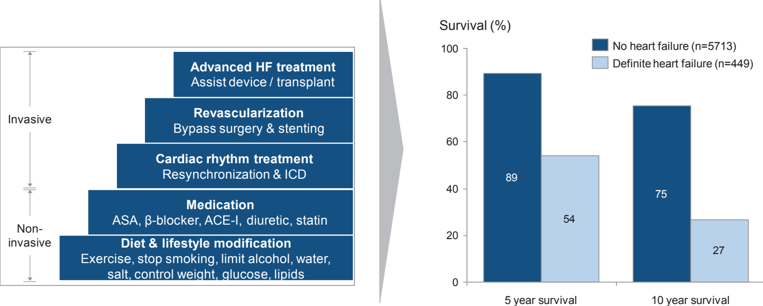

Figure 2. Despite rich treatment arsenal, heart failure remains deadly. Heart failure (HF) treatment modified from Up to date and right panel modified from data from the Echocardiographic Heart of England Screening (ECHOES) study[1].

Modulating post-infarction inflammatory response

The inflammatory response which follows acute myocardial infarction is important for healing the broken heart by replacing necrotic patches of myocardium with scar tissue. Because this response was evolutionarily honed to protect damaged skin and mucous membranes against bacterial infections, it could be excessive in the post-ischemic heart. Modulation of the innate response could benefit clinical myocardial ischemia-reperfusion injury, but promising preclinical findings were lost in translation[10]. However, a better fundamental understanding of the triggers, mediators and cellular effects of the post-infarction inflammatory response could pave the way for targeted therapy that could benefit millions of patients every year.

Theoretical Background

Myocardial ischemia/reperfusion injury

The aphorism *time is tissue* accurately describes the urgency of acute myocardial infarction[6]. After twenty minutes of ischemia[11], myocardium begins to succumb and reperfusion is the therapy of choice[7]. Paradoxically, the restoration of blood flow to post-ischemic tissue is injurious. The discovery of ischemic preconditioning[12] – tissue protection from brief non-lethal ischemia prior to prolonged ischemia – sparked hope for tissue salvage beyond that offered by early reperfusion. As one cannot deploy tissue-protective strategies prior to acute myocardial infarction, the molecular mechanisms for reperfusion injury have been intensely studied in search of clinically useful adjunct treatment to prompt reperfusion.

During prolonged ischemia, energy production is switched from oxygen-dependent oxidative phosphorylation to glycolysis with resulting lactate accumulation and acidification. This starts a dangerous spiral: The extrusion of hydrogen ions by the Na^+/H^+ exchanger and impaired function of the Na^+/K^+ pump cause intracellular

accumulation of sodium ions, which in turn are exchanged for calcium ions by reverse mode of the Na^{+}/Ca^{2+} exchanger (NCX)[13] which leads to intracellular calcium accumulation. Reperfusion injury comprises four entities: Reperfusion arrhythmias, myocardial stunning, microvascular obstruction and lethal reperfusion injury[3]. Only the latter will be discussed. Lethal myocardial ischemia-reperfusion injury is the reperfusion-induced demise of cardiomyocytes that were viable at the end of ischemia. A large body of evidence implicates some key mediators. Reactive oxygen species (ROS) generated in the early post-ischemic myocardium[14,15] can damage cellular membranes including the mitochondria which can release additional ROS[16]. ROS may also damage the sarcoplasmic reticulum which may release calcium ions that cause hypercontracture of the myofibrils and cardiomyocyte death[17]. Rapid restoration of physiological pH has also been shown to be injurious[18].

There is evidence that several pathways converge on the mitochondria and that mitochondrial permeability transition (MPT) is important in lethal reperfusion injury[19]. Mitochondrial permeability transition is an increase in the permeability of the mitochondrial membranes to molecules < 1500 Daltons[19,20] and is thought to be caused by the opening of a membrane spanning MPT pore, the MPTP[21]. The molecular composition of the MPTP remains elusive despite intensive study. Both voltage dependent anion channels (VDAC) and adenine nucleotide translocase (ANT) were likely candidates but shown non-essential for mitochondrion-dependent cell death[22,23]. However translocator protein[24] and cyclophilin-D[25] are necessary for MPT. Intriguingly, cyclophilin-D dependent MPT appears to be important for necrotic, but not apoptotic cell death[26] and the cyclophilin-D inhibitor cyclosporine A reduces infarct size in patients[27,28]. In addition to these mechanisms, the inflammatory response that follows acute myocardial infarction and reperfusion[13] is also a mediator of lethal reperfusion injury[2].

The innate immune response

The immune system constitutes two parts separated by speed and specificity: The fast-responding, all-purpose innate response, and the slower, but more precise, specific response[29]. Although this thesis only investigates aspects of innate immunity, a brief mention of the former is warranted for completeness and because the specific or adaptive immunity has also been implicated in ischemia-reperfusion injury[30]. Specific immunity comprises specific receptors on B lymphocytes and T lymphocytes. These receptors are subjected to somatic recombination and mutation, which probably produces receptors that are specific for antigens on all pathogens the host will ever encounter. Upon activation, B and T lymphocyte clones will proliferate and collectively mount a highly specific response to an invading microorganism. Because the organism might succumb to infection before a specific response is successfully mounted, an immediate, all-purpose response is also required – the innate immune response.

The innate immune response comprises mainly phagocytic cells and their mediators which plays a key role in tissue repair of damaged organs[31]. In the heart which has negligible regenerative capacity, cardiomyocytes are not replaced after myocardial infarctions and the innate immune response crucially replaces dead myocardium with scar tissue which allows the heart chambers to remain patent. Cells of the innate immune system are quickly recruited to sites of injury or infection in response to signs of injury from the body or the invading microorganism[29]. Similarly, debris from necrotic cardiac cells and extracellular matrix is released to the extracellular milieu and the circulation after myocardial infarction[2]. It has been known for a long time that immune cells infiltrate the post-infarction myocardium[32]. The signals that recruit these cells may depend on how the cell dies – as uncontrolled, unregulated necrotic demise may release more and a wider range of intracellular molecules and thereby pose a more powerful stimulus of the innate immune system than controlled necroptotic or apoptotic cell death[33]. However, microorganisms and dead cells will inevitably release clues of their presence.

Exogenous and endogenous triggers of inflammation

The innate response relies on recognition of non-self in order to respond to a limited number of structures that are highly conserved across microorganisms. Structures that are part of microorganisms and are capable of triggering the innate response are called pathogen-associated molecular patterns, or PAMPs, and include constituents of bacterial cell walls or propulsion machinery and viral and bacterial nucleic acids.

Endogenous molecules that trigger the innate immune system are called damage-associated molecular patterns (DAMPs)[34]. DAMPs released from post-ischemic cardiomyocytes may include high-mobility group box 1[35,36], heat-shock proteins[37-40], adenosine triphosphate[41], uric acid[42] and mitochondrial DNA (mtDNA)[43,44]. These beacons of danger from the outside or within are recognized by receptors of the innate immune system called pattern recognition receptors or PRRs.

Pattern recognition receptors

Germline-encoded PRRs are classified into five major groups: Absent in melanoma (AIM)-like receptors (ALRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoid acid inducible gene 1 (RIG1)-like receptors (RLRs), and toll-like receptors (TLRs)[45]. CLRs and TLRs are membrane-bound and ALRs, NLRs and RLRs are cytosolic[45] (figure 3).

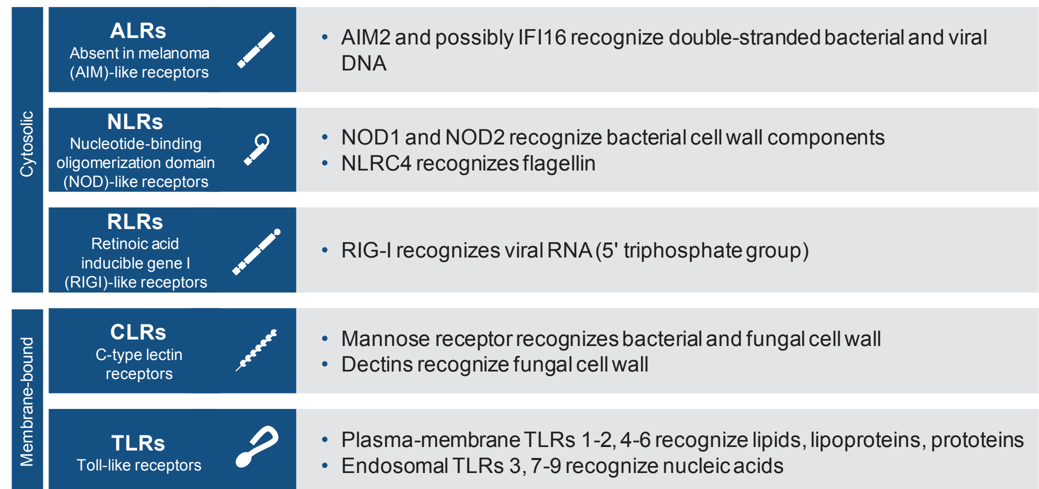

Figure 3. Cellular localization and typical microbial ligands for the five main groups of pattern recognition receptors[46]. IFI16: Gamma-interferon-inducible protein 16; NLRC4: NLR family, caspase recruitment domain (CARD) containing 4.

Toll-like receptors and signaling pathways

TLRs are among the best described PRRs[46]. Identified in the fruit fly *Drosophila melanogaster* in 1985[47], Toll-receptor was shown crucial for anti-fungal defense[48], and the following year the first human ortholog was described[49]. There are thirteen known mammalian TLRs and TLR1-10 are functional in humans[50]. mRNA for most TLRs is expressed in the murine[51] and human heart[52] and protein of TLR2[53], TLR4[54] and TLR9[55] have been found in the heart. TLRs contain a C-terminal cytoplasmic signaling domain, called toll IL-1 receptor (TIR) domain[56], a single transmembrane helix and an N-terminal ligand recognition domain[57]. The characteristically horse shoe shaped ligand recognition domains are exposed to the extracellular face of the cell or to the lumen of endosomes, topologically equivalent to the outside of the cell[58]. These domains contain leucine-rich repeats (LRRs)[57], the highly conserved building blocks of TLRs, which are also found in plants[59]. Upon activation TLRs associate to form m-shaped homodimers[59]. Heterodimerization occurs and may contribute to increased ligand repertoire[60] along with association with non-toll co-receptors like CD14[61-63], myeloid differentiation 1 (MD-1)[64] and MD-2[65].

The function of the mammalian TLRs has largely been determined by functional ablation in mice. TLR1-2 and 5-6 are expressed on the cell surface and recognize lipopeptides (TLR2), peptidoglycans (heterodimers of TLR2 and 1 or 6), lipoproteins (TLR4) and flagellin (TLR5). TLR 3 and 7-9 are found intracellularly in endolysosomes and recognize RNA (TLR3, 7-8) and DNA (TLR9)[46].

When activated, TLRs recruit adaptor proteins to trigger a signaling cascade that culminates in the transcription of pro-inflammatory genes. TLR adaptor proteins contain TIR domains which associate with the cytoplasmic TIR domains of TLRs. The intracellular cascades downstream of TLR activation are broadly myeloid differentiation primary response gene 88 (MyD88)-dependent or MyD88-independent[66,67]. The latter is also known as the TIR-domain-containing-adaptor-inducing IFNβ (TRIF)-dependent pathway. With the exception of TLR3, all TLRs can signal through the MyD88-dependent pathway[66]. Some TLRs require TIR-domain containing adaptor protein (TIRAP, also known as MAL; MyD88-adapter-like) to bind indirectly to MyD88[68]. MyD88 recruitment stimulates recruitment and phosphorylation of interleukin-1 receptor associated kinase (IRAK) 4, which recruits IRAK-1 and IRAK-2 to the complex. The IRAK family interacts with the E3 ubiquitin ligase tumor necrosis factor α associated factor 6 (TRAF6)[69], which, when activated recruits transforming growth factor β activated kinase 1 (TAK1) to the IRAK-1/TRAF6 complex by binding TAK1 binding protein 2 (TAB2) and 3 (TAB3)[69]. In plasmacytoid dendritic cells (pDCs), TRAF6 also activates interferon regulatory factor 7 (IRF7), in a TRAF3 and osteopontin (OPN) dependent manner[70-72], which then translocates to the nucleus and induces transcription of type 1 interferons[70].

Though both pathways culminate with the activation of the pro-inflammatory transcription factor nuclear factor kappa light chain enhancer of activated B cells (NF-κB), MyD88 dependent TLR signaling bifurcates at TAK1 activation: 1) The TAK1 complex phosphorylates inhibitor of κB kinase β (IKKβ)[73,74], which then phosphorylates inhibitor of NF-κB, IκB (IκBα). IκBα is bound NF-κB, but is ubiquitinated and degraded upon activation, which then releases NF-κB[75]. NF-κB translocates to the nucleus and induces transcription of pro-inflammatory cytokines like TNFα, pro-IL-1β, and IL-6. 2) The TAK1 complex activates kinase cascades which culminate in the phosphorylation of mitogen-activated protein (MAP) kinases c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinases (ERK1/2)[76,77], which have a wide range of cellular functions including cell survival, differentiation and proliferation[78]. Moreover, MAPK stabilize mRNA downstream of NF-κB and thereby increase translation of pro-inflammatory cytokines[79].

MyD88 independent/TRIF dependent signaling begins with TRIF binding to TLR3 or to TLR4 via TRIF-related adapter molecule (TRAM)[80]. TRIF interacts with TNF receptor type 1 associated DEATH domain protein (TRADD)[81], pellino-1 and receptor interacting protein 1 (RIP1)[82] to activate TAK1[83], which then activates NFκB as in the MyD88 dependent pathway. In addition, TRIF activates TANK-binding kinase 1 (TBK1) and inhibitor or κB kinase I (IKKi) via TRAF3 to phosphorylate IRF3[70,84], which then translocates to the nucleus to induce transcription of IFNβ[85]. As TLRs are capable of responding to a large number of ligands and initiate intracellular events potentially detrimental to the cell, it is crucial that TLR signaling is subject to

negative regulation[86]. TIR-TIR and other protein-protein interactions are crucial for function TLR signaling, and negative regulators exist at the level of MyD88[87,88], TIRAP[88], TRAM[89], IRAK[90,91] and TRAF[92]. As the vast majority of studies on PRRs have been conducted in B lymphocytes or dendritic cells, canonical signaling may apply for example in cardiomyocytes.

Toll-like receptor 9

TLR9 is trafficked to endoplasmic reticulum by the transmembrane protein Unc93b[93] where it resides until activation[94,95]. Activated TLR9 forms homodimers upon recognition of DNA ligands and translocates to endolysosomal compartments[94,95]. TLR9 was first shown to recognize bacterial DNA containing unmethylated CpG motifs[96], but also recognizes DNA from viruses[97] and plasmodium species[98]. The crystal structure of the unliganded LRR-containing ectodomain was recently reported[99], but the details of TLR9 ligand interaction are not clear. TLR9 signals through MyD88, IRAK and TRAF6 activation before bifurcation into NF-κB and IRF7 pathways which culminate in the transcription of proinflammatory cytokines and type 1 interferons, respectively[100] (figure3). Synthetic CpG oligonucleotides show specificity in pathway activation: CpG B potently activates the NFκB pathway, CpG A activates the IRF pathway while CpG C stimulates both[101]. CpG A contains phosphodiester palindromic CpG-containing sequences and CpG B contains multiple six nucleotide CpG motifs with the general formula: purine (A/G)-pyrimidine (C/T)-C-G-pyrimidine (C/T)-pyrimidine (C/T) and commercial CpG B commonly contains fully phosphorothioated backbone, more nuclease-resistant than phosphorodiester backbone[102]. IRF pathway preference may be driven by higher order structures as CpG A is multimeric and CpG C is dimeric, but monomeric CpG B exhibits little IRF stimulation[103]. Though TLR9 shows sequence preference it potentially recognizes a large number of sequences, and it has been suggested that the sugar backbone is also of importance[104].

