The innate immune response in reperfused myocardium

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Abstract
One of the major therapeutic challenges in the arena of interventional cardiology is to design strategies aimed at reducing myocardial tissue damage after myocardial infarction. In response to tissue injury, an innate immune response is initiated that orchestrates homeostatic responses and is a prerequisite for subsequent wound healing. An exaggerated inflammatory reaction, however, counteracts these beneficial effects and contributes to maladaptive tissue damage. Herein, we discuss the pathways involving the innate immune system that have been investigated in the setting of myocardial ischaemia and reperfusion injury. Endogenous ‘danger’ signals [danger-associated molecular patterns (DAMPs)] are expressed following tissue injury and alert the innate immune system. Toll-like receptors and the complement system are activated, resulting in an inflammatory reaction involving inflammatory cell influx and the production and release of inflammatory cytokines. A potential involvement of cell-derived microparticles in the modulation of the innate immune response following myocardial injury will also be discussed. Our future challenge lies within the counteraction of maladaptive inflammatory cascades, without interfering in the benign wound healing response, and in translating these anti-inflammatory strategies into clinical practice.

Keywords
Myocardial infarction • Ischaemia reperfusion injury • Innate immunity • Toll-like receptors

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1. Introduction
Coronary artery disease is the leading cause of morbidity and mortality worldwide, and >7 million people die of the disease annually.1 In humans, irreversible damage to the myocardium, i.e. infarction, becomes evident after 20 min of total ischaemia. Reperfusion is a requisite for cardiomyocyte salvage, and early reperfusion therapy is currently the most effective strategy to reduce infarct size in patients with acute myocardial infarction (MI).2 The restoration of blood flow, however, triggers a series of events that eventually culminates in accelerated apoptosis, which can paradoxically reduce the beneficial effects of reperfusion therapy. Since it is difficult to establish whether cells die due to ischaemia or due to reperfusion, the existence of reperfusion injury has long been debated. The observation, however, that the myocardial infarct size in humans can be reduced by cardioprotective interventions applied at the initiation of reperfusion points to the existence of lethal reperfusion injury as a distinct mediator of cardiomyocyte death.3

Myocardial ischaemia and reperfusion (I/R) injury is considered an inflammatory condition characterized by innate immune responses. Where originally it was thought that the immune system was designed to protect the body against foreign or non-self signals, evidence has been collected over the years that the innate immune system is alerted following tissue injury with the release of endogenous ligands. Ischaemic injury results in a ‘danger’ signal, composed of substances released by cells that die by necrosis and alterations to the extracellular matrix (ECM), that initiates the innate immune response. This ‘danger model’, postulated by Matzinger, describes a model of immunity that is based on the idea that the innate immune system responds to endogenous entities that cause injury, rather than only to those that are foreign.4 Herein, we discuss how endogenous signals trigger the innate immune response via Toll-like receptors (TLRs) and the complement system, and the consequences for MI healing after I/R injury. In addition, we will discuss a potential involvement of cell-derived microparticles, which are released in response to danger signals, in the innate immune response following tissue injury.
2. The inflammatory response after ischaemia and reperfusion

An inflammatory response, characterized by the recruitment of inflammatory cells, oxidative stress, and endothelial barrier dysfunction, constitutes the early phase of MI healing. This process is initiated by a cardiac release of soluble inflammatory mediators shortly after reperfusion. These cytokines activate and recruit neutrophils to the site of injury, which cause direct injury to the endothelial cells via the production of reactive oxygen species (ROS), proteases, cytokines, and lipids. The endothelial cells assume an inflammatory phenotype, characterized by enhanced production of ROS, inflammatory cytokines, and adhesion molecules that facilitate binding of leucocytes and platelets. Concomitantly, intercellular tight junctions are compromised, which leads to endothelial barrier dysfunction and increased vascular permeability. This promotes migration of neutrophils and other inflammatory cells into the injured myocardial tissue. Neutrophil influx is associated with increased cardiac injury after cardiac ischaemia and interventions targeting neutrophil influx confer cardioprotection. A full appraisal of the involvement of inflammatory cells and cytokines in cardiac repair has been described previously in an excellent review paper.

