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Toll-like receptor signaling in the ischemic heart

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1. ABSTRACT

Toll-like receptors (TLRs) have been identified as primary innate immune receptors. They distinguish among different patterns of pathogens and rapidly activate an innate immune response. However, TLRs can also be stimulated by host-derived molecules. TLRs are expressed in the cardiovascular system and could thus be a key link between cardiovascular diseases and the immune system. Increasing evidence suggests that TLR signaling promotes injury in the heart after ischemia, ischemia/reperfusion or hypertrophic stimuli. Herein we review the experimental and clinical evidence for the involvement of TLR and its downstream signaling molecules in cardiac ischemic injury.

2. INTRODUCTION

Toll like receptors (TLRs) have emerged as the primary, not antigen-specific defense mechanisms that enable *innate* immune detection of foreign pathogens and rapid anti-pathogen defense mechanisms. To date, 11 human and 13 mouse TLRs have been cloned (1). The ligands for TLRs are molecular motifs produced by pathogens and not by the host. For example TLR4 recognizes cell wall components of Gram-negative bacteria (2-6).

At first glance, pathologic mechanisms in cardiac diseases seem far from involving pathogen defense

mechanisms. However, several lines of evidence link the activation of the immune system with cardiovascular diseases like atherosclerosis, cardiac ischemia/ reperfusion injury, and even heart failure, although there is no evidence for a specific pathogen in their etiology. The activation of TLRs is not restricted to the immune system. Indeed, increasing evidence indicates that TLRs can also detect host-derived molecules under certain circumstances. For example, heat shock protein 60 (HSP60), a molecular chaperone conserved in both invertebrates and vertebrates, can activate NF-κB through both TLR2 and TLR4 (7). Moreover, upon tissue injury, fragments of hyaluronan and fibronectin are released that can activate NF-kB through TLR4 and TLR2 (8, 9). Thus, the extracellular matrix can turn on the innate immune response via TLRs when altered after tissue destruction even in the absence of pathogens. However, these results have to be interpreted with caution since agents used for TLR stimulation experiments could be contaminated e.g. with LPS and lead to false positive findings. Nevertheless, these endogenous TLR ligands may activate the innate immune response in cardiovascular diseases like ischemic cardiac injury.

3. TLR SIGNALING

TLRs are type 1 membrane-spanning receptors, with a leucine-rich repeat motif and a signaling motif similar to the interleukin-1 and interleukin-18 receptor (now termed the "toll-interleukin 1 receptor" or "TIR" homology domain) (1). TLR1, -2, -4, -5, -6 are expressed on the cell surface. TLR3, -7, and -9 are found in intracellular endosomal compartments. TLR8 appears to be localized primarily intracellularly with a small portion expressed on the cell surface. This allows recognition of plasmatic as well as intracellular ligands.

Each TLR activates similar, general signaling pathways, but also triggers its specific pathways. This differential induction pattern mainly depends on cytoplasmic adaptor molecules that can associate with the intracytoplasmic portion of TLRs (10). There are different TLR adaptor molecules (see Figure 1): MyD88 was first found to be critical for TLR signaling (*MyD88-dependent pathways*). MyD88 can associate with all TLRs except TLR3. After association of the TLRs with the adapter protein MyD88, IRAK 1 and 4 (interleukin receptor associated kinase), and TRAF6 (tumor necrosis factor receptor-activated factor-6) are recruited. The IRAK-4/IRAK-1/TRAF6 complex then interacts with another membrane complex involving TAK1 (transforming growth factor β activating kinase), TAB1 (TAK1-binding protein), and TAB2/3). This induces the phosphorylation of TAB2 and TAK1, and their translocation to the cytosol, together with TRAF6 and TAB1. TAK1 subsequently phosphorylates IKK (IκB kinase)-β and MAP kinase kinase 6, which results in the activation of MAP kinases and phosphorylation of IκB, thereby promoting NF-κB (nuclear factor kappa B) translocation to the nucleus and gene transcription (11, 12).

Subsequently, however, *MyD88-independent pathways* were discovered. TLR4 and -3 can also activate TRIF (TIR domain-containing adaptor protein inducing IFN-β)-dependent pathways without MyD88 association. TRIF then recruits TBK1 and mediates phosphorylation of the transcription factor IRF3 (interferon regulating factor) which leads to the production of interferon and co-stimulatory molecules (1).