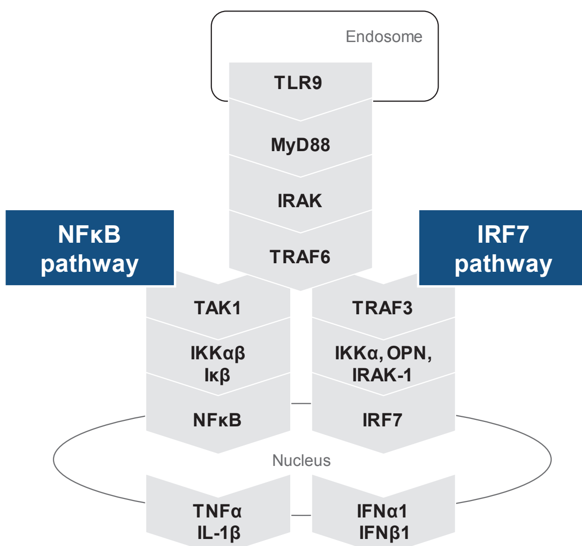

Figure 4. TLR9 signaling[105]. Mandatory MyD88, IRAK, TRAF6 activation before bifurcation into NF-κB pathway and IRF7 pathways, whose translocation to the nucleus culminates in the transcription of proinflammatory cytokines (such as TNFα, pro-IL1) and type 1 interferons (IFNα1 and IFNβ1), respectively. TLR9: Toll-like receptor 9, MyD88: myeloid differentiation primary response gene 88; IRAK: interleukin-1 receptor associated kinase; TRAF: tumor necrosis factor α associated factor; TAK: transforming growth factor β activated kinase IKK: inhibitor of κB kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; TNFα: Tumor necrosis factor alpha; IL-1β: Interleukin 1 beta; OPN: Osteopontin; IRF: Interferon regulatory factor; IFN: interferon

TLR9 in the heart

TLR9 mRNA[51,52,55,106] and protein[55] have been demonstrated in the heart. CpG DNA induces myocardial inflammation[55] and reduces contractility in isolated cardiomyocytes[55,107] via TLR9[55]. In cardiac fibroblasts, CpG induces TNFα and impairs function[108]. It has been shown that CpG pretreatment protects the mouse heart from subsequent ischemia-reperfusion injury[109,110], while another study showed no effect of CpG priming[111].

Results are similarly conflicting on the role of TLR9 in acute systemic inflammatory response syndrome (SIRS) induced cardiac dysfunction. While it has been shown that high-dose CpG B induces SIRS and cardiac dysfunction[112] and that TLR9 ablation protects against CpG induced SIRS[112] and polymicrobial sepsis[113], TLR9 stimulation via CpG has been shown to rescue cardiac dysfunction after polymicrobial sepsis[114], severe hypovolemic shock[115] and LPS-induced shock[116].

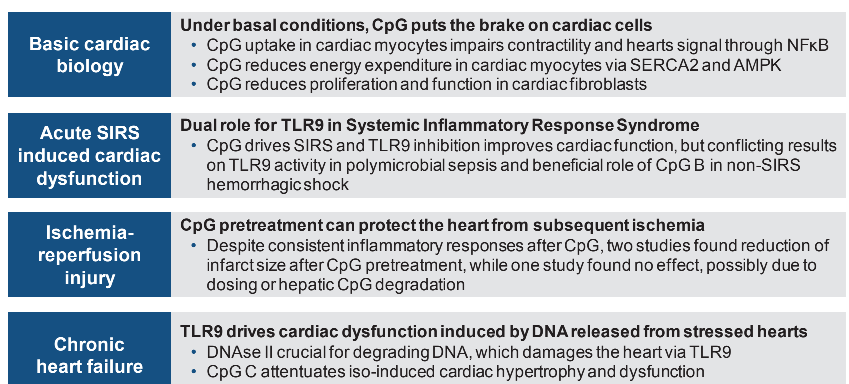

Figure 5. Summary of studies on TLR9 in the heart structured in four categories: Basic cardiac biology, acute SIRS-induced cardiac dysfunction ischemia-reperfusion injury, chronic heart failure. Citations are found in the text.

In two different models of heart failure, CpG was found to be beneficial as CpG B pretreatment reduced cardiac inflammation and attenuated transaortic constriction (TAC)-induced hypertrophy[117] and CpG C partially rescued cardiac function in isopreterenol-induced cardiac hypertrophy[118]. However, Kinya Otsu's group showed in an elegant study that DNase2a knock-out mice had more pronounced myocardial inflammation and higher mortality after TAC, that could be rescued by TLR9 inhibition[44]. Further, it was shown that myocardial inflammation was caused by undegraded mitochondrial DNA[44]. In non-immune cells, such as neurons and cardiomyocytes, it has been proposed that TLR9 activation does induce transcription of proinflammatory genes, but rather is a signal to metabolic conservation via 5' adenosine monophosphate-activated protein kinase (AMPK)[119]. TLR9 is an intracellular receptor and in order to bind to extracellular DNA, the DNA must first be internalized.

DNA sensing receptors

Though TLR9 is the focus for this thesis, more than ten cytosolic DNA sensors have been reported in recent years[120]. While Mediators of interferon responses include Z-DNA-binding protein 1/ DNA-dependent activator of IFN-regulatory factors (ZBP1/DAI)[121], RNA polymerase III[122], aspartate-glutamate-any amino acid-aspartate/histidine (DExD/H)-box helicase 36 (DHX36)[123] and gamma-interferon-inducible protein 16 (IFI15)[124]. AIM2[125] and DHX9[123] are inducers of IL-1β or TNFα. Also, stimulator of interferon genes (STING, TMEM173) is a central signaling molecule to the innate immune response to cytosolic DNA[126]. These DNA sensors have primarily been studied in dendritic cells and little is known about their expression or role in the heart.

DNA internalization in non-phagocytic cells

Though non-phagocytic cells, cardiomyocytes appear to internalize extracellular material. However, the mechanisms by which cardiomyocytes take up extracellular macromolecules has not been widely studied. It has been reported that CpG DNA can be internalized by adult primary cardiomyocytes[55], but the mechanism for uptake of DNA is not known. Phagocytosis cannot be ruled out, but other forms of endocytosis/pinocytosis; clathrin-mediated endocytosis (CME), clathrin-independent endocytosis (CIE) or macropinocytosis[127] are more likely. Both CME and CIE can be receptor-mediated (RME). Transmembrane diffusion is highly unlikely as even small DNA molecules are heavily charged. Though little is known about uptake mechanisms for CpG, it has been shown that the uptake of 20-mer oligonucleotides into human colorectal ademocarcinoma cells was inhibited by phenylarsine oxide[128], an inhibitor of macropinocytosis and phagocytosis whose mechanism of action is unknown[127].

Candidates for RME include scavenger receptors – structurally heterogeneous receptors first associated with internalization of oxidized low-density lipoprotein[129]. As well as mediating uptake of a number of different molecules, scavenger receptors also participate in cellular signaling and are considered a PRR subclass by some researchers[129]. Though the role of scavenger receptors in the uptake of DNA in the heart is not known, scavenger receptor type A was dispensable for internalization of CpG DNA in macrophages[130].

Nucleolin is a candidate for trans-sarcolemmal DNA transport. Nucleolin is abundant in the nucleolus of eukaryotic cells and implicated in ribosome maturation, RNA and DNA metabolism and shuttling of pre-RNAs from the nucleus to the cytoplasm[131,132]. Nucleolin is expressed on the cell surface of many cell types[133] and several lines of evidence underlie the hypothesis that cell-surface nucleolin is important for internalization of DAMPs, including immunogenic DNA. Cell-surface nucleolin is important for the attachment of HIV-1 to the cell surface of $CD4^+$ T cells[134] and is required for internalization of human parainfluenza virus type 3 to airway epithelial cells[135]. Cell-surface nucleolin is the receptor for a 26-mer DNA oligonucleotide[136], which is around the same size as CpG DNA. Further, cell-surface nucleolin mediates the uptake of ~8000 kDa DNA nanoparticles[137], which is slightly larger than CpG DNA[138].

Aims of the study

The purpose of this thesis is to investigate the role of cellular debris in extending cardiomyocyte injury after acute myocardial infarction. Understanding the cellular response to the sterile inflammation that follows ischemic tissue damage holds the promise for new therapy.

We hypothesized that mitochondrial DNA is a damage-associated molecular pattern and is released from the heart after acute myocardial infarction, which triggers the innate immune system via TLR9 and NF-κB. We propose that cell-surface nucleolin can internalize immunogenic DNA. Specifically, our aims were:

- Investigate the release of mitochondrial DNA from the post-ischemic human heart (paper I)
- Investigate if mtDNA activates TLR9 and contributes to cardiomyocyte injury (paper II)
- Investigate if cardiomyocytes mount an inflammatory response to mitochondrial DNA and if cell-surface nucleolin aids in internalization of DNA (paper III)

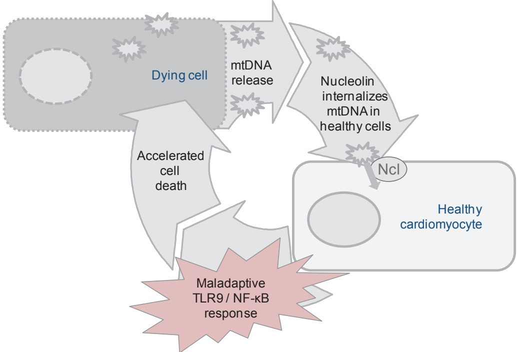

Figure 6. High-level cartoon representation of the hypotheses explored in this thesis. Dead and dying cardiomyocytes release their extracellular contents including mitochondrial DNA (mtDNA), which can be detected in the circulation. mtDNA is internalized via nucleolin in healthy cardiomyocytes and causes an unwanted inflammatory response via toll-like receptor 9 (TLR9) and NF- κB which propagates the ischemic injury.

Paper I

Summary of results

Increased circulating mtDNA after myocardial infarction

Marte Bliksøen[1], **Lars Henrik Mariero**[1], Ingrid Kristine Ohm[1], Fred Haugen, Arne Yndestad, Svein Solheim, Ingebjørg Seljeflot, Trine Ranheim, Geir Øystein Andersen, Pål Aukrust, Guro Valen[2], Leif Erik Vinge[2] *Int J Cardiol*. 2012 Jun 28;158(1):132-4

Aim: Investigate the release of mitochondrial DNA from the post-ischemic human heart

Key findings

- ST elevation myocardial infarction (STEMI) patients (n=20) had higher levels of mtDNA in cell-free plasma than stable angina pectoris (SAP) patients (n=10) three hours after PCI
- mtDNA release was higher in patients with transmural myocardial infarction than patients with non-transmural infarction as evaluated by cardiac MRI
- The release of mtDNA correlated with max troponin T release
- The heart is a likely source of circulating mtDNA, as mtDNA is detected in effluents from isolated, perfused mouse hearts

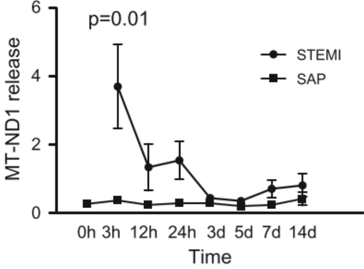

Figure 7. Cardiac mtDNA release after STEMI. mtDNA was extracted from blood samples of 20 STEMI and 10 SAP patients undergoing PCI, and amplified with real-time PCR using primers for the mtDNA-specific NADH dehydrogenase, ND1. Data are mean ± SEM

[1]These authors contributed equally to the paper
[2]These authors have a shared last authorship

Paper II

Extracellular mtDNA activates NF-κB via TLR9 and induces cell death in cardiomyocytes

Marte Bliksøen, **Lars Henrik Mariero**, Kirsti Ytrehus, Anton Baysa, Fred Haugen, Ingebjørg Seljeflot, Jarle Vaage, Guro Valen[1], Kåre-Olav Stensløkken[1]

Aim: Investigate if mtDNA activates TLR9 and contributes to cardiomyocyte injury

Key findings

- Sonicated mtDNA and plasma from STEMI patients trigger e-selectin-driven NF-κB signaling in HEK cells in a TLR9 dependent manner
- NF-κB activity was increased in luciferase reporter mice after in vivo intraperitoneal injection with mtDNA and CpG
- Isolated adult murine cardiac myocytes internalize mitochondrial DNA
- mtDNA induces cell death in isolated adult murine cardiomyocytes and reduces the time to mitochondrial membrane potential depolarization

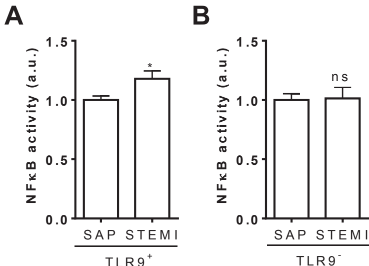

*Figure 8. TLR9 dependent NF-κB activation by mtDNA-containing patient serum. A: HEK293 co-expressing TLR9 and NF-κB luciferase reporter (TLR9$^+$) were stimulated for four hours with 10% serum from patients with ST-segment elevation myocardial infarction (STEMI, n=6) with high mtDNA levels or stable angina pectoris (SAP, n=6) with low mtDNA levels. The luciferase signal detected was detected by a CCD camera, corrected for protein content and normalized to SAP. B: Identical experiments were performed in HEK293 NF-κB luciferase reporter cells lacking TLR9 (TLR9$^-$). Data presented as mean ± SEM. To detect significant differences, student's t test was used (*p<0.05).*

[1]These authors have a shared last authorship

Paper III

Blocking cell surface nucleolin in heart cells prevents uptake of immunogenic DNA

Lars Henrik Mariero, Anton Baysa, Yuchuan Li, May-Kristin Torp, Guro Valen, Jarle Vaage, Kåre-Olav Stensløkken

Aim: Investigate if cardiomyocytes mount an inflammatory response to mitochondrial DNA and if cell-surface nucleolin aids internalization of DNA

Key findings

- mtDNA induces inflammation in cardiomyocytes during hypoxia / reoxygenation
- Extracellular DNA induces TLR9 dependent NF-κB activity during hypoxia / reoxygenation
- Nucleolin is expressed on cardiomyocyte membranes
- Nucleolin inhibition reduces uptake of CpG DNA in cardiomyocytes and cardiac fibroblasts

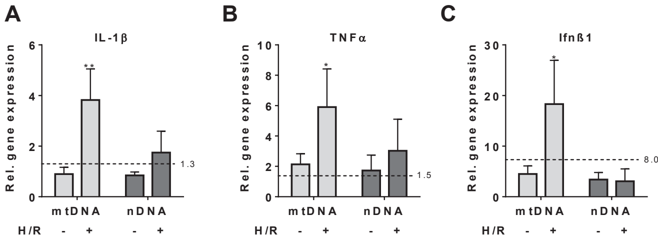

*Figure 9. mtDNA induces inflammation in cardiomyocytes during hypoxia-reoxygenation (H/R). Quantitative real-time PCR on isolated adult C57BL/6 murine cardiomyocytes (n=4-11 individuals) exposed to 40 minutes of non-lethal glucose-free hypoxia (<0.5% O_2) followed by two hours of reoxygenation (H/R +) or time-matched normoxic conditions with medium change (H/R -). Cells were either exposed to murine mitochondrial DNA (mtDNA, 20 µg/mL), nuclear DNA (nDNA, 20 µg/mL) or normal medium (data not shown). The data shows relative gene (2-ΔΔct) expression of interleukin-1β (IL-1β, A), tumor necrosis factor α (TNFα, B), and interferon β1 (Ifnβ1, E) normalized to the expression of the housekeeping gene Rpl32 with normoxic control cells as the calibrator. For each gene, the gene expression of normoxic control cells is set to 1.0. The dotted horizontal line and the numerical value to the right represents the mean gene expression of hypoxic control cells. Data is presented as mean ± SEM. To detect significant differences between normoxic control and exposure groups, the student's t-test was used (*p<0.05, **p<0.01).*

Methodological considerations

As paper I was published as a brief communication with very limited word count, full description of the methods used in that paper are included in this section. For papers II and III, only a brief mention of the method prefaces methodological consideration, as full methods are described in the papers.

Study group

The study protocol was approved by the Regional committee for medical research ethics and all patients gave written, informed consent to participate. Patient plasma and serum was obtained from previously collected material[139]. Thirty men and women between 30 and 75 years with STEMI (n=20) or stable angina pectoris (SAP, n=10), admitted to Ullevål University Hospital, Oslo, Norway, were included. All were treated successfully with percutaneous coronary intervention (PCI) in a central coronary artery obtaining normal blood flow. Heparin (70 IU/kg i.v.) was given to all patients during the PCI procedure in both groups. Four of the STEMI patients were given Gp IIb/IIIa receptor blocker. Inclusion criteria in the STEMI group were typical symptoms with ST-segment elevation in the electrocardiogram and occlusion of a central coronary artery verified by coronary angiography. Patients in the SAP group had symptoms consistent with SAP, and angiographic coronary artery stenosis. Exclusion criteria in both groups were previous transmural infarction, cardiogenic shock or co-morbidities like malignancy, stroke or significant inflammatory, endocrine or lung disease. The included patients were treated in accordance with current guidelines.

The sample size is small and concerns for heterogeneity in age, gender, co-morbidities, medications and other confounders is a possible source of error was met by strict exclusion criteria. There were no differences in baseline characteristics between the groups other than more previous PCI procedures in the stable angina pectoris patients[139]. Infarct sizes in the study population were relatively small, which might limit the extent to which DNA was released to the circulation. However, as we were able to detect mtDNA in the circulation of a small number of patients with modest infarctions suggests that the release of mtDNA after myocardial infarction is quite large. Quantification of circulating mtDNA in STEMI patients prior to PCI would allow determination of the contribution of reperfusion to the wash-out of mtDNA from the post-ischemic tissue to the circulation. Unfortunately, it was not practically possible to obtain these samples. Moreover, our first time-point in both study groups was three hours post PCI. It is conceivable that DNases in the tissue and circulation had degraded DNA by that time, and that peak levels of circulating mtDNA were higher than we were able to quantify with our study.