One of the major therapeutic challenges of modern cardiology is to design strategies that reduce infarct size and improve cardiac repair after MI. Because of its role in cardiac repair, inflammation has long been considered a promising target for therapeutic interventions. Although numerous experimental studies demonstrated that influx of leucocytes and inflammatory mediators contribute to myocardial injury, clinical trials aiming to reduce these factors were disappointing. Broad inhibition of inflammation has an adverse effect on outcome following MI. These studies emphasize that inflammation is not solely an injurious process, but it also mediates processes essential for proper MI healing.

3. Danger-associated molecular patterns

The ‘danger model’ proposed a theory that the innate immune system could be triggered by non-pathogenous signals. These endogenous alarm signals, referred to as ‘danger associated molecular patters’ (DAMPs), may be inducible or constitutive, intracellular or secreted, or part of the ECM. To date, it is unknown whether cardiac ischaemia leads to the expression other DAMPs than reperfusion and whether different signalling pathways are followed. DAMPs expressed after MI include heart shock proteins (HSPs), high-mobility group box-1 (HMGB1), low molecular hyaluronic acid, and fibronectin fragments.

HSPs were originally described to be synthesized in response to heat shock, but their expression also increases following tissue injury to act as chaperones preventing protein denaturation and loss of function. Several HSPs, such as HSP70 and HSP20, protect cells against ischaemic cardiac injury. It was therefore suggested that HSP induction in the intact heart was a naturally occurring protection mechanism against exposure to I/R, and that HSP induction following cardiac ischaemia could have therapeutic potential. However, in vivo experiments on rabbits failed to demonstrate reduction in infarct size following heat stress. In addition, HSP60 induces apoptosis via the death receptor pathway and treatment with an anti-HSP60 antibody reduces myocardial apoptosis and cytokine expression in mice. To elucidate the exact roles of specific HSPs in cardiac I/R injury, as well as the mechanisms by which HSPs influence this process, additional research is needed.

Extracellular HMGB1, a highly conserved nuclear factor involved in nucleic acid stabilization and gene transcription, is a potent innate ‘danger signal’ for the initiation of host defence or tissue repair. HMGB1 can lead to nuclear factor-kappaB (NF-kB) activation via TLR2, TLR4, and TLR9. Also the receptor for advanced glycation end products (RAGEs) has been identified as receptor for HMGB1. Andrassy et al. demonstrated that HMGB1 acts as an early mediator of inflammation and organ damage in I/R injury of the heart. Whereas HMGB1 evokes cardiac injury initially, several reports stated that HMGB1 mediates favourable effects in the chronic phase of myocardial infarct healing. Therefore, HMGB1 has to be carefully evaluated as a therapeutic target to improve clinical outcome following MI.

In addition to the signals induced by cell necrosis, dynamic alterations of the composition of the ECM can influence the innate immune response following cardiac I/R injury. ECM degradation products as well as de novo synthesized ECM molecules might act as endogenous DAMPs. Hyaluronic acid can activate the innate immune system via TLR2 and TLR4. Fibronectin is a multifunctional adhesive glycoprotein present in the ECM and is produced by cells (e.g. fibroblast, endothelial cells) in response to tissue injury. Its gene contains an alternatively spliced exon encoding type III repeat EDA that acts as an endogenous ligand for both TLR2 and TLR4. Fibronectin EDA is up-regulated following MI and enhances expansive cardiac remodelling after MI by activating circulating inflammatory cells. The involvement of EDA in lethal myocardial reperfusion injury has not been established and is questionable as new synthesis of (EDA) fibronectin might take too long to play a significant role in the first hours of reperfusion.

For a more detailed description of the different DAMPs that are involved in MI we refer to a previous review.