The TLR-class-specific signaling cascades allow different TLRs to trigger distinct signaling pathways. For example, TLR4 responses include secretion of IL-10, IFN-β and IL-12, whereas TLR2 responses involve IL-8, IL-12, and IL-23 (13). Taking into account tissue-specific expression of TLRs as well as the possibility of heterodimer or homodimer formation for TLR activation, TLR-dependent innate immunity signaling pathways do trigger responses with some degree of specificity, allowing distinct immune responses for different ligands.

4. TLRS IN ISCHEMIC HEART DISEASE

In addition to the role of TLR signaling in atherosclerosis, that has been the focus of other reviews (14, 15) and is beyond the scope of this article, several reports suggest a regulation of TLRs in patients with ischemic heart disease. For example, an increase of circulating TLR2 or -4 positive monocytes was observed in unstable angina, acute myocardial infarction and chronic heart failure (16, 17). Activation of TLR4 in monocytes is associated with the development of heart failure after acute myocardial infarction (18).

Whereas the clinical evidence is only indirect, direct hints for a role of TLRs in ischemic heart disease comes from experimental data. Indeed, TLRs are readily detectable in cardiac myocytes. TLR2, -3, -4, -6, -7 and -9 are expressed in ventricular myocytes, whereas TLR1 and -5 are not (12, 19, 20). *In vitro*, a potential role for TLR2 in the response to oxidative stress has been established in neonatal rat cardiac myocytes. Blockade of TLR2 function inhibited hydrogen peroxide-induced NF-κB activation and diminished cytotoxicity and apoptosis (19). On the other hand, TLR4 activation can reduce apoptosis of cardiac myocytes, an effect mediated by NOS2 (21). Activation of TLR2 and -4 reduces myocyte contractility and cytokine secretion in HL-1 cells, an immortalized cell line with adult cardiac myocyte properties (20). This suggests that TLR signaling may be important for myocardial diseases.

In fact, the activation of TLRs and the innate immune system plays a central role in ischemia/ reperfusion injury. $TLR4^{-/-}$ mice have reduced infarct sizes 2 hours (22) and 24 hours after reperfusion (23) (see Figure 2). Pro-inflammatory cytokines, NF-κB activation and infiltrating inflammatory cells are reduced after reperfusion, whereas neutrophil function in general is preserved in $TLR4^{-/-}$ mice. On a molecular basis this effect seems to be mediated through a phosphoinositide 3-kinase (PI3K)-dependent mechanism (24) since PI3K inhibition abrogated myocardial protection. Similarly, $TLR2^{-/-}$ mice are protected from ischemia/reperfusion injury (25) accompanied by a reduced neutrophil accumulation, lower cardiac reactive oxygen species and proinflammatory

