

Evolving role of cardiac TLR2 as therapeutic target post-ischemic injury

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Brenda B Su, Konecny F DVM. Evolving role of cardiac TLR2 as therapeutic target post-ischemic injury. *J Heart Res* December 2018;1(1): 19-22.

An inherent inflammatory response of cardiovascular system by activation of toll-like receptors (TLRs) localized in the myocardium can be set off by an increasing amount of damage-associated molecular patterns (DAMPs) in response to ischemic myocardial injury. TLR2, one of pattern recognition receptors (PRRs) interacts with an endogenous molecule initiating non-infectious cardiac inflammation cascade. Remarkably, numerous studies have demonstrated role of TLR2 as an emerging target for cardiac anti-inflammatory therapies.

Given the significance of TLR2, this short review will concentrate on DAMP-initiated TLR2 signaling and will discuss role of TLR2 absence on cardiomyocyte's cell membrane on downstream cytokine production and cell death in p38 MAPK and Akt-dependent manner. In this short review, potential of TLR2 inhibitors, which contribute to preservation of cardiac

function, will be introduced in addition to recent experimental research progress and the involvement of TLR2 in myocardial inflammation to increase the potential of TLR2 as a therapeutic target.

Key Words: TLR2, Myocardial ischemia, IL-1 β , IL-6, TNF- α , p38MAPK, pAkt, Apoptosis

Abbreviations: Akt/PKB: Protein Kinase B; AP-1: Activator Protein-1; DAMP: Damage-Associated Molecular Pattern; ERK 1/2: Extracellular Signal-Regulated Kinases; HSP: Heat Shock Protein; HMGB1: High Mobility Group Box 1; I/R: Ischemia/Reperfusion; IFN: Interferon; IkB: Inhibitory κ B; IL: Interleukin; LPS: Lipopolysaccharide; MAPK: Mitogen-Activated Protein Kinase; MI: Myocardial Infarction; MyD88: Myeloid Differentiation Factor 88; NF- κ B: Nuclear Factor- κ B; NOS: Nitric Oxygen Synthase; PI3K: Phosphatidylinositol-3-Kinase; PAMP: Pathogen Associated Molecular Pattern; PRRs: Pattern Recognition Receptors; TRAF: Family Member-Associated; NF- κ B: Activator Binding Kinase 1; TIR: Toll/Interleukin-1 Receptor; TLR: Toll-Like Receptor, TNF- α : Tumor Necrosis Factor- α ; WT: Wild Type

INTRODUCTION

Current concepts of cardiovascular research-strategies of how to reduce myocardial ischemia and reperfusion (I/R) injury discusses a) physical (pre-conditioning and post-conditioning or using hypo-/hyperthermia method) or b) using conservative pharmacological application of anti-platelet agents, statins, ACE inhibitors, beta blockers, antioxidant and anti-inflammatory strategies to inactivate conventional Toll like receptor (TLR)-NF- κ B pathway in immune cells, cardiomyocytes, endothelial cells, or c) other and its combinations [1-3]. TLR belongs to pattern recognition receptors (PRRs) which can recognize certain pattern from microbe, conserved pathogen associated molecular patterns (PAMPs) and non-communicable patterns, endogenous stress signals termed danger-associated molecular patterns (DAMP).

In many cases the school of thought is to assume that the immune response is accountable for prolonged cardiac injury following by heightened adverse cardiovascular remodelling. This notion had directed scientific investigations and search for different strategies to limit post I/R inflammatory response. At this time selective cyclooxygenase-2 inhibitors and nonselective nonsteroidal anti-inflammatory drugs were tested post-acute myocardial infarction [4], with outcome indicating that the inflammatory response is essential in initiation of multiple avenues of reparative process.

Current consensus is to limit time of the inflammatory response post myocardial ischemia rather than to suppress it. For that reason, better understanding of the inflammatory cascade and role of (PRRs) might bring us closer to shorten the time of cardiac inflammation. This would help to mitigate cardiomyocytes apoptosis, attenuate matrix degradation and improve cardiac function.

Cardiovascular innate inflammatory response to myocardial ischemia; role of TLR2

Our understanding of the evolutionarily ancient and conserved form of innate immunity system in cardiovascular tissue has evolved. A typical antigen-pathogen-associated model of e.g. bacterial lipopolysaccharides (LPS) triggering of the innate immune signal has been widened with idea of endogenous "alarm" or "danger" signal(s) coming from stressed, injured, or necrotic cardiac cells [5].

This inherent myocardial inflammatory response is set off by pattern recognition receptors (PRRs), among which, Toll-like receptors (TLRs) recognize and respond to damage-associated molecular patterns (DAMPs), consisting of exogenous pathogen-associated molecular patterns (PAMPs), structures of the pathogenic microorganisms and endogenous alarmins that are released in response to stress or tissue damage [6,7].

