Innate immunity as a target for acute cardioprotection

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Abstract

Acute obstruction of a coronary artery causes myocardial ischaemia and if prolonged, may result in an ST-segment elevation myocardial infarction (STEMI). First-line treatment involves rapid reperfusion. However, a highly dynamic and co-ordinated inflammatory response is rapidly mounted to repair and remove the injured cells which, paradoxically, can further exacerbate myocardial injury. Furthermore, although cardiac remodelling may initially preserve some function to the heart, it can lead over time to adverse remodelling and eventually heart failure. Since the size of the infarct corresponds to the subsequent risk of developing heart failure, it is important to find ways to limit initial infarct development. In this review, we focus on the role of the innate immune system in the acute response to ischaemia-reperfusion (IR) and specifically its contribution to cell death and myocardial infarction. Numerous danger-associated molecular patterns (DAMPs) are released from dying cells in the myocardium, which can stimulate pattern recognition receptors including toll like receptors (TLRs) and NOD-like receptors (NLRs) in resident cardiac and immune cells. Activation of the NLRP3 inflammasome, caspase 1 and pyroptosis may ensue, particularly when the myocardium has been previously aggravated by the presence of co-morbidities. Evidence will be discussed that suggests agents targeting innate immunity may be a promising means of protecting the hearts of STEMI patients against acute IR injury. However, the dosing and timing of such agents should be carefully determined because innate immunity pathways may also be involved in cardioprotection. This article is part of a Cardiovascular Research Spotlight Issue entitled 'Cardioprotection Beyond the Cardiomyocyte', and emerged as part of the discussions of the European Union (EU)-CARDIOPROTECTION Cooperation in Science and Technology (COST) Action, CA16225.

1. Introduction

Acute obstruction of a coronary artery causes myocardial ischaemia and may results in an ST-elevation myocardial infarction (STEMI). First-line treatment involves the rapid reperfusion of the myocardium by percutaneous coronary intervention or use of a thrombolytic agent. This can salvage nonlethally injured myocardium and improves patient outcomes, but reperfusion paradoxically causes some further damage.[1] In response to damage, a highly dynamic and co-ordinated inflammatory response is rapidly mounted to repair and remove the injured cells, but this can further exacerbate the injury. Cardiac remodelling may initially preserve some function to the heart, but over time can lead to adverse remodelling and eventually heart failure. Since the size of the infarct corresponds to the risk of developing heart failure,[2] it is important to find ways to limit infarct development in STEMI.

A number of experimental strategies have been developed that can limit ischaemia and reperfusion (IR) injury.[1, 3, 4] These strategies include ischaemic conditioning applied before ischaemia (preconditioning or IPC), after ischaemia (postconditioning or IPost) or to a distal organ or limb (remote conditioning, RIC). Ischaemic conditioning is believed to work via activation of the PI3K/Akt (Reperfusion Injury Salvage Kinase, RISK), JAK/STAT (survivor activating factor enhancement, SAFE), or cGMP/PKG protein kinase signalling pathways. These pathways can affect several critical end-effectors including the mitochondrial permeability transition pore (MPTP).[1] Numerous pharmacological treatments have been identified that induce cardioprotection by targeting the conditioning pathways.[1, 3, 4] However, the translation of these cardioprotective methods to the clinical setting of STEMI has been generally disappointing.[1] Despite the wealth of information learned over the past 30 years about how the heart responds to IR, and the mechanisms by which cardiomyocytes die,[4, 5] it is clear that more remains to be understood.[6] In particular, the many nuances of the immune system are still being elucidated.

In order to successfully ward off infection, the immune system must be poised to respond rapidly and forcefully at the first signs of invading microorganisms. This is accomplished by the most evolutionary ancient components of the immune response, the innate immune system. This includes blood components such as complement, as well as cellular components, namely mast cells, neutrophils, monocytes, macrophages and dendritic cells, which are all experts at recognising pathogen-associated antigens ("PAMPs"). However, even in the absence of any pathogen, this system can be rapidly activated by dying cells that inappropriately release damage-associated molecular patterns ("DAMPs"), and that signal can be amplified by positive feedback as more cells die. Other cell types can contribute to the response including such "non-professional" immune cells as the endothelium, fibroblasts and pericytes. The innate immune system can be thought of as the first responders at an incident, which then call in the adaptive immunity "specialised-response" team of circulating monocytes and lymphocytes etc. The latter are discussed more fully in an accompanying review article in this series (**reference to be added in proof**). While the innate immune response provides an essential function in protecting against cellular injury by invading organisms, in other cases it can be detrimental. For example, it is now recognised to play an important role in situations such as IR, where a strong inflammatory response is activated in the absence of pathogens – so-called "sterile inflammation". The innate immune response also makes an important contribution to chronic diseases of sterile inflammation including atherosclerosis and heart failure, as reviewed elsewhere.[7] Here, we focus on the role of the innate immune system in the acute response to IR and specifically cell death and myocardial infarction. We additionally consider whether it represents a promising target to protect the hearts of STEMI patients against IR injury.