Blood sampling

Venous blood was collected immediately before PCI in the SAP group and in both groups after 3 and 12 hours and after 1, 3, 5, 7 and 14 days. From day 1, all samples were obtained from fasting patients before intake of medication. Serum was prepared by centrifugation within 1 h at 2500g for 10 min. DNA was isolated from platelet-poor plasma obtained by centrifugation within 30 min at 4°C and 3000g for 20 min. Samples were stored at -80°C until analysis. Troponin T (reference values <0.03 µg/L) was analyzed in serum by routine methods at the clinical laboratory at Oslo University Hospital Ullevål (Cobas e601 assay, Roche Diagnostics, Basel, Switzerland).

Platelets or leukocytes are possible sources of mitochondrial DNA in the blood samples, but care was taken to quickly centrifuge the samples to obtain platelet-poor plasma. A single centrifugation of plasma has been shown be reliable for quantification of circulating mtDNA[140]. All samples were stored at -80°C to prevent lysis or degradation prior to analysis.

Cardiac magnetic resonance imaging (MRI)

In the STEMI group, cardiac MRI was performed with a 1.5 T whole body scanner (Philips Intera, Best, The Netherlands) and analysis was performed on a View Forum workstation (Philips Medical Systems). Short axis images were acquired for left ventricular volume and ejection fraction analysis. Infarct size by MRI was determined with the gadolinium late contrast enhancement technique[141] six weeks after PCI. SAP patients did not undergo cardiac MRI, which reduced the statistical power to detect a possible correlation between the level of circulating mtDNA and infarct size.

Extraction of mitochondrial DNA from human plasma and qPCR

DNA was extracted from cell-free platelet-poor plasma samples with Qiagen QIAamp® DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, USA). In order to control procedural variability, 50 µl of plasma was spiked with 15.63 pg/mL mtDNA-enriched murine DNA and 10 µg linear acrylamide (Ambion, Applied Biosystems, TX, USA). DNA was eluted in 300 µl nuclease-free water before a 10 µl aliquot was analyzed by qPCR with SYBR Green chemistry. DNA was analyzed using Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Life Technology Corporation, CA, USA). The primers used for these assays were mitochondrially encoded NADH dehydrogenase (MT-ND1), mitochondrially encoded cytochrome c oxidase III (MT-CO3), genomic 18S and specific murine mtDNA-primers[142] (table 1). Standard curves were made from mtDNA-enriched DNA isolated from human placental tissue and murine liver using Mitochondria Isolation Kit for Tissue (Thermo Fischer Scientific, IL, USA) and the primers targeting human mtDNA and

were proven specific for their respective species. The concentration of murine mtDNA used to spike the human plasma samples was several thousand-fold lower than that of the human mtDNA.

Ex vivo (Langendorff) perfusion

To study if the myocardium could release mtDNA, isolated mouse hearts were subjected to global ischemia and reperfusion. Male C57BL/6 mice were anesthetized with 5% sodium pentobarbital (60 mg/kg) and heparinized (500 IU i.p). After anesthesia, the heart was rapidly excised and placed in ice-cold Krebs-Henseleit buffer (KHB, containing in mmol/L: NaCl 118.5; $NaHCO_3$ 25.0; KCl 4.7; KH_2PO_2 1.2; $MgSO_4$ 1.2; glucose 11.1; $CaCl_2$ 1.8) and the aorta was cannulated and the heart mounted on a Langendorff system (ADInstruments, Castle Hill, NSW, Australia).

Hearts were retrogradely perfused with warm (37°C), oxygenated (95% O_2, 5% CO_2) KHB at a constant pressure of 70 mmHg. The heart temperature was monitored and kept constant at 37°C during the experiment. A fluid-filled balloon was inserted into the left ventricle via the left atrium to measure heart rate (HR) and ventricular pressures (Power lab system, ADInstruments). Left ventricular end-diastolic pressure (LVEDP) was set to 5—10 mmHg at the start of stabilization, and changes in LVEDP as well as left ventricular systolic pressure (LVSP) were measured throughout the experimental protocol. Left ventricular developed pressure (LVdevP=LVSP-LVEDP) and maximum and minimum derivative of left ventricular pressure development (LVdp/dtmax and LVdp/dtmin) were calculated. Coronary flow (CF) was measured by collecting the coronary effluent. Animals with aortic cannulation time > 3 min, CF >4 ml/min, LVSP <80 mmHg, HR <220 beats per minute during stabilization or irreversible reperfusion arrhythmias (asystole or ventricular fibrillation) were excluded from the study. All hearts underwent 20 min of pre-ischemic stabilization, 30 min global ischemia and 60 min reperfusion. Coronary effluents were collected during the last 5 min of stabilization and throughout reperfusion. The effluents were frozen immediately at -20°C for further analysis by qPCR. After reperfusion, hearts were sectioned perpendicular to the long axis and four central 1 mm ventricular slices were incubated at 37°C in 1% triphenyltetrazolium chloride (TTC) for 15 min. After incubation the slices were gently pressed between two glass plates and photographed (Nikon, Coolpix 5400). The infarct area was measured as percentage of total area minus cavities and calculated with Adobe Photoshop by a researcher blinded to the experimental groups.

Langendorff perfusion is a staple in heart research[143] but the denervated, cell-free perfusion is often criticized as an overly simple model. In paper I, isolated mouse hearts were retrogradely perfused to investigate if the post-ischemic myocardium is a source of mtDNA. For this application, isolated heart perfusion is an advantage as it reduces non-myocardial sources of mtDNA. However, as leukocytes contribute to the damage of the post-

ischemic heart it is imaginable that this cell-free model underestimates ischemic damage to the myocardium and subsequent mtDNA release. Importantly, our lab enforces strict exclusion criteria[144] to ensure that hearts are comparable and well functioning prior to ischemia.

Quantification of mtDNA in coronary effluents from isolated hearts

DNA was isolated from coronary effluents from nine mouse hearts using the DNeasy blood & tissue kit (Qiagen) and qPCR using mouse mtDNA primers[142] (117 bp, see table 1) was performed. mtDNA-enriched DNA from mouse livers was used for standard curves and the relative standard curve method was used to quantify mtDNA release. Samples were run in duplicate using SYBR Green chemistry and the amount of mtDNA was normalized to total coronary flow and perfusion time.

In vivo NF-κB luciferase activity following i.p. mtDNA and CpG administration

In paper II, mtDNA or CpG was administered to transgenic mice that express firefly luciferase under the control of three NF-κB response elements, enabling real-time in vivo imaging of NF-κB activity[145]. 300 µL vehicle or equal volume containing 200 µg mtDNA or 100 µg CpG C was injected i.p. and animals were imaged for luciferase reporter activity twice: immediately after injection and after six hours. Though both CpG C and mtDNA activated NF-κB in vivo, it is clear from the data that there were non- or low responders in both the treatment groups. This could be attributable to differences in the reporter system, such as varying induction of the signal, or due to failed i.p. injections. The reporter system is well tested[146] and the injections were performed by experienced experimentalists. However, despite varying responses, clear differences were seen in animals injected with mtDNA or CpG C compared to control. The relatively high CpG dose is in line with published reports[108,109,111,116]. Moreover, though the total amount of DNA injected was quite high, we have shown that the majority is of nuclear origin, so that <50 µg of the injected DNA is actually mtDNA. Also, though fragmented, each mtDNA is considerably larger than the ~22-mer CpG. Assuming that the mtDNA fragments are 100-1000 bp, the number of CpG agonists would outnumber the number of mtDNA agonists by a factor of ~5-50. Collectively, this suggests that the potency of pure mtDNA might actually rival that of CpG.

Non-lethal hypoxia / reoxygenation for primary heart cells and cell lines

In paper III, we established a model of non-lethal hypoxia / reoxygenation for primary cells and cell lines. Several publications describe glucose-free H/R for primary adult murine cardiomyocytes with hypoxia (0-1.5 per cent) from 0.5-1 hour[147-151] up to 4-5 hours[152,153] and reoxygenation from 0-17 hours. These models induce cell death as measured by trypan blue[148] or LDH release[147]. However, to investigate the immunogenicity of DNA in H/R, we sought to limit the release of possible DAMPs from dying cardiomyocytes and therefore wanted a non-lethal model. We conducted pilot experiments and found that 1 hour of hypoxia followed by 2

hours of reoxygenation induced cell death, but that 40 minutes of hypoxia and 2 hours of reoxygenation did not.

Preparation of mtDNA and nDNA agonists

Male C57BL/6 mice were euthanized by cervical dislocation and a transverse laparotomy was performed. After ligating the hepatic hilus to prevent bile contamination, the liver was quickly excised en bloc and the hepatic hilus was removed. Liver tissue was dissected into 4-6 pieces of 200-250 mg (a liver from a 25 g mouse yields ~1.2 g of liver excluding large vessels and gall bladder) which were washed in sterile PBS and kept on ice. The liver pieces were homogenized using a pre-chilled dounce tissue grinder (Kontes 2 mL, Vineland, NJ, USA) and mitochondria were isolated (Pierce Mitochondria Isolation Kit for Tissue, Thermo Fisher Scientific, MA, USA). In brief, tissue samples were dounced with pestle A and B until low resistance (about 10 times each) before centrifugation at 700g and 4°C for 20 min to pellet nuclei which were stored on ice and later used to isolate primarily nuclear DNA. The supernatant was centrifuged at 3,000g and 4°C for 15 min, resuspended in 500 µL washing solution (50 % solution C from the aforementioned mitochondria isolation kit and 50 % MQ H_2O) before centrifugation at 12,000g and 4°C for 5 min, which was repeated three times. DNA was isolated from isolated mitochondria and crude nuclear fractions with a DNA isolation kit (DNeasy Blood & Tissue Kit, Qiagen) and eluted in 2 x 100 µL AE buffer. Isolated mtDNA was pooled as was nDNA, and the DNA was precipitated overnight at 4°C in 2 x volume 96 % ethanol and 1/10 final volume 3M sodium acetate pH 5.2. Precipitated DNA was centrifuged at ~10,000 rpm for 20 minutes at 4°C, washed with 70 % ethanol and centrifuged at ~10,000 rpm for 5 minutes at 4°C. Air-dried DNA pellets were resuspended in 110 µL AE buffer, of which 100 µL was fragmented on ice using a 30 kHz/50W ultrasonic sonicator (Hielscher UPH50, Hielscher Ultrasonics, Teltow, Germany) with a 0.5 mm head at 14 µm for 2 x 30 seconds.

The relative quantities of mitochondrial and nuclear DNA in the preparations was evaluated by qPCR using primers for murine MT-ND-1 (mitochondrially encoded NADH dehydrogenase for mitochondrial DNA and NDUFV1 (NADH dehydrogenase (ubiquinone) flavoprotein 1, for nuclear DNA (see table 1 for primer sequences).

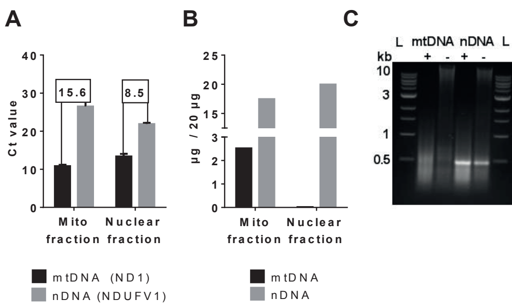

Figure 10. qPCR quality control of DNA agonists. The relative quantities of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) in the preparations were evaluated by qPCR using primers for murine MT-ND-1 (mitochondrially encoded NADH dehydrogenase 1) for mtDNA and NDUFV1 (NADH dehydrogenase (ubiquinone) flavoprotein 1) for nDNA. A; ct values for each gene in mitochondrial and nuclear fractions. B; from the equation DNA content (pg) = genome size (bp) / (0.978 x 10^9), the mass mtDNA and nDNA in our typical 20 µg/mL stimulation was estimated. C: fragmentation of sonicated (+) or non-sonicated (-) DNA was confirmed by 1 % agarose gel electrophoresis with a DNA ladder (L) and visualized with SYBR Safe.

The relative abundance of mtDNA and nDNA from isolated livers was quite stable, as were the absolute ct values. The mouse mitochondrial genome contains 16.295 kilobases[154] and its nuclear genome contains about 2.493 gigabases[155], making the latter 1.53 x 10^5 larger. DNA mass can be calculated from the number of base pairs[156].

$$DNA\ content\ (pg) = genome\ size\ (bp) / (0.978 \times 10^9)$$

From this equation, the mass of the mitochondrial genome is 1.67 x 10^{-6} pg and the nuclear 5.10 x 10^{-1} pg / genome. If the quantities of nDNA and mtDNA were equal in a sample as evaluated by real-time qPCR, mtDNA PCR products should outnumber nDNA by 1.53 x 10^5, or a ct value for mtDNA that is 17.2 ($\log_2$(1.53 x 10^5)) lower than for nDNA. We estimated the mass of mtDNA and nDNA in our typical 20 µg / ml stimulation. In the mitochondrial samples, the ratio of nDNA to mtDNA in this isolation was $2^{17.2-15.6} = 2^{1.62} =$ 3.08. This sample contains 3.08 times more nuclear DNA than mtDNA, which in 20 µg total DNA translates to 4.90 µg mtDNA and 15.10 µg nDNA. In the nuclear extracts, the ratio of nDNA to mtDNA was $2^{17.2-8.5} = 2^{8.7} =$ 423. This sample contains 423 times more nuclear DNA than mtDNA, which in 20 µg total DNA translates to 0.047 µg mtDNA and >19.95 µg nDNA.

The integrity of the isolated DNA, as well as degree of fragmentation after ultrasonification was visualized on a 1 % agarose (SeaKem, Lonza, Basel, Switzerland) gel visualized using a commercially available DNA dye (Life Technologies SYBR Safe, Thermo Fisher Scientific, Waltham, MA, USA). Fragmentation to 100-1000 bp was considered acceptable.

DNA preparations were tested by qPCR for the relative abundance of nuclear and mitochondrial DNA, and DNA fragmentation was verified by gel electrophoresis. Several methods of tissue homogenization and DNA isolation were tested, including bead mill and hand-held rotor homogenizer and MN-DNA isolation columns, but the combination of dounce homogenization and the Pierce mitochondria isolation kit for tissue and Qiagen DNeasy columns provided the best yield and purity. The first concern was to ensure reasonable yield of mitochondrial and nuclear DNA with minimal cross-contamination. mtDNA was isolated from isolated mitochondria, with additional washing steps to the manufacturer's protocol to increase purity. For nuclear DNA isolation, we used crude nuclear extracts obtained by centrifugation of tissue homogenate. A second concern was the possible contamination of non-DNA DAMPs or even PAMPs in the DNA preparations. Severe infection in donor animals is unlikely as organs were excised under clean conditions from animals that were housed in individually ventilated cages and fed autoclaved feed. Sentinel animals in the animal facility are routinely tested for infection. Further, DNA was isolated with spin column which should reduce carry-over of non-nucleic acids. Proteinase K was used and the samples were not RNAse-treated. However, incubation at 56°C for about 1 hour was performed as part of the DNA isolation protocol as well as preferential binding of DNA to the spin columns should reduce RNA contamination. Further, mtDNA and nDNA agonists were isolated from the same animals and in all experiments both mtDNA and nDNA were used, reducing possible impact from different contaminant concentrations in the two fractions. Preparations were tested for the presence of LPS retrospectively. Both liver and heart are rich sources of mtDNA, both containing about 3000 copies of the mitochondrial genome per cell[157], which show similar methylation patterns[157]. In our experience, one mouse liver yields around 1.0-1.2 g of tissue excluding the hepatic hilus, which corresponds to the mass of around five to ten mouse hearts excluding the great vessels[158]. In addition, the mitochondrial DNA yield from the soft liver tissue is much greater than the compact myocardium. Thus, the current study would have required several hundred mice for DNA extraction if we were to use the heart as a source. The liver was chosen for its large size, relatively simple homogenization and high number of mitochondria[157]. We used qPCR primers against sequences that appear only once in the nuclear or mitochondrial genome, respectively. Estimations of mass in our DNA preparations assume equal primer efficiencies (experimentally confirmed) and equal distribution of guanine, adenine, thymine and cytosine in the mitochondrial and nuclear genomes. Further, the estimation assumes that qPCR will detect two copies of NDUFV1 for each diploid nuclear genome.