4. Toll-like receptors

TLRs were identified in 1997 by Medzhitov et al. as an important part of the innate immune system. TLRs are highly conserved transmembrane receptors with a leucine-rich repeat extracellular motif, that are expressed on antigen-presenting cells and several types of parenchymal cells, including cardiomyocytes. To date, 10 functional TLRs have been identified in humans (TLRs 11–13 are only expressed in mice). TLRs were originally described as pattern recognition receptors that recognize conserved molecular motifs on pathogens, so-called pathogen-associated molecular patterns (PAMPs), such as cell-surface lipopolysaccharides (recognized by TLR4), peptidoglycans (TLR1 and TLR2), lipoproteins (TLR2 and TLR6), viral double-stranded RNA (TLR3), viral single-stranded RNA (TLR7 and TLR8), bacterial and viral CpG (cytosine-phosphate-guanine dinucleotide) oligodeoxynucleotides (TLR9), and flagellin (TLR5). TLRs therefore form the first line of defence against invading pathogenic micro-organisms. Besides their function in discriminating between ‘self’ and ‘non-self’ molecules, evidence is accumulating that TLRs are involved in many pathophysiological processes in human disease that do not imply pathogens. Endogenous ligands that are released in response to tissue injury, DAMPs as discussed above, are able to activate TLRs thereby triggering the innate immune system. Excellent reviews have been published previously dealing with the intracellular signalling pathways of different TLRs. Therefore, these will not be discussed further in this review.
5. TLR signalling in cardiovascular disease

TLRs have been implicated in a number of cardiovascular disorders. TLR2 and TLR4 are involved in the intimal hyperplasia and atherosclerotic lesion development. Additional studies suggested that TLR-induced inflammation may lead to atherosclerotic plaque destabilization and to the development of acute coronary syndromes in patients with coronary artery disease. Clinical observations in patients with loss of function TLR4 polymorphisms (of which the Asp299Gly single nucleotide polymorphism leading to a blunted TLR4-mediated inflammatory response has been studied the most) revealed that these patients had a lower rate of cardiovascular events, although others could not reproduce this associative finding between this polymorphism and MI. Aspirin, on the other hand, were protected against lethal reperfusion injury, although others could not reproduce this associative effect. Ex vivo experiments revealed that hearts from TLR2–/– mice challenged with Staphylococcus aureus or I/R injury are protected against deterioration of cardiomyocyte contractile function. Infarct size was not affected in these models. Traditional ‘loss of function’ animal studies, however, demonstrated that targeted disruption or deletion of TLR4, TLR2, and MyD88 reduces infarct size by reducing inflammation and oxidative stress. To comprehend these seemingly conflicting results, it is of importance to acknowledge that TLRs are expressed on parenchymal cells, such as endothelial cells and cardiomyocytes, and on circulating cells. The results obtained in isolated perfused heart model, in which circulating factors are eliminated from the experiment, demonstrate that parenchymal TLR2 mediates the development of cardiomyocyte dysfunction after I/R injury. Lethal reperfusion injury, on the other hand, is mediated by circulating TLR2 as became evident from an experiment with chimeric mice. Infarct size in TLR2–/– mice, that underwent bone marrow transplantation with wild-type bone marrow cells, was comparable with that of wild-type mice. Wild-type mice with TLR2–/– bone marrow, on the other hand, were protected against lethal reperfusion injury to the same extent as TLR2 knockout mice. TLR2 and TLR4 constitute potential therapeutic targets in the treatment of I/R injury. Treatment with eritoran, which reduces the binding of lipid A (the biologically active part of LPS) to TLR4, reduced infarct size in mice. Also an antibody directed against TLR2, administered after the ischaemia, just prior to reperfusion, significantly reduced infarct size. Pre-treatment with TLR antagonists may be harmful since TLRs mediate ischaemic preconditioning, as mentioned above. This may complicate the chronic use of TLR antagonists to inhibit progression of atherosclerosis, for example. Prolonged treatment with TLR antagonists after MI may have additional benefit, as both TLR2 and TLR4 contribute to expansive cardiac remodelling and functional impairment after MI. Figure 1 shows the TLR signalling pathway and summarizes the TLR-related interventions that have been investigated in the setting of MI.