2. Activation of the innate response during acute IR

Cardiovascular patients are at particular high risk if they suffer from severe inflammatory complications, which may dictate the progression and outcome of the underlying heart disease.[8] Upon cellular stress or injury, several structurally unrelated molecules called DAMPs are liberated into the extracellular space of the heart and into blood, not only serving as "alarmins" to foster an immediate innate immunity response, but also leading to severe organ complications.[8, 9]

i. Danger-associated molecular patterns (DAMPs)

DAMPs are the main mediators of sterile inflammation, but also play a crucial role in the inflammatory phase of MI.[8, 10]. DAMPs induced by vessel-wall injury also elicit further pro-fibrotic and adaptive auto-immune responses to promote atherogenesis. Significant DAMPs include several nuclear or cytosolic proteins including histones, the nuclear protein "high mobility group box 1" (HMGB1),[11] heat-shock proteins and S100-proteins,[12] as well as macromolecular DNAs (both nuclear and mitochondrial), RNAs[13, 14] and purine metabolites such as ATP.[15] Components of the extracellular matrix such as hyaluronic acid and fibronectin can act as a DAMPs via the release of either newly synthesised proteins or degradation products.

Some DAMPs not only serve as potential biomarkers in situations of cardiac IR injury, but have also been characterised as causal factors for the onset and progression of the pathology.[8, 9, 16] In particular, HMGB1 as a chromatin-associated protein is a major mediator of endotoxin shock and acts on several immune and tissue cells to trigger inflammatory responses.[17] The recently discovered antimicrobial extracellular DNA-histone complexes, derived from neutrophils and designated NETs (neutrophil extracellular traps),[18] also appear to play a role in IR injury, since NETs are a major component of arterial thrombi and may provoke cytotoxicity.[19, 20]

In general, the following therapeutic approaches could be envisaged to target DAMPs and protect against cardiac IR injury: (a) prevent the release of DAMPs; (b) neutralise or block the function of extracellular DAMPs; or (c) block the DAMP receptors and/or their intracellular signalling pathways.[21] For example, the administration of DNase may not only reduce thrombotic complications[19, 22] but could also serve as a cardioprotective regimen, provided the underlying pathomechanisms are unravelled. Likewise, application of RNase1 in experimental disease models has been shown to successfully tune down the damaging effect of extracellular RNA in IRI and other inflammatory situations.[23] Thus, targeting different DAMPs and their associated signalling machineries may provide new therapeutic ways to dampen the adverse outcome of cardiac IRI by minimizing acute cardiac injury.

ii. Cardiac-resident immune cells

The DAMPs that are released from dying and damaged cells during acute MI activate and recruit innate and adaptive immune system cells to the heart over the ensuing days (as discussed in an accompanying review in this spotlight series on inflammatory cells as target for cardioprotection).[24](**reference to be added in proof**) However, there are already a significant number of resident immune cells even in the healthy heart,[25, 26] some of which are known to influence the response of the heart to IR, and which therefore represent targets for cardioprotection.

In mouse hearts, there are ~2,300 resident leukocytes/mg tissue.[27] Although figures about their precise makeup depends somewhat on the isolation procedure used, these consist of ~25% lymphoid cells (mostly B and T cells but also some NK cells), and ~75% myeloid (mostly macrophages, some

neutrophils, and low numbers of monocytes, dendritic cells and eosinophils and mast cells)(**Figure 1**).[27-29] However, cardiovascular diseases including cardiovascular disease, diabetes, hypertension, as well as normal physiological aging may strongly increase the numbers of immune cells present in the heart.[29, 30](**Add reference during proof, to inflammatory cells article in this SLI**)

In healthy hearts, there are relatively few resident mast cells.[29] However, these respond rapidly to IR, by degranulating and releasing numerous preformed pro-inflammatory mediators including histamine, ATP, heparin, cytokines, growth factors, lipid mediators, vasoactive agents, reactive oxygen species (ROS) and proteases.[31] These can activate a number of targets, including the endothelium, resident monocytes/macrophages, and infiltrating neutrophils.[31] Mast cell-deficient (W/Wv) mice have smaller myocardial infarct sizes early after IR,[32] though interpretation of this experiment is complicated because some other hematopoietic lineages are also affected in these mice. Moreover, a number of studies have also shown that so-called "mast cell stabilisers", which inhibit mast cell activation, reduce infarct size after IR,[33] and inhibition of mast cell proteases is also cardioprotective.[34] Mast cells may also contribute to other cardioprotective strategies. For instance, the mechanism of cardioprotection induced by adenosine A2A receptor agonists in isolated, perfused mouse hearts was shown to involve the inhibition of resident cardiac mast cell degranulation.[35]

The above results raise the possibility that mast cells could be involved in ischaemic conditioning strategies. Indeed, as the major source of TNFα following IR[36, 37] mast cells could contribute to activation of the SAFE cardioprotective signalling pathway. However, the results of studies to investigate the role of mast cells in IPC are contradictory.[38, 39]

Pericytes are smooth muscle-like cells that wrap around arterioles and capillaries and can regulate blood flow and vascular permeability. Although not typically regarded as part of the immune system, pericytes express functional pattern-recognition receptors, and can modulate innate and adaptive immune responses.[40, 41] Pericyte constriction may contribute to no-reflow after MI.[42] Thus, they represent potential novel therapeutic target in ischaemic heart disease.

3. The innate immune response to acute IR

Pattern recognition receptors such as Toll like receptors (TLRs) and nucleotide-binding oligomerisation domain-like receptors, or NOD-like receptors (NLRs), are adapted to recognising PAMPs expressed by invading microorganisms. However, they also recognise a diverse range of DAMPs and inflammatory factors including those released during IR.

i. Toll like receptors (TLRs).