We were unable to produce absolutely pure mitochondrial DNA isolations from mouse livers, but found it salient to use primary adult tissue from mice for extraction as a proof of concept. Around 75 per cent of the DNA in the mitochondrial fractions was nuclear. However, the difference in mitochondrial DNA in the

mitochondrial and nuclear fractions was >100-fold (4.90 versus 0.047 µg / ml). Observed differences in treatment with mtDNA and nDNA likely represent concentration dependent effects of mtDNA, even though the primary constituent of both preparations is nuclear DNA.

Isolated adult murine cardiomyocytes and cardiac fibroblasts

In papers II and III, primary cells from mouse hearts were used for several applications. Cardiomyocytes were isolated and cultured as previously described[159,160]. Under clean conditions, hearts from non-responsive pentobarbital-anesthetized mice were quickly excised, washed in ice cold perfusion buffer (containing in mmol/L: NaCl 120.4; KCl 14.7; KH_2PO_4 0.6; Na_2HPO_4 0.6; $MgSO_4$ 1.2; Na-HEPES liquid 10.0; glucose 5.5; $NaHCO_3$ 4.6; taurine 30.0; BDM 10.0) cannulated via the aorta and subjected to retrograde perfusion in a Langendorff apparatus heated to 37°C. Hearts were perfused for 4 minutes with buffer alone to purge blood before 12 minutes perfusion with perfusion buffer containing 1.3 mg/mL collagenase II (Worthington Biochemical, Lakewood, USA, batch #42B13273, 370 U/mg) to digest the extracellular matrix; the last 8 minutes in the presence of 40 µM $CaCl_2$. The digested ventricles were cut from the perfusion apparatus just apical to the valvular plane, mechanically disrupted and suspended in perfusion buffer containing 12.5 µM $CaCl_2$ and 10 % FBS (HyClone Bovine Calf Serum, cat. #SH30073.03, lot #AVA60491, Thermo Fisher Scientific, MA, USA). Cardiomyocytes were serially centrifuged at 20g for three minutes at room temperature, gradually reintroducing Ca^{2+} to a final concentration of 1.2 mM by resuspending the cardiomyocytes in FBS-supplemented perfusion buffer containing [Ca^{2+}] of 12.5, 100, 400 and 800 µM, respectively, before resuspension in plating medium containing endotoxin-tested MEM (#M5775, Sigma-Aldrich, St. Louis, MO, USA) with 10 % FBS, 10 mM BDM (2,3-Butanedione monoxime, #B0753; Sigma-Aldrich), 100 Units/mL penicillin G; (#P7794 Sigma-Aldrich) and 2 mM L-glutamine (#25030-024, Invitrogen, Carslbad, CA, USA). Cells were plated on six-well plates pre-coated with 1µg/cm^2 laminin (#354232; BD Biosciences, East Rutherford, NJ, USA). After 1-2 hours in equilibrated plating medium, the majority of viable cardiomyocytes had attached to the culture dish and plating medium was changed to short-term culture medium containing 0.10% BSA (#A8806 Sigma-Aldrich), 100 U/mL penicillin, 2 mM L-glutamine, and 1 mM BDM in MEM. All stimulations in our experiments were performed in short term medium.

Cardiomyocytes were stimulated in short-term medium with added sonicated mtDNA (20 µg / mL), nDNA (20 µg / mL) or volume-matched AE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9, Qiagen). Cardiomyocytes were washed once with PBS before addition of 350 µl RLT buffer (Qiagen), scraping with a rubber cell scraper (#353085, BD Falcon) before snap freezing in liquid nitrogen. The cells were stored in an ultra freezer at -80°C until RNA extraction.

Isolated cardiomyocytes is an important model in our group and the technique has been refined over several years. With experience, it is a reproducible and reliable model with wide applicability. The inclusion time to excise and hang hearts for isolation of cardiomyocytes in our lab is <4 minutes and perfusion buffer is pH adjusted, sterile filtered and prepared freshly the day of the experiments, as are all the cell culture media. Through gradual reintroduction of calcium, viability and purity of the cardiomyocyte fraction are improved. Though each isolation was not tested for purity, we routinely test the quality of isolation by qPCR for contamination from fibroblasts, endothelial cells or smooth muscle cells. For studies of the innate immune system, it is important to note the perfusion procedure is not sterile and it is difficult to categorically rule out contamination of the isolated cells. However, we make every effort to produce clean, uncontaminated and healthy cardiomyocytes: Animal health is monitored, instruments are autoclaved, all solutions sterile filtered, the perfusion rig is cleaned with MQ H_2O prior to each isolation and cleaned with MQ H_2O and 70 % ethanol after every isolation. Regularly, all tubing is replaced and glass autoclaved. Cell isolation and culture is performed in a cell-culture facility in a laminar air flow bench. Incubators are regularly autoclaved and cultured cells mycoplasma tested. In stimulations of isolated cardiomyocytes, the volume of DNA or AE solute was ~1 % of final volume, reducing the likelihood of non-specific dilution effects. The presence of DNases in the medium or supplements was not evaluated and exogenous DNases could have been used as an additional control.

To assess the purity of cardiomyocyte isolation, RNA was isolated using a commercially available kit (Rneasy Mini, Qiagen) according to the manufacturer's instructions and cDNA was reverse transcribed using a commercially available kit (Quanta qScript, Quanta Biosciences, Gaithersburg, MD, USA) and real-time qPCR using SYBR Green PCR chemistry (Applied Biosystems) conducted with primers for cardiomyocytes (Tnni3, troponin I, cardiac 3) fibroblasts (Vim, vimentin) endothelial cells (Pecam1/CD31) and smooth muscle cells (Acta2, α-2 smooth muscle actin). Please refer to table 1 for primer sequences. The purity obtained was in line with previous findings in our group and considered acceptable.

Cardiomyocyte viability assessment

Isolated cardiomyocytes were stimulated with DNA added 48, 24 and 4 0.5 hours before the staining protocol. Each ligand had its own time-matched control with short-term medium plus vehicle. Cells were stained with Hoechst, propidium iodide and MitoTracker deep red. The cells were visualized and quantified using an Olympus Scan^R imaging station with inbuilt software and equal automatic settings for all experiments. Images were taken at 25 fixed positions within each well using a 10X objective and a triple-band emission filter. Individual cardiomyocytes were detected based on MitoTracker deep red intensity after a Hoechst based

autofocus. Objects larger than one cardiomyocyte were automatically excluded from the analysis. The percentage of necrotic cells (propidium iodide positive cells divided by all cells) was calculated on the basis of a fixed threshold in each well.

The method has clear advantages. First of all, fully automated image acquisition allows quantification of a large number of cells. In our setup, 25 standardized images are obtained from each well, ensuring large statistical power. Moreover, image analysis is automated, based on standardized conditions set by the researcher which allows unbiased quantification. In many regards, the technique offers the advantages of flow cytometry, but because it is based on image analysis, the images that underlie the analysis can be viewed for verification of the automated analysis.

Laser-induced mitochondrial membrane depolarization

Cardiomyocytes were exposed to DNA agonists and incubated for 20 minutes in fresh short-term medium containing 1.25 μM tetramethylrhodamine methyl ester (TMRM) and 10 mM BDM[161] and live cells were imaged using confocal microscopy in a HEPES-buffered solution[162] with 10 mM BDM 37°C and 2% CO_2. TMRM accumulation in mitochondria is proportional to the mitochondrial membrane potential ($\Delta\Psi$) and the signal is lost with mitochondrial depolarization. There is concurrent ROS production and the technique indirectly measures ROS-induced ROS release[163]. Laser-induced loss of mitochondrial membrane models mitochondrial function and MPT in isolated cardiomyocytes. It is important to note, however, that this relatively crude method does not measure ROS directly. Approximately 10-14 pairs of mitochondria were analyzed by a person blinded to the experimental groups to ensure statistical power and objective analysis.

Uptake of fluorescent DNA

In papers II and III, uptake of fluorescent DNA was studied. In paper II, mtDNA and nDNA were sonicated and amplified by PCR[164] to incorporate fluorescent nucleotide triphosphates. DNA was cleaned by silica column purification. Commercially available fluorescent CpG B served as positive control and DNA-free PCR product with fluorescent dCTP served as negative control. Cells were incubated two hours or overnight and DNA uptake was evaluated by confocal microscopy. Though very brightly positive cells invariably showed rounded morphology, there was intracellular fluorescent signal in elongated, morphologically normal cardiomyocytes incubated with fluorescent mtDNA, nDNA and CpG, suggesting that cardiomyocytes are capable of internalizing extracellular DNA.

In paper III, we used fluorescent CpG C to investigate the internalization of extracellular DNA. CpG C was chosen as it is a less potent activator of NF-κB than CpG B. Primary cardiomyocytes or cultured cardiac

fibroblasts were incubated ~16 hours in 20 µg/mL CpG with or without 200 nM midkine and fluorescent images were acquired and quantified using an Olympus Scan^R imaging station with inbuilt software as outlined above. For cardiomyocytes, the average maximum fluorescence was used as a measure for CpG uptake and for cardiac fibroblasts; the average fluorescence per spot. The analysis settings were different because the number of spots was lower in the cardiomyocytes and not all cells contained fluorescent signal. Though the uptake of CpG by different cell types could not be appropriately analyzed by the same parameters, equal automated settings were applied within each cell type. Automated fluorescent imaging offers high power, but with the disadvantage of not separating between intracellular and extracellular fluorescent signal. Cells were incubated with CpG overnight as to maximize time for uptake and reduce the likelihood of detecting extracellular CpG. Further, wells were washed prior to fixation and only fluorescent signals that were within the perimeter of the cell were quantified. Further, intracellular presence of CpG was confirmed by confocal microscopy. The cell-surface nucleolin blocker midkine[134,165] was used. Any chemical inhibitor may have off-target effects that may directly or indirectly influence experimental outcomes. Midkine did not influence morphology or viability of cardiac cells in our experiments. It is important to note that midkine is a dual-function cytokine implicated in progression of inflammatory disease, such as experimental autoimmune encephalomyelitis[166], but with protective effects in both myocardial infarction[167] and in post-infarction heart failure[168,169]. For further dissection of the role of cell-surface nucleolin in the uptake of immunogenic DNA, it is salient to understand the role of midkine in that model system, or use a more targeted approach, such as genetic modification or a more specific inhibitor.

Mouse models for inflammation research

In this thesis, C57BL/6 mice have been used for several experiments, including DNA isolation from liver tissue, in vivo stimulation with extracellular DNA in NF-κB reporter mice, ex vivo heart perfusion and isolation of cardiomyocytes and cardiac fibroblasts. Mice are commonly used in all fields of biomedical research, including studies of innate immunity. Lately, mouse models have come under fire in inflammation research as significant discrepancies between the inflammatory responses in humans and mice were reported[170]. However, reanalysis of the same data resulted in the opposite conclusion[171] and inflammation researchers professed that the mouse is still a valid model[172]. The controversy over these recent data, however, serves as a reminder to resist the temptation of direct extrapolation of findings in animal models to human disease.

Human embryonic kidney 293 NF-κB luciferase reporter cells

Two different NF-κB reporter cell lines overexpressing TLR9 and matched TLR9 deficient controls were used. In paper II, HEK293 cells stably co-expressing human TLR9 and the luciferase reporter construct pELAM-luc

($HEK293^{TLR9pELAM}$) were used with HEK293 cells without the TLR9 as controls ($HEK293^{pELAM\text{-}luc}$)[173]. Cells were incubated six hours with normal medium supplemented with 10 % serum from patients with STEMI or angina pectoris and NF-κB luciferase reporter activity was quantified. mtDNA containing serum from STEMI patients induced TLR9 dependent NF-κB activity. The cells could have been treated with DNase to show that it was in fact DNA in the serum samples which induced NF-κB activity. Unfortunately, those experiments could not be performed due to scarcity of the clinical material. However, the observation that the NF-κB activity was TLR9 dependent is evidence of a DNA mediated effect. Further, it suggests that the samples were not contaminated by significant amounts of other PRR agonists, like LPS, which would have induced NF-κB activity independent of TLR9. Importantly, in vitro incubation with patient serum was performed shortly after the quantification of mtDNA in the same samples, so the risk of additional degradation was minimal. Further, as the study patients had modest infarctions, samples used for in vitro stimulation probably did not contain uncommonly high concentrations of mtDNA.

Human embryonic kidney 293 NF-κB SEAP reporter cells

Because the agonists used in paper III were all murine, an NF-κB reporter system with mouse TLR9 was used. HEK-Blue mTLR are commercially available HEK293 cells co-transfected with mouse TLR9 and an inducible embryonic alkaline phosphatase reporter coupled to NF-κB[174]. The cells were exposed to mtDNA, DNA and CpG and hypoxia / reoxygenation or normoxia. Though HEK293 is a cancer cell line and in many respects far from cardiomyocytes, it offers a relatively simple model to study loss-and-gain of function and concurrent reporter activity in living cells. As such, the model is a tool for molecular dissection of cellular phenomena. Cell lines of cardiac origin exist and the murine atrial tumor-derived HL-1 line[175] is among the most widely used. We have previously used HL-1 cells[176] but chose commercially HEK293 lines as the advantages of cardiomyocyte-like properties of HL-1 cells did not outweigh the disadvantages, such as loss of contractility with passaging and low genetic stability, in studies of cellular signal transduction. The effects of extracellular DNA on NF-κB reporter activity were not corroborated by experiments with DNase treatment to degrade DNA. However, the dependence on TLR9 was shown by inhibition at the receptor (CpGi) and endosomal processing (chloroquine) level as well as in cells lacking TLR9.

Quantitative polymerase chain reaction (qPCR)

qPCR was used in all the papers in this thesis. In paper II, qPCR was used to determine the mRNA expression of TLR9 in mouse hearts and isolated cardiomyocytes, cardiac fibroblasts and cultured cardiac fibroblasts and here, the data is presented as ct values. In paper III, the gene expression of nucleolin and inflammatory genes downstream of TLR9 activation was determined. Data is shown as relative expression ($2^{-\Delta\Delta ct}$) normalized to the endogenous control Rpl32 (60S ribosomal protein L32). As cells from each animal were seeded on separate

cell culture plates and the variation between the individuals was quite large, delta ct values were calibrated to normoxic control cells from each animal. Primers were designed to produce amplicons that span exon junctions where possible and tested for efficiency and primer dimerization. Water and cDNA reactions without reverse transcriptase were included.

Target	Species	Primer sequences	Accession number	Application
MT-ND1 Mitochondrially encoded NADH dehydrogenase 1	Human	(+) 5'-ATA CCC ATG GCC AAC CTC CT-3' (-) 5'-GGG CCT TTG CGT AGT TGT AT-3'	NC_012920.1	Quantify circulating mtDNA in patients in paper I
MT-CO3 Mitochondrially encoded cytochrome c oxidase III	Human	(+) 5'-ATG ACC CAC CAA TCA CAT GC-3' (-) 5'-ATC ACA TGG CTA GGC CGG AG -3'	NC_012920.1	Quantify circulating mtDNA in patients in paper I
18S Genomic 18 S	Human	(+) 5'-CGG CTA CCA CAT CCA AGG AA-3' (-) 5'-GCT GGA ATT ACC GCG GCT-3'	NR_003286	Quantify circulating nDNA in patients in paper I
mtDNA 117 bp amplicon	Mouse	(+) 5'-CCC AGC TAC TAC CAT CAT TCA AGT-3' (-) 5'-GAT GGT TTG GGA GAT TGG TTG ATG T-3'	NC-005089.1	Quantify mtDNA in coronary effluent from isolated mouse hearts in paper I
Tnni3 Troponin I, cardiac 3	Mouse	(+)-5'-GAG ATG GAA CGA GAG GCA GAA-3' (-)-5'-CGG CAT AAG TCC TGA AGC TCT T-3')	NM_009406.3	Assess purity of isolated cardiomyocytes (CM)
Vim Vimentin	Mouse	(+)-5'-CCC TGA ACC TGA GAG AAA CTA ACC-3' (-)-5'-GTC TCA TTG ATC ACC TGT CCA TCT-3')	NM_011701.4	Assess purity of isolated cardiac fibroblasts, contamination in CM fraction
Pecam1 platelet/endothelial cell adhesion molecule 1 / CD31	Mouse	(+)-5'-TCC CCG AAG CAG CAC TCT T-3' (-)-5'-ATG ACA ACC ACC GCA ATG AG-3'	NM_001032378.1	Assess contamination from endothelial cells in isolated CM and fibroblasts (CF)
Acta2 α-2 smooth muscle actin	Mouse	(+)-5'-TCC TGA CGC TGA AGT ATC CGA TA-3' (-)-5'GGT GCC AGA TCT TTT CCA TGT C-3'	NM_007392.3	Assess contamination from smooth muscle cells in isolated CM and CF
Tlr9 Toll-like receptor 9	Mouse	(+)-5'-CCT GGC ACA CAA TGA CAT TCA T-3' (-)-5'-GCT GAA GTC AAG AAA CCT CAC TGA-3'	NM_031178.2	Assess contamination from endothelial cells in isolated CM and CF
Rpl32 Ribosomal protein L32	Mouse	(+) 5'-TCG TCA AAA AGA GGA CCA AGA AG-3' (-) 5'-CCG CCA GTT TCG CTT AAT TT -3'	NM_172086.2	Housekeeping gene / endogenous control
Ifna1 Interferon alpha 1	Mouse	(+) 5'-CCT GAA CAT CTT CAC ATC AAA GGA-3' (-) 5'-AGC TGC TGG TGG AGG ATC AAA-3'	NM_010502.2	Quantify type 1 interferon response in paper III
Ifnb1 Interferon beta 1	Mouse	(+) 5'-GAA AGG ACG AAC ATT CGG AAA T-3' (-) 5'-CGT CAT CTC CAT AGG GAT CTT GA-3'	NM_010510.1	Quantify type 1 interferon response in paper III
Il1b Interleukin 1 beta	Mouse	(+) 5'-TGA CAG TGA TGA GAA TGA CCT GTT C-3' (-) 5'-GGA CAG CCC AGG TCA AAG G-3'	NM_008361.3	Quantify pro-inflammatory cytokine response in paper III
Il6 Interleukin 6	Mouse	(+) 5'-TTC CTC TCT GCA AGA GAC TTC CA-3' (-) 5'-GGG AGT GGT ATC CTC TGT GAA GTC-3'	NM_031168.1	Quantify pro-inflammatory cytokine response in paper III
Tnf Tumor necrosis factor (alpha)	Mouse	(+) 5'-GAC CCT CAC ACT CAG ATC ATC T-3' (-) 5'-TCC TCC ACT TGG TGG TTT GC-3'	NM_001278601.1	Quantify pro-inflammatory cytokine response in paper III
mt-Nd1 NADH dehydrogenase 1, mitochondrial	Mouse	(+) 5'-TCG ACC TGA CAG AAG GAG AAT CA-3' (-) 5'-GGG CCG GCT GCG TAT-3'	NC_005089.1	Quantify mtDNA content in DNA agonists
Ndufv1 NADH dehydrogenase (ubiquinone) flavoprotein 1	Mouse	(+) 5'-GAG CAG GAC TTC TCC TTC ACA TC-3' (-) 5'-CCC GTC TCA GGG CAC CTT-3'	NC_000085.6	Quantify nDNA content in DNA agonists
Ncl Nucleolin	Mouse	(+) 5'-AGC ACC TGG AAA ACG GAA GA-3' (-) 5'-GGT GTA GTT GGT TCT GAG CCT TCT A-3'	NM_010880.3	Quantify nucleolin expression in various cells and tissues in paper III