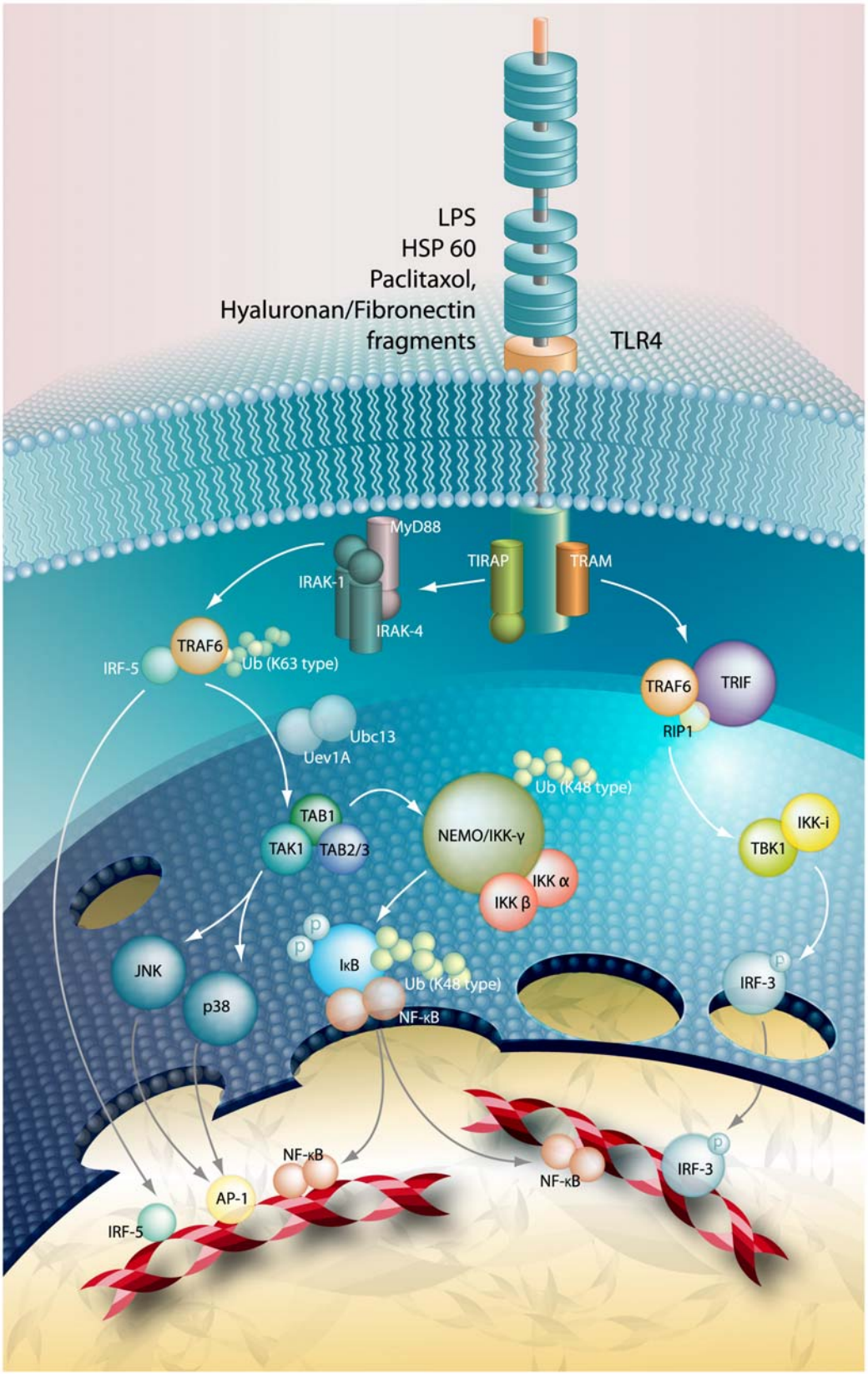

Figure 1. Toll signaling pathway. TLR signaling pathway in general. For details and abbreviations see the text.

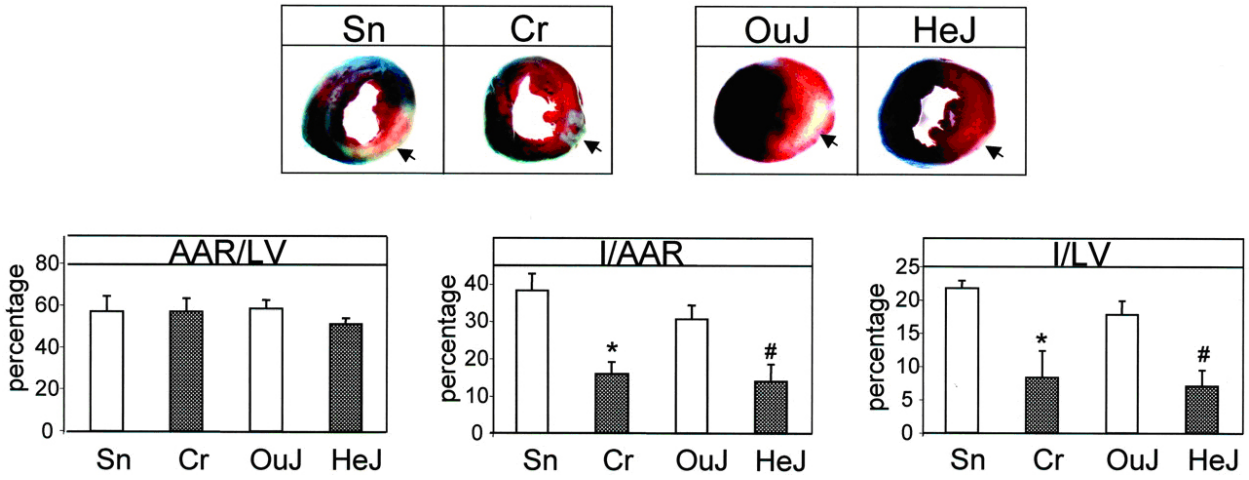

Figure 2. Myocardial infarct size after 1-hour ischemia and 24-hour reperfusion in mice that lack functional TLR4 signaling (Cr and HeJ) versus wild-type controls (Sn and OuJ, respectively). Top, representative sections distal to the site of ligation. Blue area depicts perfused tissue; red plus white, at-risk tissue; white (arrows), infarcted tissue. Lower bar graphs from left to right depict the AAR as a percent of the LV area, infarct size (I) as a percent of the AAR, and infarct size as a percent of the LV area (I/LV). Results are mean±SEM from Cr (n=7), Sn (n=8), HeJ (n=4), and OuJ (n=5). *Significant difference between Sn and Cr; #Significant difference between OuJ and HeJ. Comparisons made by Student's *t* test at $P<0.05$. (Figure reprinted with permission of (23)).