TLRs are transmembrane receptors that recognise PAMPs coming from microorganisms and enable the host defence mechanism against invading pathogens. TLRs, however, are also able to recognise DAMPs,[43, 44] including several cardiac DAMPs that are produced and released after ischaemia. For example, heat shock protein 60 (HSP60) escapes from cells after cardiac ischaemia and induces apoptosis in cardiomyocytes partly via TLR4.[45, 46] Endogenous nucleic acids are released after apoptosis and necrosis and can be recognised by TLR3, 7, 8 and 9 which are important in the response to viral infection.[47, 48] The nuclear protein HMGB1 is also released in response to ischaemic injury of the heart,[49] and stimulates not only the receptor for advanced glycation end product (RAGE), but also TLR2, TLR4 and TLR9.[50, 51]

TLRs and their endogenous ligands appear to have great potential as targets for treatment of cardiac IR injury (**Table 1**). In particular, the strong but short inflammatory burst following reperfusion allows for a one-hit approach to dampen this adverse early inflammatory response without risking detrimental long-term effects on the immune system.

Unfortunately, the results of experiments targeting HMGB1 in IR injury are highly contradictory: while pre-treatment of mice with recombinant HMBG1 before ischaemia increased infarct size by 30%,[52] treatment with neutralising antibody against HMGB1 also increased infarct size, revealing a complex signalling for HMGB1.[17] Furthermore, any potential treatment targeting HMGB1 would need careful safety evaluation since HMGBs may promote cancer by inducing angiogenesis.[53]

After cardiac ischaemia, hyaluronic acid is fragmented into low-molecular-weight hyaluronic acid[54] that is a ligand for both TLR2 and TLR4,[55] with pro-inflammatory effects.[56] A splice variant of fibronectin known as fibronectin-EDA, which is normally only transcribed during embryogenesis, can be detected in the infarct area and acts as an endogenous ligand for both TLR2 and TLR4.[57] However, the mRNA for fibronectin-EDA is only detected from three days after MI, making this an appropriate target for adverse remodelling but not relevant to early infarct size.[58]

Interestingly, cardiomyocytes also express TLRs despite their having no direct role in host innate immune responses.[59] A variety of TLR agonists and antagonists have been developed, including TLR-targeted antibodies, small molecules and nucleic acid based drugs. Although none have achieved clinical success in treating any pathology to date, there remains great hope for their potential, as has been extensively reviewed.[60] With regards to cardiac IR injury, TLR2 has received the most attention. Administration of a blocking antibody (OPN301 & 305) shortly after reperfusion reduced infarct size in both mouse and pig models.[61, 62] However, bone marrow transplantation experiments revealed that TLR2 on the circulating leukocytes but not those in the heart determines infarct size after reperfusion.[62] Interestingly, low level stimulation of TLR2 can reduce infarct size, and IPC is ineffective in mice lacking TLR2, which suggest that the innate immune response is also capable of initiating a cardioprotective pathway under certain circumstances.[63]

TLR2 is not the only TLR receptor involved in IR injury. In fact, infarct size was also reduced in TLR3-deficient[64] and TLR4-defective[65, 66] mice subjected to cardiac IR. Furthermore, *in vitro* administration of mitochondrial DNA to the cardiomyocyte-derived cell line H9C2 suggested that the TLR9 pathway might also play a role in reperfusion injury.[47]

A wide range of TLR antagonist and agonist therapeutics have already been developed that are ready to be investigated in the setting of IR injury. One of them, the TLR4 antagonist Eritoran has been shown to reduce infarct in mice.[67]

ii.   Nod-like receptors (NLRs)

The innate immune response is regulated by caspase-activating complexes called inflammasomes, which consist of NLRs and proteins such as Apoptosis-associated speck-like protein containing a CARD (ASC),[68] although the exact constituents depend on the activating DAMPs.[69] These promote caspase activation, and the release of the inflammatory cytokines interleukin (IL)-1β and IL-18 via pores in the plasma membrane formed by gasdermin D. A lytic mode of cell death called pyroptosis ensues.[70] Although most well characterised in myeloid cells, pyroptosis also contributes to the death of other cell types and to IR injury.[5, 71-73]

The NLRs are a family of intracellular sensors of PAMPs and DAMPs with over 20 members. NLRP3 (NACHT, LRR and PYD domains-containing protein 3) is the most studied in the setting of cardiac IR (**Table 1**). Within the healthy heart NLRP3 is not - or is very marginally - expressed.[74] This is in contrast to NLRX1, for example, which is constitutively expressed and was shown to affect kidney IR damage,[75] but has not yet been studied in cardiac IR. The low level of NLRP3 expression explains why acute cardiac IR damage (within 1 to 3 h of reperfusion) is not affected by deficiency of NLRP3 or ASC in either *ex vivo* and *in vivo* "healthy" mouse models.[74, 76-78] While NLRP3 expression levels were not significantly increased up to 3 h reperfusion, by 6 h the increase became significant.[71, 77] NLRP3 is mainly expressed in fibroblast and endothelial cells, but also in cardiomyocytes,[71, 79-81] and is increased as a result of activation of the transcription factor nuclear factor-κB (NF-κB). NF-κB is engaged through ligand activation of TLR or cytokine receptors by the release of DAMPs (**Figure 2**).[82] As a consequence, in conditions where these ligands are elevated (e.g.: in diabetic/hyperglycaemic conditions with elevated glycocalyx breakdown products[83]) or with extensive surgery before the actual IR intervention (e.g.: open-chest versus closed-chest models of IR), NLRP3 expression may already be elevated before the ischaemic insult.[74] In these conditions, more acute effects of the NLRP3 on cardiac IR injury are anticipated. Interestingly, alarmins, microRNAs, mitochondrial-bound hexokinase, impaired mitochondrial function and autophagy - all factors known to be involved in cardiac IR injury[84, 85] - affect NLRP3 expression,[82] which may partly explain their effects on IR injury.