Table 1: Real-time qPCR primer sequences. All real-time qPCR primers were ordered for SYBR Green chemistry from Eurofins (Hamburg, Germany) at final concentrations of 10 pM, with the exception of Unc93b1 (accession number NM_001161428.1, not listed in the table as primer sequence is proprietary to Life Technologies) an inventoried Taqman 20x primer/probe set (#Mm00457643_m1, Life technologies)

Protein immunoblotting

In paper II, the protein expression of TLR9 was investigated by protein immunoblotting. Our main objective was to investigate the expression of TLR9 in cardiomyocytes and cardiac fibroblasts. Two different antibodies were used and LS-B756 (LifeSpan BioSciences, rabbit anti-human and –mouse polyclonal) proved best suited. Protein immunoblotting is a semi-quantitative technique which only detects relatively large differences in protein expression. There is potential for both false positive and negative findings. Therefore, both positive and negative controls were included in the form of HEK293 cells with or without the expression of murine TLR9.

The sensitivity of the band in the positive control was confirmed as its intensity increased when cells were treated with the TLR9 agonist CpG B and its specificity was confirmed by the absence of signal in protein lysates from $TLR9^{null}$ cells. Protein extracts from mice of the same strain with genetic deletion of the protein of interest are often used as negative controls. However, the most widely used TLR9 knock-out model expresses a c-terminal protein fragment[96] detectable by our antibody.

Protein immunoblotting was used to investigate the expression of nucleolin in paper III. To identify the sub-cellular localization of the protein we investigated pure sub-cellular fractions and showed by immunoblotting the presence of nucleolin on the membrane of cardiomyocytes, a finding also confirmed by immunocytochemistry. It has been reported that nucleolin protein of about 110 kDa appears to be cleaved during in vivo ischemia-reperfusion, as the intensity diminishes simultaneously with the appearance of a 80 kDa band[177], whereas we predominantly found a band of ~76 kDa in the membranes of cardiomyocytes. It has been shown in Jurkat cells and human T lymphocytes that glycosylation is important for directing nucleolin to the cell-surface, and that cell-surface nucleolin in these cell types has a weight of ~110 kDa[178,179]. However, as the expression of nucleolin in the membranes of cardiomyocytes has not previously been investigated, species differences, cell type differences as well as the use of different primary antibodies can account for high abundance of ~76 kDa nucleolin in cardiomyocyte membranes in our study.

Statistical analysis

GraphPad Prism 5.0 or 6.0 (GraphPad Software, CA, USA) was used for statistical analyses. Student's t-test, ANOVA or paired t-test was used, where appropriate. $p<0.05$ was considered statistically significant and $p\leq0.1$ was regarded a tendency. Significance is denoted by p value or by symbols where $*p<0.05$, $**p<0.01$, $***p<0.001$. Unless otherwise stated, data is presented as mean ± SEM.

Discussion

We have shown that the heart is the likely source of mtDNA released to the circulation after myocardial infarction[180], that mtDNA triggers TLR9 dependent NF-κB activation in two different cell lines and in vivo. Further, we have shown that non-lethal hypoxia/reoxygenation and TLR9 ligands synergistically activate NF-κB and that cardiomyocytes express pro-inflammatory cytokines in response to mtDNA and non-lethal hypoxia/reoxygenation. Finally, we have shown that nucleolin is expressed on cardiomyocyte membranes and that it might be important for internalization of immunogenic DNA.

Inhibition of post-infarction inflammation in acute myocardial infarction patients

It has long been known that acute myocardial infarction causes inflammatory changes in the myocardium[32]. The consensus model is that the myocardium contains relatively few resident leukocytes[181], and neutrophils infiltrate the myocardium within twenty-four hours and macrophages within seven days[2]. In our model of acute H/R in cardiomyocytes, we have modeled the inflammatory effects in the cardiomyocyte as the influx of inflammatory cells is sparse at this time point. The influx of innate immune cells to the post-ischemic myocardium is the organism's go-to response to initiate tissue repair. However, the processes that ensue were not evolutionarily honed to repair the broken heart[10]. Ischemic heart disease typically occurs after reproductive age and the response to sterile cardiac inflammation should therefore not be under evolutionary pressure. It is therefore a reasonable hypothesis that the intense innate response whose main function is to repair injured cells and protect against bacterial infections, could be excessive in the post-ischemic heart. As a corollary, modulation of the innate response could benefit clinical myocardial ischemia-reperfusion injury.

Glucocorticoids are powerful but non-specific anti-inflammatory drugs used for a wide range of acute and chronic inflammatory conditions[182]. On one hand, glucocorticoids should reduce the pro-inflammatory signaling after myocardial infarction, but at the same time blunting of this signaling could delay the normal healing process[31]. There are concerns of delayed healing and aneurism formation in patients treated with glucocorticoids following myocardial infarction[183]. A meta-analysis of 11 controlled trials including more than 2,600 patients showed a 26% decrease in mortality with acute corticosteroid treatment after acute myocardial infarction (odds ratio 0.74 with 95% confidence interval 0.59 to 0.94, $p<0.05$)[184]. However, chronic corticosteroid use for inflammatory disease might increase the risk of acute myocardial infarctions[185]. Moreover, high-dose i.p. methylprednisolone given to rats with transmural myocardial infarctions reduced the number of cardiomyocytes in the infarct area leading to a thinner ventricular wall three days post infarction[186]. Thus, untargeted disruption of the normal healing process in sterile inflammation following myocardial infarction might unwise and a more focused approach is warranted.

One more focused strategy that has been investigated is antibodies against integrins CD11 and CD18[10], which are instrumental for leukocyte adhesion and rolling[187]. This strategy was shown to reduce infarct size by 90 per cent in the cat[188], 50 per cent in the dog[189] and 40 per cent in the baboon[190]. However, the promising preclinical findings were lost in translation. In two studies of around 400 AMI patients, anti-CD18[191] or anti-CD11/CD18[192] antibodies failed to improve post-thrombolysis coronary blood flow or infarct size. In fact, there were indications of increased susceptibility to bacterial infections in both studies[191,192]. A smaller study at the same time showed no beneficial effects of anti-CD11/CD18 antibodies administered as an adjunct to primary PCI[193]. Similarly, the SELECT-ACS trial did not show benefit of inhibiting P-selectin[194], another leukocyte adhesion molecule shown to benefit experimental myocardial ischemia-reperfusion injury[195] and the FIRE trial showed no effect of inhibition of VE-cadherin[196]. Similarly, small studies targeting the complement system and matrix metalloproteinases have been disappointing[197]. Inhibition of interleukin 1 receptor improved post-infarction outcomes in preclinical studies, but in pilot patient studies using the IL1 receptor antagonist as an adjunct to PCI, results have been equivocal[198,199]. Chronic TNFα inhibition appears to reduce the incidence of AMI in patients with psoriasis[200] or rheumatoid arthritis[201] and reduces experimental ischemia-reperfusion injury by reducing post-infarction inflammation[202]. The acute effects of TNFα inhibition in AMI have not been subject to rigorous investigation. In a first-in-human trial of 26 NSTEMI patients, the TNFα inhibitor etanercept reduced post-infarction inflammatory response, but increased platelet-monocyte aggregation[203]. To date, no studies have evaluated functional endpoints of TNFα inhibition in AMI[197].

The track-record of translating preclinical studies of immune modulation during acute myocardial infarction into the clinical setting is poor. The earlier trials of anti-inflammatory strategies were studied as an adjunct to thrombolysis, and it is possible their effectiveness would be greater as an adjunct to PCI. Michel Ovize's group has demonstrated clinical benefit of cyclosporine in AMI[27,28], an inhibitor of mitochondrial permeability transition. Though this might be the mechanism of action, cyclosporine is foremost a post-transplantation anti-rejection drug, whose main function is to inhibit production of interleukin 2, instrumental for T cell activation[204]. As a side note, T cells have been shown to infiltrate the post-ischemic myocardium and to be important for the propagation of ischemia-reperfusion injury in a mouse model[30]. Despite the failures of clinical trials, the role of the post-infarction inflammatory response in myocardial ischemia-reperfusion injury remains a valid research question because some of the early clinical trials may have been premature. There is evidence for benefit of targeted approaches and the important basic science questions that remain independently legitimize further inquiry. Moreover, addressing the triggers of inflammation, such as mtDNA, represents a potent strategy for modulating the post-infarction inflammatory response.

Circulating cell-free nucleic acids are markers and makers of damage

During myocardial ischemia-reperfusion injury, cardiomyocytes die by necrosis, necroptosis, autophagy and apoptosis[205]. While the former is unregulated, passive process, the latter three are regulated and energetically demanding. Necroptosis was discovered relatively recently[206] and is a form of regulated necrosis[207]. Autophagy is a survival mechanism whereby damaged organelles can be recycled in controlled manner[208], but it can also contribute to cell death in the heart[209]. Apoptosis is a highly regulated form of programmed cell death[210]. Depending on the route of demise, the contents of a dead cell may be released to the extracellular space where it may come in contact with other, viable cells. The question then becomes if extracellular, or even circulating, cell-free nucleic acids are injury-inducing or merely the smoking gun in a battle that has died down?

On a cellular scale, the havoc created by acute myocardial infarction is likely to lead to the release of *all* intracellular components to the extracellular milieu. Large infarctions release the nucleic acids from a large number of cells, which may be detected in the circulation if the capacity of tissue and circulating DNases is exceeded. At least five studies have shown cell-free circulating DNA in acute myocardial infarction patients[211-215]. Our study was the first to report circulating mtDNA in patients, which was detectable in patients with relatively modest infarcts three hours after reperfusion[180]. Thus, circulating cell-free nucleic acids likely represent markers of damage. Other studies have used non-sequence specific methods to detect DNA[211,213-215], but we used qPCR which is both sensitive and specific. However, as mtDNA is not tissue specific, cell-free mtDNA can arise from any damaged tissue, or even be released from neutrophils[216,217]. Therefore, though circulating cell-free DNA or mtDNA are likely markers of damage they are non-specific to myocardial injury and may therefore be of limited diagnostic or prognostic value. As sequence-specific qPCR-based assays are relatively time-consuming, these are unlikely to compete with current ELISA-based technology for the detection of circulating myocardial proteins for the diagnosis of AMI. However, the quantification of post-infarction circulating, cardiac-enriched micro-RNAs[218] using specific point-of-care testing for nucleic acids[219] represents a possible avenue for DNA-based AMI diagnostics in the future. Moreover, what separates mtDNA from other circulating DNA is its capacity to also inflict damage on viable tissue.

Mitochondrial DNA is a damage-associated molecular pattern

Mitochondria are prokaryotic endosymbionts[220] whose DNA and proteins exhibit chemical and structural differences to nuclear DNA and genomically encoded proteins. For example, mtDNA resembles bacterial DNA, which contains pro-inflammatory unmethylated CpG motifs[221,222]. Because mitochondria occupy 25% of the volume of the human heart[223], severe tissue damage can release substantial amounts of mitochondrial components. Per cell, the heart contains more than ten times more mtDNA than the spleen, three times more than kidney and nearly twice that of the brain[157].

CpG DNA injected intra-articularly (i.a.) in mice was shown to recruit innate immune cells and cause TNFα mediated arthritic changes[224] and TLR9 was demonstrated as a receptor for CpG DNA[96]. CpG-containing mtDNA but not nDNA induced NF-κB-mediated TNFα release when injected i.a. in mice and oxidative damage to the mtDNA appeared to be important for its inflammatogenic properties[222]. Further, extracellular mtDNA was found in the synovial fluids[222] and serum[225] of patients suffering from rheumatoid arthritis. Interestingly, the pro-inflammatory effects of mtDNA were mediated by cells of the innate immune system and independent of B or T lymphocytes[222]. More recently, mtDNA has been shown to induce sterile inflammation through TLR9 in human neutrophils[43] and in pressure-overloaded heart failure in mice, undegraded mtDNA induces inflammation and worsens outcome after transaortic constriction[44]. We have shown that mtDNA fragmented mtDNA triggers NF-κB activation in a TLR9 dependent mechanism in an in vivo mouse model and in HEK293 cells and that fragmented mtDNA induces the transcription of pro-inflammatory cytokines in isolated adult murine cardiomyocytes during H/R. Collectively, there is convincing evidence that mtDNA is inflammatogenic and act as a DAMP.

However, the molecular patterns present in mitochondrial DNA that are responsible for its immunostimulatory properties are not fully clarified. Bacterial, but not vertebral DNA activates NK cells[226]. In bacterial DNA, the prevalence of CpG dinucleotides is as expected by chance, but in vertebral genomes the prevalence is about one quarter of predicted, a phenomenon dubbed CpG suppression[227]. B cells are stimulated by oligonucleotides that contain CpG dinucleotides, but not non-CpG oligonucleotides[228]. It appears that methylation of CpG motifs is of importance as methylation of bacterial DNA abolished its ability to stimulate B cells[228] and demethylation of murine genomic DNA increased immunostimulatory effects[229]. However, demethylation of genomic DNA did not increase the immunogenicity to the level of bacterial DNA, suggesting that CpG methylation alone does not account for the differences in bacterial and vertebral DNA[229]. Although unmethylated CpG dinucleotides probably contribute to the immunogenicity of mtDNA versus nDNA, there are most likely other contributing factors than CpG suppression and CpG methylation. It has been proposed that inhibitory motifs may play a role, which may be more common in nDNA[229]. DNA-protein interactions might also be important, as binding of CpG DNA to HMGB1[230,231] or mtDNA to TFAM[232] (mitochondrial transcription factor A) enhance the immunogenicity of mtDNA[230,232]. In our study, DNA was extracted using silicon columns which reduces the risk of contamination of other DAMPs or PAMPs, suggesting that the observed effects were mediated by mtDNA. Regardless of the quantitative impact of different molecular motifs on the immunogenicity, we show in paper II and paper III that mtDNA does indeed function as a DAMP, activating NF-κB via TLR9 in two different HEK293 lines, and that this activation does not occur

with nDNA. This supports the hypothesis that differences in structure or sequence or other properties between mtDNA and nDNA.