6. Complement

The complement system is a first line of defence, which functions on the interface of innate and adaptive immunity. It consists of many plasma- and cell membrane proteins, many of which circulate as pro-enzymes. Pathogen-antigens (PAMPs) that are bound to antibodies lead to activation of these pro-enzymes by pathogenous antigens through proteolytic cleavage, inducing an activation cascade. Like TLRs, also the complement system can be activated by endogenous signals (DAMPs) that are released in response to tissue injury. Subcellular substances released from cardiomyocytes after ischaemia, such as cardiolipin, can activate complement. C-reactive protein and IgM co-localize with activated complement in human infarcted myocardium, indicating that they both play a role in activation of the complement system. The complement system is activated through three pathways: the classical pathway, the lectin pathway, and the alternative pathway. All pathways lead to cleavage of C3 with subsequent activation of C5 and formation of the membrane attack complex (MAC; C5b-9) Figure 2. Activation causes opsonization of pathogens, phagocytosis, augmentation of inflammation via anaphylatoxins, and direct cell lysis. Activation of the complement cascade is regulated by various regulatory proteins, including C1-inhibitor (C1-INH) and complement receptor 1 (CR1). Involvement of the complement system in myocardial ischaemia was first demonstrated in 1971 in a rat MI model. Since then a role of the complement system in tissue injury following ischaemia has been well established. The classical, lectin and alternative pathways have all been implicated in the pathogenesis of I/R injury. The most recent evidence points to involvement of the lectin pathway to a greater extent than the other pathways. The lectin complement pathway is initiated by recognition of infectious non-self and altered self by mannose-binding lectin (MBL), a circulating pathogen recognition molecule. After myocardial I/R injury, the lectin pathway is activated in an antibody independent manner and its inhibition by using antibodies against MBL reduces neutrophil infiltration and attenuates myocardial I/R damage. Mice lacking MBL, and hence are devoid of MBL-dependent lectin pathway activation but have fully active alternative and classical pathways, are protected from cardiac I/R injury. Mice that lack C1q, a major component of the classical complement pathway initiation complex, but have intact MBL, are not protected from injury. It was recently shown that IgM binds to self-antigen on injured cells providing a binding site for MBL. Infarct size, leukocyte infiltration and C3 deposition were dependent on the combined presence of circulating soluble IgM and MBL, suggesting this pathway is not totally antibody-independent. Cobra venom factor (CVF), a substance capable of depleting complement factor C3, has been shown to reduce I/R injury in rats. However, due to the immunogenicity of CVF and its capability of
cleaving C5, it was not suitable for clinical application. Subsequently, a recombinant humanized CVF was developed, which was able to attenuate injury in a mouse model without influencing C5 titres, making it an interesting potential therapeutic for clinical use.

CR1 can inhibit both the classical and the alternative pathway by dissociation of C3- and C5 convertases and serving as a co-factor for factor I-mediated degradation of C3b and C4b. The recombinant human soluble CR1 (sCR1) and C1-INH are potent complement inhibitors, reducing infarct size in various animal models.

Given the promising results in animal models, several clinical studies have been conducted concerning C5 inhibition in humans. Most studies have focused on pexelizumab, a single-chain fragment of a humanized monoclonal antibody against complement component C5. The results, however, were disappointing. A recent meta-analysis, concerning 15,196 patients either undergoing percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG), showed no benefit of pexelizumab administration in patients with acute MI undergoing PCI, but there was a significant reduction in...
mortality in the setting of CABG.70 Favourable results were obtained clinically with C1-INH. In a small pilot study consisting of 31 patients with acute MI effectively treated with thrombolytic reperfusion therapy, continuous administration of C1-INH reduced cardiac injury as measured by cardiac enzyme release.71 In patients with acute MI who underwent emergent CABG, C1-INH treatment resulted in lower cardiac enzyme release and improved haemodynamics.72 Larger randomized studies should be performed to support these promising initial findings.