Following the increase in NLRP3 expression, activation of the NLRP3 inflammasome by the formation of active NLRP3-ASC-Caspase 1 oligomers is needed for final production of active caspase 1, IL-1β, IL-18 and pyroptosis. Despite many efforts, the exact molecular scheme of activation is still incomplete, but many factors intertwine (**Figure 3**). Some of the important activators of the NLRP3 inflammasome include: cellular potassium efflux (e.g.: via activation of P2X7 receptors by extracellular ATP or IR); cathepsin released from disrupted lysosomes (initiated by aggregates such as cholesterol, uric acid or proteins); metabolic disturbances and mitochondrial dysfunction (e.g.: hexokinase II detachment from mitochondria, release of cardiolipin, oxidised mitochondrial DNA, ROS from mitochondria); Ca^{2+}-induced calpain activation resulting in the release of caspase-1 from actin[86]; and impaired autophagy/mitophagy.[82] Following the generation of the active NLRP3 inflammasome, active caspase-1 cleaves gasdermin D and pro-IL-1β into gasdermin D-NT (GSDMD-NT) and IL-1β. GSDMD-NT then forms oligomers that locate to the plasma membrane forming pores (pyroptosis) through which IL-1β leaves the cell.[87] Posttranslational modifications such as phosphorylation (PKA, PKB), ubiquitination and SUMOylation also control NLRP3 inflammasome activity.[82, 88]

The IL-1β, IL-18 and active caspase-1 produced as a consequence of reperfusion-induced NLRP3 inflammasome activation may contribute to the progression of cardiac IR injury. Inhibition or deficiency of one of the components of the NLRP3 inflammasome reduced infarct size and improved cardiac function in a variety of experimental settings.[71, 79-81] It is apparent, however, that there is no reduction at early timepoints (i.e. 1 to 3 h) and that the benefit of NLRP3 inhibition only becomes apparent after 6 to 24 h reperfusion.[71, 79-81]

Cardioprotection has been seen with the use of several NLRP3 inhibitors and gene silencing. NLRP3 KO mice, however, were not protected in the *ex vivo* IR model, or *in vivo* models of coronary artery ligation.[74, 89] This suggests either that there is a compensatory developmental mechanism for the lack of NLRP3 or that the protective role of NLRP3 inhibitors may be independent of the inflammasome. These possibilities deserve further attention. Inhibition of ASC, another key component of the inflammasome, seems to reproduce the reduction in myocardial injury at 24 h.[89]

Of interest, NLRP3 appears to have a protective role in IPC, since IPC was ineffective in NLRP3-deficient mice, but not in ASC-deficient mice.[76] This was related to diminished IL-6 signalling and STAT3 phosphorylation.[76] The role of NLRP3 in IR may therefore be more complex than originally considered: on one hand it may serve as a trigger for IPC, leading to preservation of viable myocardium during IPC, while on the other hand it may promote further cardiomyocyte loss by inducing pyroptosis, thus leading to greater infarct size, and favour adverse remodelling by inducing the release of IL-1β and IL-18.

The results of preclinical studies using NLRP3 inflammasome inhibitors in models of IR consistently showed a reduction in infarct size and preservation of cardiac function. These inhibitors did not reduce the expression of NLRP3 but rather inhibited the aggregation of the inflammasome by inhibiting the ATPase activity or interfering with NLRP3-ASC binding. Bay 11-7082, a compound initially developed to inhibit NF-κB, was shown to block NLRP3 ATPase[90] and to reduce infarct size when administered prior to reperfusion.[81, 91] Another inhibitor of NLRP3 ATPase, an acrylamide derivative, was also shown to reduce infarct size in an *ex vivo* model of global IR.[92, 93] OLT1177 is also another NLRP3 ATPase inhibitor that prevents aggregation,[94] and reduces infarct size following ischemia reperfusion.[95] OLT1177 is of particular interest due as it is already in clinical testing in phase II trials in gout (EudraCT 2016-000943-14) and phase IB trial in heart failure.(ClinicalTrials.gov Identifier: NCT03534297) A small molecule derived from glyburide, but free of the insulin-secreting function, was demonstrated to selectively inhibit the NLRP3 inflammasome formation, without inhibiting the ATPase nor interfering with other types of inflammasomes, and to reduce infarct size after ischemia reperfusion by inhibiting the late phases of injury.[77, 96] Another inhibitor, MCC950, was also shown to inhibit NLRP3-ASC aggregation independent of ATPase activity and reduced infarct size in a porcine model of acute IR.[78] Taken together these data support the concept that while NLRP3 may have intrinsic protective signalling in the myocardium, the inhibition of the NLRP3 inflammasome aggregation, by either interfering with the ATPase or with the binding of NRLP3-ASC, limits the loss of myocardium after IR.

iii.   Priming of NLRs by co-morbidities

The majority of animal studies have shown that the increase in expression of the NLRP3 inflammasome takes hours to days following IR, which appear to make it an unlikely contributing factor to acute IR injury.[71, 77] However, it is important to recognize that the majority of these studies have been performed in healthy, juvenile animals, in which the NLRs are expressed at a low basal level. In animals or humans with co-morbidities, in which the NLRs are already primed, their response may be greatly accelerated.