The abundance of mtDNA in the heart hints at importance for cardiac inflammation

As ischemia and reperfusion inevitably destroys myocardium, the post-ischemic cardiomyocyte is immersed in intra- and extracellular DAMPs including high-mobility group box 1[35,36], heat-shock proteins[37-40], adenosine triphosphate[41] and uric acid[42], all of which can activate PRRs. Also, polymorphonuclear leukocytes whose proteolytic enzymes directly damage cardiomyocytes quickly infiltrate the tissue[2]. It is therefore important to note that although this thesis is devoted to a single pathway from mtDNA via nucleolin to TLR9 to NF-κB and IRF to proinflammatory cytokines and type I interferons, there are many other DAMPs and many other receptors which contribute to the post-infarction inflammatory response. This thesis investigates only one pathway among several which contribute to post-infarction inflammation. However, the abundance of mtDNA in the heart underpins the importance of identifying its receptors and the impact of their activation on cardiac inflammation.

The cardiomyocyte as an immune cell

Under normal conditions the heart is less immunologically active than many other tissues which function to continuously survey for microorganisms or other signs of danger. The presence of innate immune receptors in the heart may represent vestigial receptors which have eluded evolutionary pressure. The heart can be exposed to exogenous triggers of innate immunity when normal defenses break down and to endogenous triggers when large-scale tissue damage occurs. For the human heart, the most common trigger of innate immunity is ischemia-reperfusion injury[233]. For a long time, the heart has been recognized as a target of immune reactions after infection[234] or infarction[32]. However, the heart might also play and active role in immunity[235]. The first prerequisite for the heart as an immunological organ is the expression of PRRs. The heart expresses TLRs mRNA for TLRs 1-10 with TLR4 and TLR2 as the most abundant[52]. The presence of TLRs would suggest that cardiomyocytes are capable of mounting a response to TLR ligands, and for ischemia-reperfusion[236,237] and LPS-induced shock[237], myocardial TLR4, not just that expressed in immune cells, is important for the immunological response[236,237]. Similarly for TLR9, stimulation with bacterial DNA or CpG rich DNA induces myocardial inflammation[55] and reduces cardiomyocyte contractility[55,107]. It has been shown that TLR9 activation by CpG does not cause prototypical pro-inflammatory signaling in normoxic neonatal rat cardiomyocytes, but rather induce an AMPK-dependent energy-conservation[119]. We hypothesized, however, that cardiomyocytes during normoxia respond differently to danger signals than during hypoxia-reoxygenation. In paper III, we show that the gene expression of the pro-inflammatory cytokines interleukin-1β, tumor-necrosis factor α and interferon α1 was upregulated by mtDNA, but not nDNA in cardiomyocytes exposed to

40 minutes of non-lethal hypoxia and two hours of reoxygenation, showing a clear pro-inflammatory phenotype to mtDNA when under cellular stress. Importantly, the immunological functions of the cardiomyocytes in situations of cellular stress are likely due to interaction with other cell types, including immune cells and cardiac fibroblasts, which may function as sentinel cells in the heart[108]

Cardiomyocytes internalize DAMPs

For ligands whose receptors reside inside the cell, ligand uptake is a prerequisite for activation. We have shown in papers II and III that cardiomyocytes internalize DNA, which has also been shown by other investigators in normal[55] and pathological circumstances[44,55]. However, the mechanism for internalization of DNA is unknown, as is its function. First, there might be a difference in the rate of internalization under normal and pathological situations – possibly the stressed cardiomyocyte is more likely to internalize extracellular material, which could be regulated at the level of the receptor or mechanism responsible for internalization. Second, the cardiomyocyte's capacity to respond to internalized DAMPs could also be context-specific. Under normal circumstances, DAMPs could signal low-grade danger and induce metabolic adaptation in the cardiomyocyte[119]. As we have shown in paper III, cardiomyocytes mount an inflammatory response to extracellular DNA when exposed to H/R but not under normal circumstances. Further, under chronic stress, DAMPs cause a maladaptive response in the cardiomyocyte[44]. Therefore, it is tempting to speculate that inhibition of internalization of DAMPs such as mtDNA is a viable strategy for reducing receptor activation.

Can cell-surface nucleolin be responsible for DNA internalization?

TLRs recognize nucleic acids in endosomes[238], but endosomal localization is probably not important for ligand recognition. Rather, compartmentalization appears to be a mechanism to avoid unwanted activation and probably autoimmunity as chimeric TLR9 expressed on the cell-surface recognizes self-DNA[239]. Although cell-surface TLR9 has been described[240], its primary location is in the endosome. Therefore, the ligand – CpG DNA or mtDNA – must be internalized for recognition. We initially hypothesized that endocytosis was responsible for the uptake of DNA, but found in paper III that two different inhibitors of endocytosis did not inhibit the uptake of fluorescent CpG in cardiomyocytes. Transmembrane diffusion and phagocytosis are unlikely. Other investigators have provided evidence of receptor-mediated endocytosis of CpG in B cells without implicating a specific receptor[241]. Candidates for RME include scavenger receptors, which are considered a PRR subclass by some[129]. RAGE has been implicated in CpG internalization[230], as have eight different RNA-binding proteins in microglia[242]. We investigated the role of another RNA-binding protein, nucleolin, in the internalization of DNA in cardiac cells. Nucleolin shuttles pre-RNAs from the nucleus to the cytoplasm[131,132] and facilitates the attachment of HIV-1 to the cell surface of $CD4^+$ T cells and is required for internalization of human parainfluenza virus type 3 to airway epithelial cells[135]. Cell-surface nucleolin is the

receptor for a 26-mer DNA oligonucleotide[136] and mediates the uptake of DNA nanoparticles[137]. We show in paper III that nucleolin is expressed in the membranes of cardiomyocytes and that its inhibition reduces the uptake of fluorescent CpG. Although we do not show direct evidence of CpG or mtDNA interaction with cell-surface nucleolin, it does represent a possible route for internalization of immunogenic DNA in cardiomyocytes and cardiac fibroblasts. It is important to note that midkine is a dual-function cytokine implicated in progression of inflammatory disease, such as experimental autoimmune encephalomyelitis[166], but with protective effects in both myocardial infarction[167] and in post-infarction heart failure[168,169]. It is tempting to speculate that one of the protective roles of midkine in these studies could be via reduced uptake of immunogenic mtDNA.

Concluding remarks

Experimental evidence in this thesis is in line with our main hypothesis that cellular debris from dead cardiomyocytes cause inflammation which contributes to post-ischemic damage to the heart. Specifically, the main findings were:

- Mitochondrial DNA is released from the post-ischemic human heart
- Mitochondrial mtDNA activates TLR9 and NF-κB and contributes to cardiomyocyte injury
- Cardiomyocytes mount an inflammatory response when exposed to mitochondrial DNA during H/R and cell-surface nucleolin may be responsible for internalization of DNA

If an experiment answers one question, two more arise. These, and more, will be the subject of future experiments in our group:

- Is mitochondrial DNA also released from the human heart during heart surgery?
- Is circulating mitochondrial DNA free or bound to proteins or packed in vesicles?
- Which parts of mitochondrial DNA are immunogenic?
- Are there other mitochondrial components that may drive inflammation if released?
- Does mitochondrial DNA cause more potent inflammation in men than in women?
- Which other receptors in the heart are important for extending post-ischemic cardiomyocyte injury?
- How do cardiomyocytes and other cell types interact during post-ischemic inflammation?
- How does cell-surface nucleolin internalize DNA?
- What are the end effects of the inflammatory response of cardiomyocytes?

The over-reaching aim of this line of research is to improve post-infarction outcomes for patients. Better understanding of the molecular mechanisms that drive the maladaptive post-ischemic inflammatory response in the heart will allow future therapy to strike the right balance between maladaptive inflammation and critical tissue repair. The abundance of mitochondria in the heart hints at the importance of their once-bacterial components as triggers of inflammation and as potential therapeutic targets.

References

1.Taylor, C. J., Roalfe, A. K., Iles, R. & Hobbs, F. D. R. Ten-year prognosis of heart failure in the community: follow-up data from the Echocardiographic Heart of England Screening (ECHOES) study. *Eur. J. Heart Fail.* **14,** 176-84-184 (2012).

2.Frangogiannis, N. G. The inflammatory response in myocardial injury, repair, and remodelling. *Nature reviews. Cardiology* **11,** 255-65 (2014).

3.Yellon, D. M. & Hausenloy, D. J. Myocardial reperfusion injury. *The New England Journal of Medicine* **357,** 1121-35 (2007).

4.Pedersen, A. G., Westby, G., Østevold, G., Kramer-Johansen, Ø. & Borgan, J. Causes of death 2012. Statistics Norway, Division for Health Statistics. (2013).

5.Mendis, S., Puska, P. & Norrving, B. *Global Atlas on cardiovascular disease prevention and control. World Health Organization*. (2011).

6.de Luca, G., Suryapranata, H., Ottervanger, J. P. & Antman, E. M. Time delay to treatment and mortality in primary angioplasty for acute myocardial infarction: every minute of delay counts. *Circulation* **109,** 1223-5 (2004).

7.Antman, E. M. *et al.* 2007 Focused Update of the ACC/AHA 2004 Guidelines for the Management of Patients With ST-Elevation Myocardial Infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines: developed in collaboration With the Canadian Cardiovascular Society endorsed by the American Academy of Family Physicians: 2007 Writing Group to Review New Evidence and Update the ACC/AHA 2004 Guidelines for the Management of Patients With ST-Elevation Myocardial Infarction, Writing on Behalf of the 2004 Writing Committee. *Circulation* **117,** 296-329 (2008).

8.Antman, E. M. *et al.* ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction--executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 1999 Guidelines for the Management of Patients With Acute Myocardial Infarction). *Circulation* **110,** 588-636 (2004).

9.Moran, A. E., Forouzanfar, M. H., Roth, G. & Mensah, G. A. The global burden of ischemic heart disease in 1990 and 2010: the Global Burden of Disease 2010 Study. *Circulation* (2014).

10.Christia, P. & Frangogiannis, N. G. Targeting inflammatory pathways in myocardial infarction. *European Journal of Clinical Investigation* **43,** 986-995 (2013).

11.Reimer, K. A., Lowe, J. E., Rasmussen, M. M. & Jennings, R. B. The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. *Circulation* **56,** 786-794 (1977).
12.Murry, C. E., Jennings, R. B. & Reimer, K. A. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* (1986).
13.Hausenloy, D. J. & Yellon, D. M. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *The Journal of Clinical Investigation* **123,** 92-100 (2013).
14.Zweier, J. L., Flaherty, J. T. & Weisfeldt, M. L. Direct measurement of free radical generation following reperfusion of ischemic myocardium. *Proceedings of the National Academy of Sciences* **84,** 1404-7-1407 (1987).
15.Zweier, J. L. *et al.* Measurement and characterization of postischemic free radical generation in the isolated perfused heart. *Journal of Biological Chemistry* **264,** 18890-5-18895 (1989).
16.Becker, L. B. New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovascular research* **61,** 461-470 (2004).
17.Miyamae, M., Camacho, S. A., Weiner, M. W. & Figueredo, V. M. Attenuation of postischemic reperfusion injury is related to prevention of [Ca2+]m overload in rat hearts. *Am. J. Physiol.* **271,** H2145-53 (1996).
18.Fujita, M. *et al.* Prolonged transient acidosis during early reperfusion contributes to the cardioprotective effects of postconditioning. *American Journal of Physiology - Heart and Circulatory Physiology* **292,** H2004-8 (2007).
19.Halestrap, A. P. A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. *Biochemical Society transactions* **38,** 841-860 (2010).
20.Lemasters, J. J., Theruvath, T. P., Zhong, Z. & Nieminen, A. Mitochondrial calcium and the permeability transition in cell death. *Biochimica et Biophysica Acta (…* **1787,** 1395-401 (2009).
21.Halestrap, A. P., Clarke, S. J. & Javadov, S. A. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. *Cardiovascular research* **61,** 372-85 (2004).
22.Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E. & Cai, J. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature* (2004).
23.Baines, C. P., Kaiser, R. A., Sheiko, T. & Craigen, W. J. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nature Cell Biology* (2007).
24.Sileikyte, J. *et al.* Regulation of the inner membrane mitochondrial permeability transition by the outer membrane translocator protein (peripheral benzodiazepine receptor). *J. Biol. Chem.* **286,** 1046-53 (2010).
25.Baines, C. P., Kaiser, R. A., Purcell, N. H., Blair, N. S. & Osinska, H. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* (2005).

26.Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* (2005).
27.Mewton, N. *et al.* Effect of cyclosporine on left ventricular remodeling after reperfused myocardial infarction. *J. Am. Coll. Cardiol.* **55,** 1200-5 (2010).
28.Piot, C. *et al.* Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *The New England Journal of Medicine* **359,** 473-81 (2008).
29.Parkin, J. & Cohen, B. An overview of the immune system. *The Lancet* (2001).
30.Yang, Z. *et al.* Myocardial infarct-sparing effect of adenosine A2A receptor activation is due to its action on CD4+ T lymphocytes. *Circulation* **114,** 2056-64 (2006).
31.Frangogiannis, N. G. Regulation of the inflammatory response in cardiac repair. *Circulation research* **110,** 159-73 (2012).
32.Mallory, G. K., White, P. D. & Salcedo, J. The speed of healing of myocardial infarction: a study of the pathologic anatomy in seventy-two cases. *American Heart Journal* **18,** 647-671 (1939).
33.Davidovich, P., Kearney, C. J. & Martin, S. J. Inflammatory outcomes of apoptosis, necrosis and necroptosis. *Biological chemistry* **395,** 1163-71 (2014).
34.Seong, S. & Matzinger, P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. *Nature Reviews Immunology* **4,** 469-78 (2004).
35.Andrassy, M. *et al.* High-mobility group box-1 in ischemia-reperfusion injury of the heart. *Circulation* **117,** 3216-26 (2008).
36.Ding, H. S. *et al.* The HMGB1–TLR4 axis contributes to myocardial ischemia/reperfusion injury via regulation of cardiomyocyte apoptosis. *Gene* (2013). doi:10.1016/j.gene.2013.05.041
37.Tian, J. *et al.* Extracellular HSP60 induces inflammation through activating and up-regulating TLRs in cardiomyocytes. *Cardiovascular research* **98,** 391-401 (2013).
38.Knowlton, A. A. & Gupta, S. HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. *American Journal of Physiology - Heart and Circulatory Physiology* **292,** H3052-6 (2007).
39.Dybdahl, B. *et al.* Myocardial ischaemia and the inflammatory response: release of heat shock protein 70 after myocardial infarction. *Heart* **91,** 299-304 (2005).
40.Zou, N. *et al.* Critical role of extracellular heat shock cognate protein 70 in the myocardial inflammatory response and cardiac dysfunction after global ischemia-reperfusion. *American Journal of Physiology - Heart and Circulatory Physiology* **294,** H2805-13 (2008).
41.la Sala, A. *et al.* Alerting and tuning the immune response by extracellular nucleotides. *Journal of Leukocyte Biology* **73,** 339-43 (2003).

42.Shi, Y., Evans, J. E. & Rock, K. L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425,** 516-21 (2003).
43.Zhang, Q. *et al.* Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* **464,** 104-7 (2010).
44.Oka, T. *et al.* Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature* **485,** 251-5 (2012).
45.Hansen, J. D., Vojtech, L. N. & Laing, K. J. Sensing disease and danger: a survey of vertebrate PRRs and their origins. *Dev. Comp. Immunol.* **35,** 886-97 (2011).
46.Takeuchi, O. & Akira, S. Pattern recognition receptors and inflammation. *Cell* (2010).
47.Anderson, K. V., Jürgens, G. & Nüsslein, C. Establishment of dorsal-ventral polarity in the Drosophila embryo: Genetic studies on the role of the Toll gene product. *Cell* (1985).
48.Lemaitre, B., Nicolas, E., Michaut, L. & Reichhart, J. M. The Dorsoventral Regulatory Gene Cassette spätzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults. *Cell* (1996).
49.Medzhitov, R. & Preston, P. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* (1997).
50.Kawai, T. & Akira, S. The roles of TLRs, RLRs and NLRs in pathogen recognition. *International immunology* **21,** 317-37 (2009).
51.Boyd, J. H., Mathur, S., Wang, Y., Bateman, R. M. & Walley, K. R. Toll-like receptor stimulation in cardiomyoctes decreases contractility and initiates an NF-kappaB dependent inflammatory response. *Cardiovascular research* **72,** 384-93 (2006).
52.Nishimura, M. & Naito, S. Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. *Biological and Pharmaceutical Bulletin* **28,** 886-92 (2005).
53.Frantz, S., Kelly, R. A. & Bourcier, T. Role of TLR-2 in the activation of nuclear factor kappaB by oxidative stress in cardiac myocytes. *Journal of Biological Chemistry* **276,** 5197-203-5203 (2000).
54.Frantz, S. *et al.* Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium. *The Journal of Clinical Investigation* **104,** 271-80-280 (1999).
55.Knuefermann, P. *et al.* Bacterial DNA induces myocardial inflammation and reduces cardiomyocyte contractility: role of toll-like receptor 9. *Cardiovascular research* **78,** 26-35 (2008).
56.O'Neill, L. & Bowie, A. G. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature Reviews Immunology* (2007).
57.Bell, J. K. *et al.* Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends Immunol.* **24,** 528-33 (2003).