7. Microparticles

Microparticles are circular membrane vesicles that are produced and secreted by many different cell types in response to cell activation or apoptosis. Different types of microparticles exist, including exosomes, exosome-like vesicles, microvesicles, ectosomes, membrane particles, and apoptotic bodies, but the nomenclature is still confusing.73 Although first considered cellular disposal, it is now becoming increasingly evident that microparticles facilitate a sophisticated mode of intercellular communication, as they can assist in the intercellular transfer of soluble proteins, membrane proteins, lipids, RNA and miRNA. Important roles in processes relevant for cardiovascular disease, such as coagulation, inflammation, and endothelial function can be ascribed to microparticles.74 Increasing evidence suggests that microparticles modulate the innate and acquired immune system. Activation of TLR4 by LPS, complement and ROS have been described to accelerate the shedding of microparticles by different cell types.75–77 This suggests that the release of microparticles is stimulated by danger signals. Endogenous danger signals that are expressed following myocardial injury may induce the release of microparticles. Higher levels of circulating microparticles are detectable in the blood after MI.78 Microparticles contribute to an inflammatory innate immune response by various mechanisms. Platelet-derived microparticles contribute to inflammatory cell influx by delivering arachidonic acid to endothelial cells, which results in a up-regulation of adhesion molecules and adhesion of monocytes.79 Also oxidized phospholipids that are present in microparticles that are shedded in response to oxidative stress, are able to activate endothelial cells.
and leucocytes.80,81 Microparticles shed by infected macrophages contain PAMPs and stimulate a pro-inflammatory response in a TLR and MyD88-dependent manner.85 In patients with rheumatoid arthritis, inflammation is amplified by microparticles, and in patients with septic shock, microparticles contribute to myocardial dysfunction.83,84 Also anti-inflammatory actions of microparticles have been described, e.g., the interaction between microparticles and B-cells or monocytes induces a transition towards a more anti-inflammatory phenotype.85 Like other mediators of innate immunity, also microparticles appear to have a dual role in the inflammatory response. There is evidence that the composition of microparticles, and thus their function, changes with the pathophysiological context in which they are released.86 The exact involvement of endogenous microparticles in I/R injury and cardiac repair remains to be investigated, but the innate immune response following I/R injury can be beneficially influenced by administration of exogenous microparticles. Infusion of media conditioned by mesenchymal stem cells (MSCs) reduces I/R injury and improves cardiac repair by reducing inflammation, oxidative stress, and apoptosis, and by stimulating angiogenesis.87,88 Exosomes were identified as the cardioprotective factor in MSC conditioned media.89 This suggests that the protective effects of stem cell delivery following MI are driven by paracrine immunomodulatory mechanisms mediated by exosomes. Very low concentrations of exosomes resulted in an impressive reduction in infarct size. Exosomes form an efficient transport vehicle to deliver cardioprotective substances (proteins, miRNA) to endangered cardiomyocytes, protecting these substances from degradation by proteases and ribonucleases. Many factors can be delivered to endangered cardiomyocytes simultaneously in order to coordinate a homeostatic response, eventually resulting in cell survival. Further studies are needed to elucidate the precise role of endogenous microparticles in the regulation of the inflammatory response following MI, as well as the mechanisms of exogenous MSC-exosome-mediated cardioprotection.

8. Conclusions and therapeutic implications

The innate immune system has been implicated in the pathogenesis of cardiovascular disease, including MI, whereas the innate immune system orchestrates beneficial homeostatic responses in the heart following MI and contributes to tissue repair, an exaggerated response of specific parts of the innate immune system causes additional injury. Endogenous signals (DAMPs) that appear after tissue injury are able to trigger the innate immune system via TLRs and the complement system, which can lead to detrimental inflammatory responses. Our growing knowledge of the inflammatory mechanisms involved in I/R injury increases the options for therapeutic interventions.