The activators of NLRP3 inflammasome are typically polymers or crystalline moieties produced by cells under pathophysiological conditions. Recently, triggering factors related to metabolic abnormalities have been identified as NLRP3 inflammasome activators, including uric acid, glucose, extracellular amyloids, fatty acids and ceramides, lipoperoxidation compounds, as advanced glycation end-products (AGE) and oxidised low-density lipoprotein particles.[97-101] These findings suggest that the gluco- and lipo-toxic environments of metabolic disorders are crucial for NLRP3 inflammasome activation. For example, fat-laden cells undergoing lipotoxicity release microvesicles, which may activate NLRP3 inflammasome following internalisation by either macrophages or cells of hepatocellular origin.[102]

In this regard, the NLR family has been reported to be one of the major intracellular molecular machineries able to induce metabolic inflammation ("metaflammation") leading to the development of metabolic diseases and the exacerbation of related cardiovascular dysfunctions.[103, 104] Furthermore, NLRP3 activation is detectable in the subcutaneous adipose tissue of diabetic, dyslipidaemic, and obese

patients and is linked to the severity of coronary atherosclerosis.[105] The local over-activation of the NLRP3 inflammasome due to organ metabolic impairments may exacerbate cardiovascular ischaemic injury in obesity and diabetes.[71, 106, 107] These data support the premise of NLRP3 inflammasome as an innovative pharmacological target for post-ischaemic recovery in patients with metabolic abnormalities. Using a combination strategy to target both the inflammatory and the pro-survival signalling pathways may be necessary for satisfactory protection against myocardial IR injury.

iv.   Caspase 1

As discussed above, inflammasomes are an important part of the innate immune system and there is strong evidence that activation of at least the NLRP3 inflammasome, possibly by the release of oxidised DNA fragments from damaged mitochondria,[108, 109] contributes to myocardial infarction during cardiac IR. IR causes the activation of calpain, a calcium-dependent protease, which releases a pool of caspase-1 from the actin cytoskeleton (**Figure 3**).[86] Several inflammasomes including AIM2, pyrin, NLRC4, and NLRP3 also converge on caspase-1 which then is a common target accounting for their bactericidal activity.[110] The canonical function of caspase-1 is to proteolytically transform pro-IL-1β and pro-IL-18 into their active moieties, and these interleukins have been proposed to contribute to much of the tissue injury in the sterile inflammation following myocardial IR. However, caspase-1 can also injure the cell membrane through cleavage of gasdermin D.[111] The liberated n-terminal segment of gasdermin D attaches to the inner surface of the cell membrane and creates large pores through which the interleukins escape, a process termed pyroptosis. Unfortunately, LDH and other vital enzymes will also escape, thus killing the cell. Conventional necrosis also involves sarcolemmal failure, but that is not caspase-1-dependent. Whether caspase-1-mediated injury results directly from pyroptotic cell membrane destruction of the reperfused cells or whether pyroptosis is confined to a very few sentinel cells which then release a lethal dose of interleukins into tissue is unknown. Finally, caspase-1 is known to proteolytically attack a number of vital cytosolic enzymes.[112]

Regardless of its exact mechanism of killing, inhibition of caspase-1 should be an effective anti-inflammatory target. Knocking out caspase-1 resulted in smaller infarcts in mice,[79] and a caspase-1 inhibitor was cardioprotective in in human atrial trabeculae exposed to simulated IR.[113] The highly selective caspase-1 inhibitor VX-765 profoundly reduced infarct size when administered to rats immediately before the end of a 60 min period of coronary occlusion, and only 2hr of reperfusion, revealing that a large amount of myocardial reperfusion injury is caspase-1-dependent.[114] This observation is clinically relevant because VX-765 is a pharmaceutical-grade drug available for human testing. Furthermore, VX-765 can add its protection to that from the platelet P2Y$_{12}$ inhibitor ticagrelor[114] that is routinely given to patients with acute myocardial infarction prior to percutaneous intervention. Ticagrelor, itself, is cardioprotective[115-117] and the finding that combining VX-765 with ticagrelor yields additive protection suggests their mechanisms of protection are fundamentally different. As mentioned above, NLRP3 is not strongly expressed in healthy hearts but is thought to appear several hours after reperfusion. However, in *in situ* rat hearts infarct size was dramatically reduced after only 2 h of reperfusion and there was no further increase in infarct sizes between 2 h and 72 h reperfusion in either the untreated or VX-765-protected hearts[114] suggesting that an inflammasome other than NLRP3, or perhaps calpain activation as discussed above,[86] might be the source of caspase-1. The topic of platelets as a target for IR injury is addressed in an accompanying review.(**reference to add during proof**)

Caspase-1 inhibition is equally protective when administered to blood-free, isolated hearts indicating that the lethal inflammasomes are located in cardiac tissue rather than the blood.[73, 114] Surprisingly, the caspase-1-dependent loss of membrane integrity occurs in the initial few minutes of reperfusion.[114] Inhibition of caspase-1 prior to ischaemia[72] is no more protective than inhibition at reperfusion[114] which suggests that pro-caspase-1 is not converted to active caspase-1 until after reperfusion. In summary, caspase-1 inhibition appears to be an anti-inflammatory strategy with a high potential for salvage of ischaemic myocardium in patients with acute myocardial infarction treated with primary angioplasty.