cytokines. Moreover, the ischemia/reperfusion-induced coronary endothelial dysfunction is abolished in TLR2$^{-/-}$ mice. To examine the cellular compartment involved in endothelial injury, bone-marrow chimeric mice were created by transplantation of TLR2$^{-/-}$ bone marrow in WT mice or WT bone marrow in TLR2$^{-/-}$ mice. Both chimeric mice displayed similar protection as TLR2$^{-/-}$ mice indicating a role of non-bone-marrow cells and cells of bone marrow origin in endothelial dysfunction.

Inhibition of TLR signaling components can also attenuate ischemia/ reperfusion injury. E.g. blockade of MyD88 by myocardial transfection of a dominant-negative MyD88 significantly reduces myocardial ischemic injury in the rat (26). As pointed out above, TLR signaling converges on the activation of the transcription factor NF-κB. The role of NF-κB in ischemia/ reperfusion injury is well defined by now (27). After ischemia/reperfusion injury, NF-κB activation is biphasic, with peaks after 15 minutes and 3 hours attributed to the release of reactive oxygen species and the production of inflammatory cytokines, respectively (28, 29). As for TLR, ischemia/ reperfusion injury is reduced by inhibition of NF-κB using molecular inhibition of p65 by double-stranded oligonucleotides (30), use of an IκB triple mutant (S32A, S36A, Y42A) completely abrogating NF-κB activation (31), as well as pharmacological methods (IKK inhibition (32)). We recently demonstrated that mice with targeted deletion of the NF-κB subunit p50 are protected against ischemia/ reperfusion injury (30). KO and WT animals underwent 30 minutes of coronary artery ligation and 24 hours of reperfusion *in vivo*. Ischemia-reperfusion damage was significantly attenuated in the p50 KO compared to WT mice. Although adhesion molecules such as ICAM were upregulated in left ventricles of p50 KO animals, fewer neutrophils infiltrated the infarct area, suggesting leukocytes as a potential mediator of the protection observed in the p50 KO. This was confirmed in adoptive transfer experiments: Whereas transplantation of KO bone marrow in KO animals sustained the protective effect on ischemia-reperfusion injury, transplantation of WT bone marrow in KO animals abolished it. Thus impaired NF-κB activation in p50 KO leukocytes attenuated cardiac damage. Collectively, these data suggest that activation of TLR/MyD88/NF-κB-dependent signaling pathways in myocardial ischemia/ reperfusion injury plays a critical role in the pathophysiology of acute heart diseases.

Heart failure is a complex disorder with several contributing pathophysiologic systems including inflammatory responses (33, 34). TLRs and their signaling components are upregulated in experimental or clinical heart failure. TLR4 expression is increased in the myocardium of patients with advanced heart failure (12, 35). In addition, there is a change in the TLR expression pattern: whereas in normal murine and human myocardium TLR4 expression is diffuse and predominantly confined to cardiac myocytes, myocardium from patients with advanced heart failure displays focal areas of intense TLR4 staining (see Figure 3). The reason for this change in TLR4 expression in the remodeled failing myocardium is not yet known (12). TLR signaling converges on the activation of IRAK-1 and the transcription factor NF-κB (see Figure 1). In line with the previous findings, IRAK-1 as well as NF-κB are activated by cardiac ischemia or in experimental and human heart failure (see Figure 4) (36-38); NF-κB is also increased in peripheral leukocytes of patients with stable heart failure (39). Taken together, the evidence is strong that TLR and its signaling components are activated by ischemic heart failure.