DAMPs constitute promising therapeutic targets, since they are induced after injury. In theory, however, every intracellular molecule released after tissue injury and matrix molecules that are altered after tissue injury are potential DAMPs. TLRs can be activated by different DAMPs and therapeutic targeting of one DAMP could still result in TLR activation via other DAMPs. Investigating the role of multiple ligands together in initiating innate immune responses may therefore be relevant area to study.

Direct inhibition of TLR2 and TLR4 represents another potential therapeutic option, although preclinical evidence is still limited. Most experiments were performed using knockout mice, which excluded the possibility to study the effect of TLR inhibition at a clinically relevant time point, i.e., at the end of ischaemia or at the beginning of reperfusion. Antagonists of TLR2 and TLR4 are available and some have been tested for reduction in I/R injury. Eritoran blocks binding of LPS to TLR4 and infusion of eritoran before I/R injury reduces infarct size in mice.51 As eritoran is a competitive inhibitor of LPS binding to TLR, it is uncertain whether eritoran blocks binding of all endogenous activating TLR4 ligands, preventing intracellular TLR4 signalling. Novel TLR4 ligands are being developed and should be tested in highly translational animal models of I/R injury. OPN-301 is a mouse monoclonal antibody against TLR2. Administration at the end of ischaemia reduced infarct size in mice,49 which encourages further testing of OPN-301 and other TLR2 antagonists in larger animals and in humans.

The involvement of the complement system in I/R injury has been widely investigated at the preclinical level. Clinical study results using complement inhibitors, however, have been disappointing. Different reasons can be proposed for this incongruence, ranging from the use of models with low translational value (e.g. knockout animals) to the fact that negative results tend to remain unpublished. It is also of importance to realize that in human disease, in contrast to animal models, the relative importance of pro-inflammatory mediators and pathways varies between individuals, depending on factors such as the duration and severity of the ischaemia, co-morbidity, and medication. The most promising results were obtained with C1-INH.71,72 These were small pilot studies, however, that require confirmation in larger randomized placebo-controlled clinical trials. Our expanding knowledge of the involvement of the complement system in I/R injury may lead to better complement-related therapies.

Translation of therapeutic anti-inflammatory strategies to reduce myocardial I/R injury into clinical practice appears a challenging task. General inhibition of the innate immune system is associated with adverse outcome after MI. The challenge is to inhibit those specific parts of the innate immune system that cause injury, without affecting proper myocardial infarct healing. Our current body of knowledge is insufficient to fully comprehend the spatial and temporal functions of endogenous ligands and their receptors, inflammatory cells, and inflammatory mediators with pleiotrophic and synergistic or antagonistic effects in myocardial I/R injury. The myocardial tissue within the perfusion territory of an occluded coronary artery consists of a central necrotic core that expands from the endocardium to epicardium during myocardial ischaemia, surrounded by a border area of endangered cardiomyocytes with yet uncertain fate. Reperfusion limits progression of the central necrotic core, but sets into motion the molecular events causing lethal reperfusion injury in the border area. The necrotic core, ischaemic border area and reperfused myocardiun are distinct sources of mediators that can be recognized by pattern recognition pathways. The fact that all areas have been studied simultaneously in the setting of I/R injury complicates differentiation between the signals and the subsequent signalling pathways in the different areas and their contribution to outcome. It is unknown whether reperfusion induces the expression of different triggers of the inflammatory response than those that arise during ischaemia. Then there is also the systemic response that consists of mediators released from the injured myocardium and activated circulating cells. A better understanding of such issues is necessary before specific interventions are pursued on a therapeutic basis. We have to keep in mind that most studies that gave us insight into the inflammatory mechanisms involved in cardiac repair have been performed in...
knock-out animal models that impose serious limitations as to how they extrapolate into the human system. The use of highly translation- al large animal models that more closely reflect the human pathologic al situation might be an appropriate intermediate step to select large animal models that more closely reflect the human pathologic al situation. The use of highly translation- al large animal models that more closely reflect the human pathologic al situation might be an appropriate intermediate step to select large animal models that more closely reflect the human pathologic al situation might be an appropriate intermediate step to select large animal models that more closely reflect the human...
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