4. Other factors influencing the innate immune response to IR

A number of additional factors can influence infarct development and the response to IR via a mechanism that may involve the innate immune system. For example, co-morbidities such as obesity, diabetes, age and hypertension are increasingly prevalent in the target STEMI patient population, and impair cardioprotective strategies.[118, 119] These co-morbidities are also increasingly recognised as increasing inflammation, including via effects on the innate immune system.[120]

For many years it was thought that only the adaptive immune system could respond to previously seen challenges with an immunological memory. However, in recent years, it has been shown that the innate immune response, too, has a type of trained immunological memory that can be activated in monocytes.[121, 122] Trained immunity can be induced by a range of stimuli, including various PAMPs and DAMPs, and involves the activation of monocytes and macrophage and their epigenetic reprogramming. One such stimulus capable of inducing trained immunity is β-glucan (from bacterial and fungal cell walls).[121, 122] Interestingly, i.v. or i.p. pre-treatment of rats and dogs with β-glucan has been shown to reduce infarct size[123, 124] as has 10 days oral administration to pigs.[121] However, whether monocytes or trained immunity is responsible for this effect has not yet been investigated.

Over the past ~10 years, the existence of a neuro–immune circuit has emerged which allows the central nervous system to regulate the innate immune response in peripheral tissues (reviewed in [125]). This circuit is believed to explain some of the long-recognised links between psychosocial stress and disease.[125] For example, the hypothalamic–pituitary–adrenal axis produces glucocorticoids, which circulate throughout the body and can alter a variety of processes, including immune response gene programmes.[125] Furthermore, adrenaline and noradrenaline released by the sympathetic nervous system can modulate the innate immune system. Interestingly, neutrophils, NK cells and dendritic cells all express adrenergic receptors, and the β-blocker metoprolol reduces infarct size partly via its effects on neutrophils.[126]

Another topic of great interest currently is the gut microbiota. Gut microbiota-dependent metabolites, in particular trimethylamine N-oxide, increase risk of cardiovascular events by promoting atherosclerosis and thrombosis.[127, 128] The mechanism for this seems to be an increase in numbers of proinflammatory monocytes.[129] Recently, MI was shown to increase gut permeability and causes the translocation of gut microbes into the circulation.[130] The resultant systemic inflammation predicted adverse cardiovascular events in STEMI patients.[130] In mice subjected to MI, three days treatment with the antibiotic polymyxin B decreased bacterial translocation, plasma LPS, the proportion of inflammatory (Ly6C^{hi}) monocytes and decreased infarct size.[130]

5. Conclusion and future perspectives

The evidence described above indicates that at least part of the myocardial cell death that occurs during myocardial infarction is due to the rapid activation of an inflammatory response further exacerbating that very same injury. This appears to be a consequence of inappropriate activation of the innate immune system responding rapidly to DAMPs as if they were PAMPs from invading microorganisms. Therefore, interruption of this feedback cycle with drugs designed to target key components of the innate immune system is a promising approach for limiting IR injury. A word of caution relates to the fact that mild activation of the TLR-NLRP3 axis can also be protective against IR damage, necessitating optimal dosing before embarking on large clinical trials. It remains to be seen whether these approaches can be effective at preventing injury to the hearts of patients with co-morbidities, where there is already low-level inflammation, elevated expression of the NLRP3 inflammasome and activation of the innate immune response even before MI has occurred. The reduction in cardiovascular events seen in the recent CANTOS trial of Canakinumab, an antibody targeting Il-1β, is very encouraging, but it is not yet clear to what extent this benefit can be ascribed to suppression of the innate immune response in the heart as opposed to systemic antiinflammatory effects.[131] Longer term following IR, innate immunity is also believed to contribute to myocardial repair and remodelling, as discussed in a recent systematic review of post-MI immunomodulation.[132] As more specific and targeted drugs are developed, it may be possible to limit background activation without impairing the response to infection. Ultimately, a combination of therapies targeting different aspects of the myocardium's response to MI may be necessary for effective cardioprotection in patients.

6. Funding

7. Conflicts of Interest

SMD, HACF, MT MCV, JMD, KTP, CJZ declare no conflict of interest. Dr. Abbate has served as a consultant for Glaxo Smith Kline, Janssen, Merck, Novartis, Olatec Therapeutics, Serpin pharma, and Swedish Orphan Biovitrum.

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9. Figure legends

Figure 1. The resident immune cell types and their numbers in the mouse heart.

Data was obtained by flow cytometric analysis of digested mouse hearts.[28]

Figure 2. The priming step of NLRP3 expression

Priming, i.e. initiating the transcription of the NLRP3 gene, is the first and most important step in NLRP3 activation, because the quantity of NLRP3 in healthy, non-stimulated cells is very low. Priming can occur through ligand-mediated activation of different pattern recognition receptors (PRRs), of which the Toll-like receptor (TLR) 2 and 4, the IL-1β receptor, and the tumour necrosis factor (TNF) receptor 1 and 2, are the most relevant for IR-induced priming of the NLRP3. Following ligand-association, the transcription factor nuclear factor- kappa β (NF-kβ) is activated, resulting in the transcription of Nlrp3 and pro-IL-1β.