Released post-ischemic DAMPs could be e.g. lipid and/or carbohydrate moieties found on cardiac cell surface or proteins not normally found in the outer cell membrane. Some of post-ischemia released substances belong to endogenous ligands, such as heat shock proteins (HSP 60 and 70), high-mobility group box 1 (HMGB-1), fibronectin and other cellular matrix breakdown products such as hyaluronan, nitric oxygen synthase (NOS) from stressed, wounded or death cells [8].

These ligands activate enzymatic (e.g. protein and lipid kinases) and transcription factors, including activation of extracellular signal-regulated kinases (ERK 1/2), c-Jun N-terminal kinases, p38 mitogen-activated protein kinase (p38MAPK), phosphatidylinositol-3-kinases (PI3K), Bruton's tyrosine kinase, nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK)/activator protein-1 (AP-1) which induce a number of inflammatory cytokines, including interleukin-1 (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interleukin-8 (IL-8) and interferon- γ (IFN- γ) [9-15].

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Received: November 23, 2018, Accepted: December 17, 2018, Published: December 25, 2018



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Stimulus of myocardial TLR2 by DAMPs leads to the dimerization of the intracellular receptor domain, which plays a crucial role in recognizing substances and ligands by recruiting their adaptor proteins such as myeloid differentiation factor (MyD88) and Toll-interleukin 1 receptor adaptor protein (TIRAP) to initiate signaling cascade [7]. For more in-depth review of the initial cascade, please refer to [7,16]. This short review will concentrate on DAMP-initiated TLR2 signaling and will discuss role of TLR2 absence on cardiomyocyte's cell membrane on downstream cytokine production and cell death in p38 MAPK and Akt-dependent manner.

Role of DAMPs in the TLR2-initiated cardiomyocyte signaling

Under ischemic conditions, the initial DAMPs-associated trigger leads to a sterile inflammation, which propagates non-infectious inflammation. Numerous recent reports assembled evidence what role TLR2 plays in the ischemic myocardium and what is the role of different DAMPs in the TLR2 initiated sterile inflammation. Several studies found that TLR2 respond to DAMPs in forms of endogenous proteins, as e.g. heat shock proteins (HSP) and high mobility group box 1 (HMGB1), released from stressed and/or injured cells [17].

In 2005 interesting submission discussed dying stem cells hypothesis by claiming that local immune reactions ensue in response to apoptosis of dying stem cell releasing variety of DAMPs, which in turn improve cardiac function post-MI [18]. In 2007, Chen et al. [19] published study showing that products of necrotic cells, but not an apoptotic, stimulate the immune system through an IL-1RI-dependent manner rather than through TLR signaling pathway.

Although both IL-ligands (α and β) initiate the pathway through MyD88 activation culminating in NF- κ B induced transcription of inflammatory genes, role of both is different. IL-1 β that is the predominant ligand in the late stages of a sterile inflammation thus further acts as a propagator of inflammation by recruiting mono-macrophages into the region [20], while IL ligand α was recently linked to an early stage of ischemia-reperfusion with its role as a cardiomyocyte alarmin [21].

In our hands, significant upregulation of cytokine levels happened at day 3 in the ischemic post-infarcted areas as e.g. levels of IL-1 β , IL-6 and TNF- α . Interestingly, all measured cytokines were augmented while levels of IL-1 β were persistent in our model, and not significantly declining at day 7 in wild type (WT) mice, supporting our earlier observation of the role of this ligand as late-perpetual inflammatory propagator [22]. We have later confirmed that the activation of the IL-1 β ligand helped to significantly increase mono-macrophage cells carrying CD11b epitope, which has been detected at day 3 in WT animals, further promoting recruitment of other white blood cell lineages through activation of TLR2 (authors unpublished observations).

Although the assumption that most cytokines during an early stage of an ischemic infarct embracing consistently negative role as e.g. in case of TNF α activation, TNF α has displayed rather interesting cardiac signaling dichotomy in heart. As e.g. shown by [23], its surface receptors i.e. TNFR1 and TNFR2 are up-regulated during progression of remodeling in murine HF, indicating its heightened signaling intensity.

In 2007, the role of TNF- α was further scrutinized using isolated perfused heart system in TLR2 knock out mice (TLR2 $^{-/-}$). Using 30 min of ischemia (30I) and 30 or 60 min of reperfusion (30R or 60R), Sakata et al. [24] showed that levels of TNF- α were significantly attenuated in the hearts at 30I/30R TLR2 $^{-/-}$ as compared to WT. Additionally, levels of ligand beta (IL-1 β), downstream of TNF- α /TRAF6, at 30I/30R and 30I/60R were also increasing during longer reperfusion in WT, which once more shows its role as an inflammatory propagator in the sterile environment [19,24,25].