58.Alberts, B. *et al. Molecular Biology of the Cell 4th edition The Compartmentalization of Cells. New York: Garland Science*. (2002).
59.Botos, I., Segal, D. M. & Davies, D. R. The structural biology of Toll-like receptors. *Structure* (2011).
60.Farhat, K. *et al.* Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *Journal of Leukocyte Biology* **83,** 692-701 (2007).
61.Janot, L. *et al.* CD14 works with toll-like receptor 2 to contribute to recognition and control of Listeria monocytogenes infection. *J. Infect. Dis.* **198,** 115-24 (2008).
62.Baumann, C. L. *et al.* CD14 is a coreceptor of Toll-like receptors 7 and 9. *The Journal of experimental medicine* **207,** 2689-701 (2010).
63.Raby, A. C., Holst, B., Le Bouder, E. & Diaz, C. Targeting the TLR Co-Receptor CD14 with TLR2-Derived Peptides Modulates Immune Responses to Pathogens. *Science translational …* (2013).
64.Nagai, Y., Shimazu, R., Ogata, H. & Akashi, S. Requirement for MD-1 in cell surface expression of RP105/CD180 and B-cell responsiveness to lipopolysaccharide. *Blood* **99,** 1699-1705 (2002).
65.Nagai, Y. *et al.* Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature immunology* **3,** 667-72 (2002).
66.Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011).
67.Akira, S. & Takeda, K. TLR signaling pathways. *Seminars in immunology* (2004).
68.Kawai, T. & Akira, S. TLR signaling. *Cell Death & Differentiation* (2006).
69.Lin, S. C., Lo, Y. C. & Wu, H. Helical assembly in the MyD88–IRAK4–IRAK2 complex in TLR/IL-1R signalling. *Nature* (2010).
70.Oganesyan, G., Saha, S. K., Guo, B., He, J. Q. & Shahangian, A. Critical role of TRAF3 in the Toll-like receptor-dependent and-independent antiviral response. *Nature* (2005).
71.Konno, H., Yamamoto, T., Yamazaki, K. & Gohda, J. TRAF6 establishes innate immune responses by activating NF-κB and IRF7 upon sensing cytosolic viral RNA and DNA. *PloS one* (2009).
72.Shinohara, M. L., Lu, L., Bu, J. & Werneck, M. Osteopontin expression is essential for interferon-α production by plasmacytoid dendritic cells. *Nature* (2006).
73.Takaesu, G. *et al.* TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. *Journal of molecular biology* **326,** 105-15 (2003).
74.Fraczek, J. *et al.* The kinase activity of IL-1 receptor-associated kinase 4 is required for interleukin-1 receptor/toll-like receptor-induced TAK1-dependent NFkappaB activation. *J. Biol. Chem.* **283,** 31697-705 (2008).
75.Gilmore, T. D. Introduction to NF-κB: players, pathways, perspectives. *Oncogene* (2006).

76.Müller, M. R. *et al.* Immunostimulation by the synthetic lipopeptide P3CSK4: TLR4-independent activation of the ERK1/2 signal transduction pathway in macrophages. ... *immunology* **103,** 49-60 (2001).
77.Ulevitch, R. J., Lee, J. & Mira-Arbibe, L. TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. *Journal of Leukocyte Biology* **68,** 909-15 (2000).
78.Arthur, J. & Ley, S. C. Mitogen-activated protein kinases in innate immunity. *Nature Reviews Immunology* (2013).
79.Saklatvala, J. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Current opinion in pharmacology* (2004).
80.Fitzgerald, K. A. *et al.* LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll adapters TRAM and TRIF. *The Journal of experimental medicine* (2003).
81.Pobezinskaya, Y. L., Kim, Y. S., Choksi, S. & Morgan, M. J. The function of TRADD in signaling through tumor necrosis factor receptor 1 and TRIF-dependent Toll-like receptors. *Nature* (2008).
82.Meylan, E. *et al.* RIP1 is an essential mediator of Toll-like receptor 3–induced NF-κB activation. *Nature immunology* **5,** 503-507 (2004).
83.Cusson-Hermance, N., Khurana, S., Lee, T. H., Fitzgerald, K. A. & Kelliher, M. A. Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. *J. Biol. Chem.* **280,** 36560-6 (2005).
84.Tatematsu, M. *et al.* A molecular mechanism for Toll-IL-1 receptor domain-containing adaptor molecule-1-mediated IRF-3 activation. *J. Biol. Chem.* **285,** 20128-36 (2010).
85.Fitzgerald, K. A. *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nature immunology* **4,** 491-6 (2003).
86.Kondo, T., Kawai, T. & Akira, S. Dissecting negative regulation of Toll-like receptor signaling. *Trends Immunol.* (2012).
87.Burns, K. *et al.* Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. *The Journal of experimental medicine* **197,** 263-8 (2003).
88.Brint, E. K. *et al.* ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nature immunology* **5,** 373 (2004).
89.Palsson-McDermott, E. M. *et al.* TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway. *Nature immunology* **10,** 579-86 (2009).
90.Kobayashi, K., Hernandez, L. D., Galán, J. E. & Janeway, C. A., Jr. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* (2002).

91.Zhang, G. & Ghosh, S. Negative regulation of toll-like receptor-mediated signaling by Tollip. *Journal of Biological Chemistry* (2002).
92.Kawagoe, T. *et al.* TANK is a negative regulator of Toll-like receptor signaling and is critical for the prevention of autoimmune nephritis. *Nature immunology* **10,** 965-72 (2009).
93.Brinkmann, M. M. *et al.* The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. *The Journal of Cell Biology* **177,** 265-75 (2007).
94.Latz, E. *et al.* TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature immunology* **5,** 190-8 (2004).
95.Leifer, C. A. *et al.* TLR9 is localized in the endoplasmic reticulum prior to stimulation. *J. Immunol.* **173,** 1179-83 (2004).
96.Kawai, T. *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* **408,** 740-5 (2000).
97.Boehme, K. W. & Compton, T. Innate sensing of viruses by toll-like receptors. *Journal of virology* (2004).
98.Parroche, P. *et al.* Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 1919-24 (2007).
99.Collins, B. & Wilson, I. A. Crystal structure of the C-terminal domain of mouse TLR9. *Proteins* **82,** 2874-8 (2014).
100.Valen, G. Innate immunity and remodelling. *Heart failure reviews* **16,** 71-8 (2010).
101.Leifer, C. A., Rose, W. A. & Botelho, F. Traditional biochemical assays for studying Toll-like receptor 9. *J Immunoass Immunochem* (2013).
102.Vollmer, J. & Krieg, A. M. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Advanced drug delivery reviews* **61,** 195-204 (2009).
103.Krieg, A. M. Therapeutic potential of Toll-like receptor 9 activation. *Nature reviews Drug discovery* (2006).
104.Haas, T. *et al.* The DNA sugar backbone 2′ deoxyribose determines toll-like receptor 9 activation. *Immunity* (2008).
105.Kawai, T. & Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology* **11,** 373-84 (2010).
106.Riad, A. *et al.* Myeloid differentiation factor-88 contributes to TLR9-mediated modulation of acute coxsackievirus B3-induced myocarditis in vivo. *American Journal of Physiology - Heart and Circulatory Physiology* **298,** H2024-31 (2010).
107.Bulicheva, N. *et al.* Effect of cell-free DNA of patients with cardiomyopathy and rDNA on the frequency of contraction of electrically paced neonatal rat ventricular myocytes in culture. *Annals of the New York Academy of Sciences* **1137,** 273-7 (2008).

108.Ohm, I. K. *et al.* Toll-Like Receptor 9 Mediated Responses in Cardiac Fibroblasts. *PloS one* **9,** e104398 (2014).
109.Markowski, P., Boehm, O., Goelz, L. & Haesner. Pre-conditioning with synthetic CpG-oligonucleotides attenuates myocardial ischemia/reperfusion injury via IL-10 up-regulation. *Basic Res Cardiol* (2013).
110.Cao, Z. *et al.* CpG-ODN, the TLR9 agonist, attenuates myocardial ischemia/reperfusion injury: involving activation of PI3K/Akt signaling. *Biochimica et Biophysica Acta (…* **1832,** 96-104 (2012).
111.Ohm, I. K. *et al.* Toll-like receptor 9-activation during onset of myocardial ischemia does not influence infarct extension. *PloS one* **9,** e104407 (2014).
112.Boehm, O., Markowski, P. & van der Giet, M. In vivo TLR9 inhibition attenuates CpG-induced myocardial dysfunction. *Mediators of …* (2013).
113.Lohner, R., Schwederski, M., Narath, C. & Klein, J. Toll-Like Receptor 9 Promotes Cardiac Inflammation and Heart Failure during Polymicrobial Sepsis. *of inflammation* (2013).
114.Gao, M. *et al.* The Toll-like receptor 9 ligand, CpG oligodeoxynucleotide, attenuates cardiac dysfunction in polymicrobial sepsis, involving activation of both phosphoinositide 3 kinase/Akt and extracellular-signal-related kinase signaling. *J. Infect. Dis.* **207,** 1471-9-1479 (2013).
115.Zhang, X. *et al.* The toll-like receptor 9 agonist, CpG-oligodeoxynucleotide 1826, ameliorates cardiac dysfunction after trauma-hemorrhage. *Shock* **38,** 146-52 (2012).
116.Mathur, S., Walley, K. R. & Boyd, J. H. The Toll-like receptor 9 ligand CPG-C attenuates acute inflammatory cardiac dysfunction. *Shock* (2011).
117.Velten, M., Duerr, G. & Pessies, T. Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice. *Cardiovascular* (2012).
118.Yang, L. *et al.* CpG-ODN attenuates pathological cardiac hypertrophy and heart failure by activation of PI3Kα-Akt signaling. *PloS one* **8,** e62373 (2013).
119.Shintani, Y. *et al.* TLR9 mediates cellular protection by modulating energy metabolism in cardiomyocytes and neurons. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 5109-14 (2013).
120.Paludan, S. R. & Bowie, A. G. Immune sensing of DNA. *Immunity* **38,** 870-80 (2013).
121.Takaoka, A. *et al.* DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **448,** 501-5 (2007).
122.Chiu, Y., Macmillan, J. B. & Chen, Z. J. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* **138,** 576-91 (2009).
123.Kim, T. *et al.* Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells. *Proceedings of the National Academy of Sciences* **107,** 15181-6-15186 (2010).

124.Unterholzner, L. *et al.* IFI16 is an innate immune sensor for intracellular DNA. *Nature immunology* **11,** 997-1004 (2010).
125.Hornung, V. *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* **458,** 514-8 (2009).
126.Burdette, D. L. & Vance, R. E. STING and the innate immune response to nucleic acids in the cytosol. *Nature immunology* (2013).
127.Dutta, D. & Donaldson, J. G. Search for inhibitors of endocytosis: Intended specificity and unintended consequences. *Cell Logist* **2,** 203-208 (2013).
128.Nakai, D. *et al.* Cellular uptake mechanism for oligonucleotides: involvement of endocytosis in the uptake of phosphodiester oligonucleotides by a human colorectal adenocarcinoma cell line, HCT-15. *J. Pharmacol. Exp. Ther.* **278,** 1362-72 (1996).
129.Canton, J., Neculai, D. & Grinstein, S. Scavenger receptors in homeostasis and immunity. *Nature Reviews Immunology* **13,** 621-34 (2013).
130.Zhu, F. G., Reich, C. F. & Pisetsky, D. S. The role of the macrophage scavenger receptor in immune stimulation by bacterial DNA and synthetic oligonucleotides. ... *immunology* **103,** 226-34 (2001).
131.Tajrishi, M. M., Tuteja, R. & Tuteja, N. Nucleolin: The most abundant multifunctional phosphoprotein of nucleolus. *Communicative & Integrative Biology* **4,** 267-75 (2011).
132.Medina, F. & González-Camacho, F. Nucleolin, a major conserved multifunctional nucleolar phosphoprotein of proliferating cells. *Journal of Applied Biomedicine* **8,** 141-150 (2010).
133.Fujiki, H., Watanabe, T. & Suganuma, M. Cell-surface nucleolin acts as a central mediator for carcinogenic, anti-carcinogenic, and disease-related ligands. *J. Cancer Res. Clin. Oncol.* **140,** 689-99 (2014).
134.Said, E. A. *et al.* The anti-HIV cytokine midkine binds the cell surface-expressed nucleolin as a low affinity receptor. *J. Biol. Chem.* **277,** 37492-502 (2002).
135.Bose, S., Basu, M. & Banerjee, A. K. Role of nucleolin in human parainfluenza virus type 3 infection of human lung epithelial cells. *Journal of virology* **78,** 8146-58 (2004).
136.Soundararajan, S. *et al.* Plasma membrane nucleolin is a receptor for the anticancer aptamer AS1411 in MV4-11 leukemia cells. *Mol. Pharmacol.* **76,** 984-91 (2009).
137.Chen, X., Kube, D. M., Cooper, M. J. & Davis, P. B. Cell surface nucleolin serves as receptor for DNA nanoparticles composed of pegylated polylysine and DNA. *Mol. Ther.* **16,** 333-42 (2007).
138.Ribes, S. *et al.* Intraperitoneal prophylaxis with CpG oligodeoxynucleotides protects neutropenic mice against intracerebral Escherichia coli K1 infection. *J Neuroinflammation* **11,** 14 (2014).

139.Grøgaard, H. K., Hoffmann, P., Arnesen, H. & Seljeflot, I. Inflammatory responses after percutaneous coronary intervention in patients with acute myocardial infarction or stable angina pectoris. *Scand. J. Clin. Lab. Invest.* **68,** 555-62 (2008).
140.Chiu, R., Chan, L., Lam, N. & Tsui, N. Quantitative Analysis of Circulating Mitochondrial DNA in Plasma. *Clinical Chemistry* **49,** 719-726 (2003).
141.Hoffmann, P. *et al.* Myocardial perfusion in ST-elevation myocardial infarction treated successfully with primary angioplasty. *Scand. Cardiovasc. J.* **40,** 96-104 (2006).
142.Bliksøen, M. *et al.* Mitochondrial DNA damage and repair during ischemia-reperfusion injury of the heart. *Journal of molecular and cellular …* **78,** 9-22 (2014).
143.Bell, R. M., Mocanu, M. M. & Yellon, D. M. Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *Journal of molecular and cellular …* **50,** 940-50 (2011).
144.Stensløkken, K. O., Rutkovskiy, A. & Kaljusto, M. L. Inadvertent phosphorylation of survival kinases in isolated perfused hearts: a word of caution. *Basic Res Cardiol* (2009).
145.Carlsen, H., Moskaug, J. Ø., Fromm, S. H. & Blomhoff, R. In vivo imaging of NF-kappa B activity. *J. Immunol.* **168,** 1441-6 (2002).
146.Carlsen, H., Alexander, G., Austenaa, L. M. I., Ebihara, K. & Blomhoff, R. Molecular imaging of the transcription factor NF-kappaB, a primary regulator of stress response. *Mutation Research/ …* **551,** 199-211 (2004).
147.Shang, L., Ananthakrishnan, R., Li, Q. & Quadri, N. RAGE modulates hypoxia/reoxygenation injury in adult murine cardiomyocytes via JNK and GSK-3β signaling pathways. *PloS one* (2010).
148.Das, A., Xi, L. & Kukreja, R. C. Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. *J. Biol. Chem.* **283,** 29572-85 (2008).
149.Das, A., Xi, L. & Kukreja, R. C. Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. *J. Biol. Chem.* **280,** 12944-55 (2005).
150.Patel, H. H., Tsutsumi, Y. M., Head, B. P. & Niesman, I. R. Mechanisms of cardiac protection from ischemia/reperfusion injury: a role for caveolae and caveolin-1. *The FASEB Journal* **21,** 1565-1574 (2007).
151.Papanicolaou, K. N. *et al.* Mitofusin-2 maintains mitochondrial structure and contributes to stress-induced permeability transition in cardiac myocytes. *Molecular and Cellular Biology* **31,** 1309-28 (2011).
152.Tao, R., Zhang, J., Vessey, D. A., Honbo, N. & Karliner, J. S. Deletion of the sphingosine kinase-1 gene influences cell fate during hypoxia and glucose deprivation in adult mouse cardiomyocytes. *Cardiovascular research* **74,** 56-63 (2007).