Figure 3. Factors involved in the activation step of the NLRP3 inflammasome

Activation steps consist of potassium-dependent and independent pathways. Potassium efflux, but also excessive potassium influx, (with a possible common downstream pathway being changes in membrane potential[86]), together with an increase in cytosolic Ca^{2+}, activate calpain that then releases a pool of caspase-1 from the actin cytoskeleton. The released caspase-1 contributes to the oligomerization of the inflammasome. Potassium efflux can also be the result of lysosomal destabilisation, releasing cathepsin that induces membrane pores for K^+-efflux, or through extracellular ATP-stimulated opening of purinergic P2X7 channels. Auto-inhibited NLRP3 can be activated by deubiquitination, TXNIP and NEX7 binding to NLRP3, and through an array of factors stemming from mitochondrial dysfunction. The most prominent mitochondrial factors are: the release of oxidized mitochondrial DNA and cardiolipin; the detachment of hexokinase II from the outer mitochondrial membrane; and the generation of mitochondrial ROS. These mitochondrial factors can also be the result of impairments in mitophagy, resulting in an increased number of dysfunctional mitochondria. The active NLRP3 inflammasome results in activated inflammatory caspases that cleave gasdermin D in an N-terminal cleavage product (GSDMD-NT) and pro-IL-1β into active IL-1β. GSDMD-NT oligomerises and is incorporated into the plasma membrane forming pores for IL-1β release, a process called pyroptosis.[87, 111]

10. Table.

Target	Treatment	Effects	Study	Reference
TLR2	antibody TLR2 at ischemia	↓ IS	open-chest pig (75 min I/24 h R)	Arslan et al (2012)[61]
TLR2	antibody TLR2 at ischemia or KO TLR2 circulating cells	↓IS	Open-chest mouse (30 min I/24 h R)	Arslan et al (2010)[62]
TLR2	KO	loss of IPC	Isolated mouse heart (30 min I/60 min R)	Dong et al (2010)[63]
TLR2	Activation TLR2 by ligand	↓ IS	Isolated mouse heart (30 min I/60 min R)	Dong et al (2010)[63]
TLR3	KO	↓ IS	Open-chest mouse (45 min I/3 d R)	Lu et al (2014)[64]
TLR4	KO	↓ IS	Open-chest mouse (30 min I/6 h R)	Ding et al (2013)[65]
TLR4	KO	↓ IS	Open-chest mouse (60 min I/24 h R)	Oyama et al (2004)[66]
TLR4	Antagonist TLR4 before ischemia	↓ IS	Open-chest mouse (30 min I/24 h R)	Shimamoto et al (2006)[67]
ASC Casp-1	KO	↓ IS	Open-chest mouse (30 min I/ 48 h R)	Kawaguchi et al (2011)[79]
NLRP3	KO	no LDH effect loss of IPC	Isolated mouse heart (35 min I/45 min R)	Zuurbier et al (2012)[76]
ASC	KO	no LDH effect intact IPC	Isolated mouse heart (35 min I/45 min R)	Zuurbier et al (2012)[76]
NLRP3	KO	↓ IS	Isolated mouse heart (35 min I/60 min R)	Sandanger et al (2013)[80]
ASC	KO	unchanged IS	Isolated mouse heart (35 min I/60 min R)	Sandanger et al (2013)[80]
NLRP3	siRNA or BAY11-7028 against NLRP3	↓ IS	Open-chest mouse (30 min I/24 h R)	Liu et al (2014)[81]
NLRP3	INF4E	↓ IS	Isolated rat hearts (30 min I/20-60 min R)	Mastracola et al (2016)[93]
NLRP3	KO	no ↓ IS	Closed-chest mouse (30 min I/3 h R)	Jong et al (2014)[74]
NLRP3	NLRP3 inhibitor 16673-34-0	↓ IS	Open-chest mouse (30 min I/24 h R)	Marchetti et al (2014)[96]
NLRP3	NLRP3 inhibitor 16673-34-0	Unchanged IS at 3 h R; ↓ IS at 24 h R	Open-chest mouse (30 min I/3 or 24 h R)	Toldo et al (2016)[77]
NLRP3	NLRP3 inhibitor MCC950	↓ IS	Closed-chest pig (75 min I/7 d R	Van Hout et al (2017)[78]
NLRP3	KO	↑IS no effect TnI	Open-chest mouse (30 min I/24 h R)	Sandanger et al (2016)[89]
ASC	KO	unchanged IS	Open-chest mouse (30 min I/24 h R)	Sandanger et al (2016)[89]
NLRP3 ASC	KO treated with protective TLR2 ligand activation	unchanged IS	Open-chest mouse (30 min I/24 h R)	Sandanger et al (2016)[89]

Table 1 Effects of genetic or pharmacologic manipulations of TLRs and NLRs on acute cardiac ischaemia – reperfusion injury. IS: infarct size; I; ischaemia; R: reperfusion; IPC: ischaemic preconditioning; KO: knockout; LDH: lactate dehydrogenase release during reperfusion; TnI: troponin I release during reperfusion.