Apoptosis of cardiac cell prevented *in vitro* as well *in vivo* using TLR2 $^{-/-}$ mice MI-model

The upregulation of various cytokines and activation of TLRs is part of an activation of the innate immune response in post-MI and ischemia-reperfusion (I/R) injury. One of the mechanisms is through conserved

membrane immune TLR2 receptor and MAPK pathway [22]. We have observed a significant over-expression of phosphorylation of p38 MAPKs occurring mainly in the infarcted area of infarction border zone of WT mice. As this protein kinase is implicated in the immune reactions, promoting further expression of pro-inflammatory cytokines such as IL-1 β , TNF- α , IL-6, and cell adhesion molecules (VCAM-1) [26], it is also linked to myocardial apoptosis [27].

Additionally, as being pro-apoptotic, Nishida et al. [27], reported another important role of p38 as being highly pro-fibrotic. By using LV pressure overload model and cardiac specific p38 knock-out mouse under MHC promoter this group showed that p38 plays an essential role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. Additionally, overexpression of activated p38 reduced Bcl-2 protein levels in neonatal myocytes [28]. Taken together, in our *in vitro* and *in vivo* experiments, the upregulation of p38 MAPK lead in WT, but not in TLR2 $^{-/-}$ mice, to heightened apoptosis that has occurred with a maximal intensity at day 3 post-cardiac ischemia.

As further confirmed *in vitro* using cardiac myofibroblasts lacking TLR2 in the same heart samples, reduction of p38 MAPK was detected at dose dependent increase of phosphorylated Akt (pAkt) [22]. Akt belongs to serine/threonine kinases, which in myocardium promotes cell survival [29].

Our results using specific Akt (473) antibody, similar to an earlier I/R study, an elegant contribution by Krieg et al. [30], where the Akt 473 phosphorylation reduced apoptotic cardiomyocyte death in response to I/R in rabbits, directly illuminating of a role of this tyrosine kinase in the upstream activation of Phosphoinositide 3-kinase (PI3K).

Others showed that PI3K/Akt signaling pathway might be involved in the endogenous negative feedback mechanism using TLR2 ligands Pam3CSK4 injected prior to I/R thus limiting pro-inflammatory and pro-apoptotic events while using this Ligand-I/R preconditioning method and generating cardio-protection against the otherwise injurious TLR2-NF κ B [31,32].

PI3K at early phase of TLR2 signaling might modulate the magnitude of the primary pathway activation or as reported by Dong et al. [33], TLR2-TIRAP summons an early myocardial warning, a cyto-protection through dependent signaling pathways upstream of IRAK1 before an activation of NF κ B. Taken together PI3K/Akt constitutes a survival signaling pathway that can be influenced by several cardio-protective ligand-receptor systems; further reviewed by [34].

Strategies should be explored to limit activation of DAMPs such as protein and peptides: (HSP 22, 60, 70, 72), HMGB1, fibronectin, fibrinogen, S100 proteins and other ligands [1], released from stressed and/or injured cells that lead to activation of TLR2-NF κ B and followed by cytokine overproduction, in our case IL-1 β . This IL-1 ligand observed by many [24,25,35] in different model of heart failure, shows that both, an early and as well as the late responses are able to activate tissue-resident mono-macrophage cell lineages, which activated by upstream TRAF6 negatively influence MAPK [36], namely p38MAPK [22], leading to apoptosis.

These considerations support hypothesis that different DAMPs might play significant but distinctive role in activation of sterile inflammatory response and are involved in progression of sterile DAMP-originated cardiovascular inflammation. Development of pharmacological interventions are underway to interfere with an expression and/or activity of these receptors and will lead to new treatments as e.g. recently tested TLR2 inhibitors (OPN-305) and similar NLR family, pyrin domain-containing 3 (NLRP3), validated for clinical testing [37-39].

Interestingly, restraining effect of activated cytokine IL-1 β signaling using interference with the TLR2 and the NLRP3 receptor's pathways showed some early interest during clinical/translational studies; namely the clinical trial of the ligand beta (IL-1 β) inhibition post-MI was very encouraging [40,41].

CONCLUSION

We have discussed in this short review the role of innate immune system, specifically the role of TLR2 during sterile inflammation activated by myocardial ischemia. Moreover, our data indicated the role of DAMPs in TLR initiated cardiomyocyte signaling, the activity of cytokine IL-1 β and its persistent high levels during ischemic injury promoted recruitment of white blood cell lineages in heart. This has led to increase of MAPKp38 promoting further the cytokine storm while increasing cardiac apoptosis that was attenuated in TLR2-/- mice. Later using isolated from TLR2-/- mice, pro-survival dose dependent increase of phosphorylated Akt 473 (pAkt) was detected, revealing role of TLR2-mediated cell survival signaling during acute inflammation post-myocardial ischemia.

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