153.Zhang, J. *et al.* Signals from type 1 sphingosine 1-phosphate receptors enhance adult mouse cardiac myocyte survival during hypoxia. *American Journal of Physiology - Heart and Circulatory Physiology* **293,** H3150-H3158 (2007).
154.Bibb, M. J., Van Etten, R. A., Wright, C. T. & Walberg, M. W. Sequence and gene organization of mouse mitochondrial DNA. *Cell* **26,** 167-180 (1981).
155.Chinwalla, A. T., Cook, L. L., Delehaunty, K. D. & Fewell, G. A. Initial sequencing and comparative analysis of the mouse genome. *Nature* (2002).
156.Dolezel, J., Bartos, J., Voglmayr, H. & Greilhuber, J. Nuclear DNA content and genome size of trout and human. *Cytometry A* **51,** (2003).
157.Kelly, R., Mahmud, A. & McKenzie, M. Mitochondrial DNA copy number is regulated in a tissue specific manner by DNA methylation of the nuclear-encoded DNA polymerase gamma A. *Nucleic acids* ... (2012).
158.Doevendans, P. A., Daemen, M. J., de Muinck, E. D. & Smits, J. F. Cardiovascular phenotyping in mice. *Cardiovascular research* **39,** 34-49 (1998).
159.O'Connell, T. D., Rodrigo, M. C. & Simpson, P. C. Isolation and culture of adult mouse cardiac myocytes. *Methods Mol. Biol.* **357,** 271-96 (2006).
160.Lei, P. *et al.* Activation of Liver X receptors in the heart leads to accumulation of intracellular lipids and attenuation of ischemia-reperfusion injury. *Basic Res Cardiol* **108,** 323 (2013).
161.Zorov, D. B., Juhaszova, M. & Sollott, S. J. Mitochondrial ROS-induced ROS release: an update and review. *Biochimica et Biophysica Acta (...* **1757,** 509-17 (2006).
162.Johansen, D. *et al.* Heptanol triggers cardioprotection via mitochondrial mechanisms and mitochondrial potassium channel opening in rat hearts. *Acta Physiologica* **201,** 435-444 (2011).
163.Zorov, D. B., Filburn, C. R., Klotz, L. O. & Zweier, J. L. Reactive oxygen species (Ros-Induced) Ros release a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *The Journal of experimental medicine* (2000).
164.Rigby, P., Dieckmann, M., Rhodes, C. & Berg, P. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *Journal of molecular biology* (1977).
165.Hovanessian, A. G. Midkine, a cytokine that inhibits HIV infection by binding to the cell surface expressed nucleolin. *Cell Res.* **16,** 174-81 (2006).
166.Wang, J. *et al.* Inhibition of midkine alleviates experimental autoimmune encephalomyelitis through the expansion of regulatory T cell population. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 3915-20 (2008).
167.Horiba, M. *et al.* Midkine plays a protective role against cardiac ischemia/reperfusion injury through a reduction of apoptotic reaction. *Circulation* **114,** 1713-20 (2006).

168.Takenaka, H. *et al.* Midkine prevents ventricular remodeling and improves long-term survival after myocardial infarction. *American Journal of Physiology - Heart and Circulatory Physiology* **296,** H462-9 (2008).
169.Fukui, S. *et al.* Therapeutic effect of midkine on cardiac remodeling in infarcted rat hearts. *Ann. Thorac. Surg.* **85,** 562-70 (2008).
170.Seok, J. *et al.* Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci. U.S.A.* **110,** 3507-12 (2013).
171.Takao, K. & Miyakawa, T. Genomic responses in mouse models greatly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci. U.S.A.* **112,** 1167-72 (2014).
172.Osuchowski, M. F., Remick, D. G., Lederer, J. A. & Lang, C. H. Abandon the Mouse Research Ship? Not Just Yet! *Shock* (2014).
173.Latz, E., Verma, A., Visintin, A., Gong, M. & Sirois, C. M. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. *Nature* (2007).
174.Kandimalla, E. R. *et al.* Design, synthesis and biological evaluation of novel antagonist compounds of Toll-like receptors 7, 8 and 9. *Nucleic Acids Research* **41,** 3947-3961 (2013).
175.Claycomb, W. C. *et al.* HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 2979-84 (1998).
176.Czibik, G. *et al.* Cardioprotection by hypoxia-inducible factor 1 alpha transfection in skeletal muscle is dependent on heme oxygenase activity in mice. *Cardiovascular research* (2009). doi:10.1093/cvr/cvp035
177.Jiang, B. *et al.* Nucleolin protects the heart from ischaemia-reperfusion injury by up-regulating heat shock protein 32. *Cardiovascular research* **99,** 92-101 (2013).
178.Losfeld, M. *et al.* N-Glycosylation influences the structure and self-association abilities of recombinant nucleolin. *FEBS J.* **278,** 2552-64-2564 (2011).
179.Losfeld, M. *et al.* The cell surface expressed nucleolin is a glycoprotein that triggers calcium entry into mammalian cells. *Experimental cell research* **315,** 357-69 (2008).
180.Bliksøen, M. *et al.* Increased circulating mitochondrial DNA after myocardial infarction. *International journal of cardiology* **158,** 132-4 (2012).
181.Kumar, V., Abbas, A. K., Fausto, N., Robbins, S. L. & Cotran, R. S. *Pathologic basis of disease. Philadelphia: Elsevier Saunders*
. (2005).
182.Rhen, T. & Cidlowski, J. A. Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *The New England Journal of Medicine* **353,** 1711-23 (2005).

183.Bulkley, B. H. & Roberts, W. C. Steroid therapy during acute myocardial infarction. A cause of delayed healing and of ventricular aneurysm. *Am. J. Med.* **56,** 244-50 (1974).

184.Giugliano, G. R., Giugliano, R. P., Gibson, C. M. & Kuntz, R. E. Meta-analysis of corticosteroid treatment in acute myocardial infarction. *Am. J. Cardiol.* **91,** 1055-9 (2003).

185.Varas-Lorenzo, C., Rodriguez, L. A. G., Maguire, A., Castellsague, J. & Perez-Gutthann, S. Use of oral corticosteroids and the risk of acute myocardial infarction. *Atherosclerosis* **192,** 376-83 (2006).

186.Mannisi, J. A., Weisman, H. F., Bush, D. E., Dudeck, P. & Healy, B. Steroid administration after myocardial infarction promotes early infarct expansion. A study in the rat. *The Journal of Clinical Investigation* **79,** 1431-9-1439 (1987).

187.Albelda, S. M., Smith, C. W. & Ward, P. A. Adhesion molecules and inflammatory injury. *The FASEB Journal* **8,** 504-12-512 (1994).

188.Ma, X. L., Tsao, P. S. & Lefer, A. M. Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. *The Journal of Clinical Investigation* **88,** 1237-43-1243 (1991).

189.Arai, M. *et al.* An anti-CD18 antibody limits infarct size and preserves left ventricular function in dogs with ischemia and 48-hour reperfusion. *J. Am. Coll. Cardiol.* **27,** 1278-85 (1996).

190.Aversano, T., Zhou, W., Nedelman, M., Nakada, M. & Weisman, H. A chimeric IgG4 monoclonal antibody directed against CD18 reduces infarct size in a primate model of myocardial ischemia and reperfusion. *J. Am. Coll. Cardiol.* **25,** 781-8 (1995).

191.Baran, K. W. *et al.* Double-blind, randomized trial of an anti-CD18 antibody in conjunction with recombinant tissue plasminogen activator for acute myocardial infarction: limitation of myocardial infarction following thrombolysis in acute myocardial infarction (LIMIT AMI) study. *Circulation* **104,** 2778-83-2783 (2001).

192.Faxon, D. P., Gibbons, R. J., Chronos, N. A. F., Gurbel, P. A. & Sheehan, F. The effect of blockade of the CD11/CD18 integrin receptor on infarct size in patients with acute myocardial infarction treated with direct angioplasty: the results of the HALT-MI study. *J. Am. Coll. Cardiol.* **40,** 1199-204 (2002).

193.Rusnak, J. M. *et al.* An anti-CD11/CD18 monoclonal antibody in patients with acute myocardial infarction having percutaneous transluminal coronary angioplasty (the FESTIVAL study). *Am. J. Cardiol.* **88,** 482-7 (2001).

194.Tardif, J. *et al.* Effects of the P-selectin antagonist inclacumab on myocardial damage after percutaneous coronary intervention for non-ST-segment elevation myocardial infarction: results of the SELECT-ACS trial. *J. Am. Coll. Cardiol.* **61,** 2048-55 (2013).

195.Wang, K. *et al.* Recombinant soluble P-selectin glycoprotein ligand-Ig (rPSGL-Ig) attenuates infarct size and myeloperoxidase activity in a canine model of ischemia-reperfusion. *Thromb. Haemost.* **88,** 149-54 (2002).
196.Atar, D. *et al.* Effect of intravenous FX06 as an adjunct to primary percutaneous coronary intervention for acute ST-segment elevation myocardial infarction results of the F.I.R.E. (Efficacy of FX06 in the Prevention of Myocardial Reperfusion Injury) trial. *J. Am. Coll. Cardiol.* **53,** 720-9 (2009).
197.Seropian, I. M., Toldo, S., Van Tassell, B. W. & Abbate, A. Anti-inflammatory strategies for ventricular remodeling following ST-segment elevation acute myocardial infarction. *J. Am. Coll. Cardiol.* **63,** 1593-603 (2014).
198.Abbate, A. *et al.* 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,** 1394-400 (2013).
199.Abbate, A. *et al.* 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.e1 (2010).
200.Wu, J. J., Poon, K. T., Channual, J. C. & Shen, A. Y. Association between tumor necrosis factor inhibitor therapy and myocardial infarction risk in patients with psoriasis. *Arch Dermatol* **148,** 1244-50-1250 (2012).
201.Roubille, C. *et al.* The effects of tumour necrosis factor inhibitors, methotrexate, non-steroidal anti-inflammatory drugs and corticosteroids on cardiovascular events in rheumatoid arthritis, psoriasis and psoriatic arthritis: a systematic review and meta-analysis. *Ann. Rheum. Dis.* annrheumdis-2014-206624 (2015). doi:10.1136/annrheumdis-2014-206624
202.Yang, M., Chen, J., Zhao, J. & Meng, M. Etanercept attenuates myocardial ischemia/reperfusion injury by decreasing inflammation and oxidative stress. *PloS one* **9,** e108024 (2014).
203.Padfield, G. J. *et al.* Cardiovascular effects of tumour necrosis factor α antagonism in patients with acute myocardial infarction: a first in human study. *Heart* **99,** 1330-5-1335 (2013).
204.Matsuda, S. & Koyasu, S. Mechanisms of action of cyclosporine. *Immunopharmacology* **47,** 119-25 (2000).
205.Smith, C. C. T. & Yellon, D. M. Necroptosis, necrostatins and tissue injury. *Journal of Cellular and Molecular Medicine* **15,** 1797-806-1806 (2011).
206.Holler, N. *et al.* Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nature immunology* **1,** 489-95 (2001).
207.Zhou, W. & Yuan, J. Necroptosis in health and diseases. *Semin. Cell Dev. Biol.* **35,** 14-23 (2014).

208.Levine, B., Mizushima, N. & Virgin, H. W. Autophagy in immunity and inflammation. *Nature* **469,** 323 (2011).
209.Matsui, Y. *et al.* Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circulation research* **100,** 914-22-922 (2007).
210.Elmore, S. Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology* **35,** 495-516 (2007).
211.Chang, C. P. *et al.* Elevated cell-free serum DNA detected in patients with myocardial infarction. *Clin. Chim. Acta* **327,** 95-101 (2002).
212.Antonatos, D., Patsilinakos, S., Spanodimos, S., Korkonikitas, P. & Tsigas, D. Cell-free DNA levels as a prognostic marker in acute myocardial infarction. *Annals of the New York Academy of Sciences* **1075,** 278-81-281 (2006).
213.Jing, R. *et al.* A sensitive method to quantify human cell-free circulating DNA in blood: relevance to myocardial infarction screening. *Clin. Biochem.* **44,** 1074-9 (2011).
214.Cui, M. *et al.* Cell-Free circulating DNA: a new biomarker for the acute coronary syndrome. *Cardiology* **124,** 76-84 (2013).
215.Shimony, A. *et al.* Cell free DNA detected by a novel method in acute ST-elevation myocardial infarction patients. *Acute Card Care* **12,** 109-11 (2010).
216.Brinkmann, V. *et al.* Neutrophil Extracellular Traps Kill Bacteria. *Science* **303,** 1532-1535 (2004).
217.Yousefi, S., Mihalache, C., Kozlowski, E., Schmid, I. & Simon, H. U. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death & Differentiation* **16,** 1438-44 (2009).
218.Cheng, C., Wang, Q., You, W., Chen, M. & Xia, J. MiRNAs as biomarkers of myocardial infarction: a meta-analysis. *PloS one* **9,** e88566 (2014).
219.Pernagallo, S. *et al.* Novel biochip platform for nucleic acid analysis. *Sensors (Basel)* **12,** 8100-11-8111 (2012).
220.Dyall, S. D., Brown, M. T. & Johnson, P. J. Ancient invasions: from endosymbionts to organelles. *Science* (2004).
221.Pollack, Y., Kasir, J., Shemer, R., Metzger, S. & Szyf, M. Methylation pattern of mouse mitochondrial DNA. *Nucleic Acids Research* **12,** 4811-24 (1984).
222.Collins, L. V., Hajizadeh, S., Holme, E., Jonsson, I. & Tarkowski, A. Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses. *Journal of Leukocyte Biology* **75,** 995-1000 (2004).
223.Barth, E., Stämmler, G., Speiser, B. & Schaper, J. Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man. *Journal of molecular and cellular ...* **24,** 669-81 (1992).

224.Deng, G. M., Nilsson, I. M., Verdrengh, M., Collins, L. V. & Tarkowski, A. Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. *Nat. Med.* **5,** 702-5 (1999).
225.Hajizadeh, S., DeGroot, J., TeKoppele, J. M., Tarkowski, A. & Collins, L. V. Extracellular mitochondrial DNA and oxidatively damaged DNA in synovial fluid of patients with rheumatoid arthritis. *Arthritis Research & Therapy* **5,** R234-40 (2003).
226.Yamamoto, S. *et al.* DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* **36,** 983-97 (1992).
227.Bird, A. P. CpG islands as gene markers in the vertebrate nucleus. *Trends in Genetics* **3,** 342-347 (1987).
228.Krieg, A. M. *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374,** 546-9 (1995).
229.Stacey, K. J. *et al.* The molecular basis for the lack of immunostimulatory activity of vertebrate DNA. *J. Immunol.* **170,** 3614-20 (2003).
230.Tian, J. *et al.* Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nature immunology* **8,** 487-96 (2007).
231.Ivanov, S. *et al.* A novel role for HMGB1 in TLR9-mediated inflammatory responses to CpG-DNA. *Blood* **110,** 1970-81 (2007).
232.Julian, M. W. *et al.* Mitochondrial transcription factor A serves as a danger signal by augmenting plasmacytoid dendritic cell responses to DNA. *J. Immunol.* **189,** 433-43 (2012).
233.Taqueti, V. R., Mitchell, R. N. & Lichtman, A. H. Protecting the pump: controlling myocardial inflammatory responses. *Annu. Rev. Physiol.* **68,** 67-95 (2006).
234.Cooper, L. T. Myocarditis. *The New England Journal of Medicine* **360,** 1526-38 (2009).
235.Lin, L. & Knowlton, A. A. Innate immunity and cardiomyocytes in ischemic heart disease. *Life sciences* **100,** 1-8 (2014).
236.Ao, L., Zou, N., Cleveland, J. C., Fullerton, D. A. & Meng, X. Myocardial TLR4 is a determinant of neutrophil infiltration after global myocardial ischemia: mediating KC and MCP-1 expression induced by extracellular HSC70. *American Journal of Physiology - Heart and Circulatory Physiology* **297,** H21-8 (2009).
237.Avlas, O., Fallach, R., Shainberg, A., Porat, E. & Hochhauser, E. Toll-like receptor 4 stimulation initiates an inflammatory response that decreases cardiomyocyte contractility. *Antioxid. Redox Signal.* **15,** 1895-909 (2011).
238.Núñez, G. Intracellular sensors of microbes and danger. *Immunological reviews* **243,** 5-8 (2011).
239.Barton, G. M., Kagan, J. C. & Medzhitov, R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nature immunology* **7,** 49-56 (2005).

240.Eaton-Bassiri, A. *et al.* Toll-like receptor 9 can be expressed at the cell surface of distinct populations of tonsils and human peripheral blood mononuclear cells. *Infect. Immun.* **72,** 7202-11-7211 (2004).

241.Liang, H., Reich, C. F., Pisetsky, D. S. & Lipsky, P. E. The role of cell surface receptors in the activation of human B cells by phosphorothioate oligonucleotides. *J. Immunol.* **165,** 1438-45 (2000).

242.Zhang, Z., Weinschenk, T. & Schluesener, H. J. Uptake, intracellular distribution, and novel binding proteins of immunostimulatory CpG oligodeoxynucleotides in microglial cells. *J. Neuroimmunol.* **160,** 32-40 (2005).