Figure 1
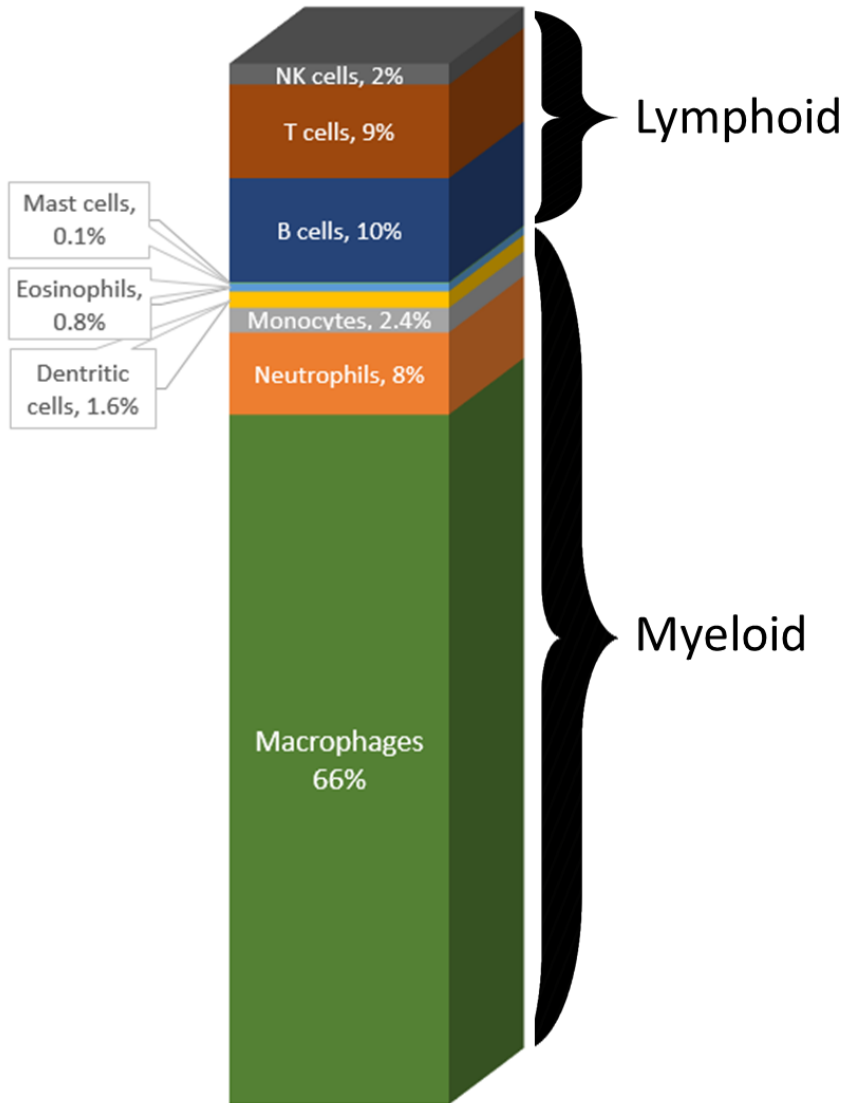
Figure 1. The resident immune cell types and their numbers in the mouse heart.
Data was obtained by flow cytometric analysis of digested mouse hearts.[30]
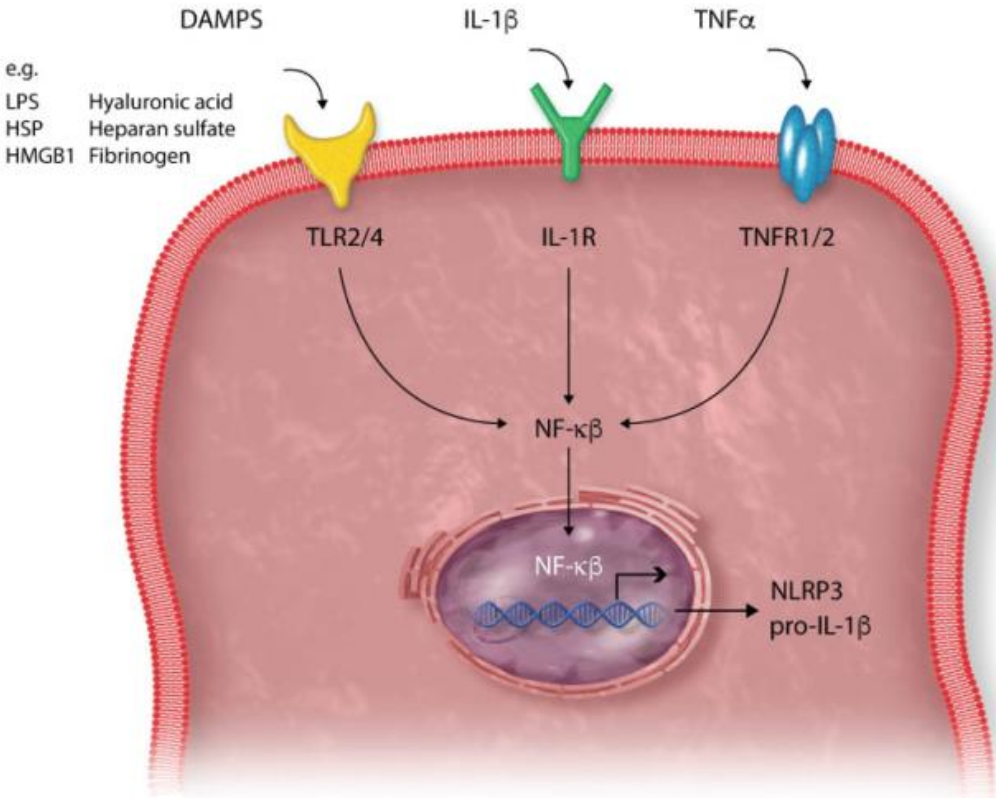
Figure 2

Figure 3