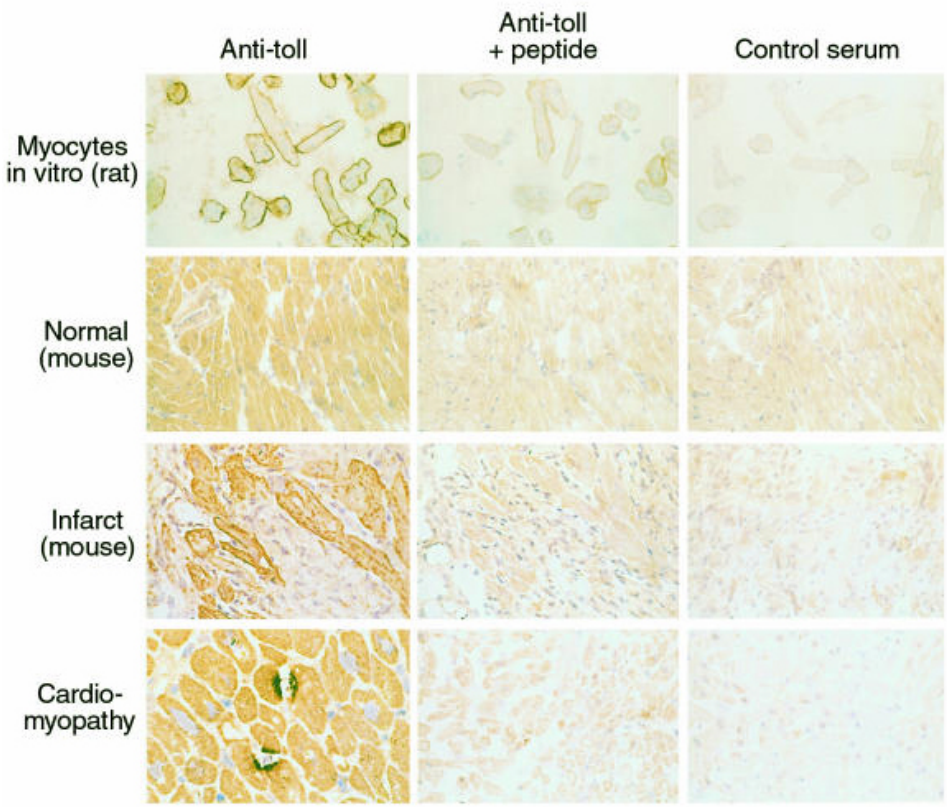

Figure 3. TLR4 in rat, murine and human myocardium. Primary isolates of adult rat ventricular myocytes 24 hr after isolation, stained with a polyclonal antibody targeted to a TLR4-specific epitope adjacent to the cytoplasmic TIR domain of hTLR4 (upper panel). Normal murine cardiac muscle (magnification 200x; second panel) exhibited diffuse, homogeneous myocyte staining. However, cardiac myocytes adjacent to an area of ischemic injury induced by coronary artery ligation exhibited intense sarcolemmal TLR4 staining. Finally, cardiomyocytes from humans with dilated cardiomyopathy (lower panel) displayed intensely stained focal expression of TLR4 (Figure reprinted with permission of (12)).

The coronary artery ligation model is the best-established and clinically most relevant experimental heart failure model. After coronary artery ligation, mortality and left ventricular dilatation were significantly reduced, and left ventricular function was preserved in TLR2$^{-/-}$ mice compared to WT mice. These effects may be mediated by TLR-dependent changes in extracellular matrix remodeling (40). Furthermore, left ventricular remodeling is improved in mice with TLR4 defectiveness together with a reduction of pro-inflammatory cytokines, alterations of extracellular matrix remodeling, but no change in the rate of apoptosis (41). The influence of TLR signaling on extracellular matrix remodeling is supported by the fact that TLR4-MyD88-NF-κB activation can enhance fibrosis in the liver (42).

In parallel, mice with targeted deletion of the NF-κB subunit p50 are protected from left ventricular dilatation after myocardial infarction and have preserved left ventricular function. Collagen content and matrixmetalloproteinase (MMP)-9 expression are significantly lower in KO mice after myocardial infarction and may account for improved left ventricular remodeling (36). Thus, TLRs and their downstream signaling components are important in left ventricular remodeling after myocardial infarction.

The TLR signaling cascade (see Figure 1) also plays a role in cardiac hypertrophy. TLR4$^{-/-}$ mice develop less cardiac hypertrophy following pressure overload by aortic banding when compared to matching WT animals (43). Inhibition of mTOR resulted in an additional decrease in the development of cardiac hypertrophy, indicating separate pathways for TLR- and mTOR-mediated effects. In line with these findings, cardiac-specific overexpression of TAK-1 results in cardiac hypertrophy (44). Blockade of MyD88, a downstream signaling component of the TLR signaling cascade, by adenoviral overexpression of a dominant negative construct significantly reduced left ventricular hypertrophy after aortic banding and improved cardiac function potentially mediated by a reduction of apoptosis (45). *In vitro* studies further suggest that activation of NF-κB is required for hypertrophic growth of neonatal rat ventricular myocytes in response to angiotensin II, phenylephrine, and endothelin-1 (46, 47). In mice with targeted disruption of the p50 NF-κB subunit cardiac hypertrophy induced by angiotensin was significantly attenuated (48). Accordingly, mice with

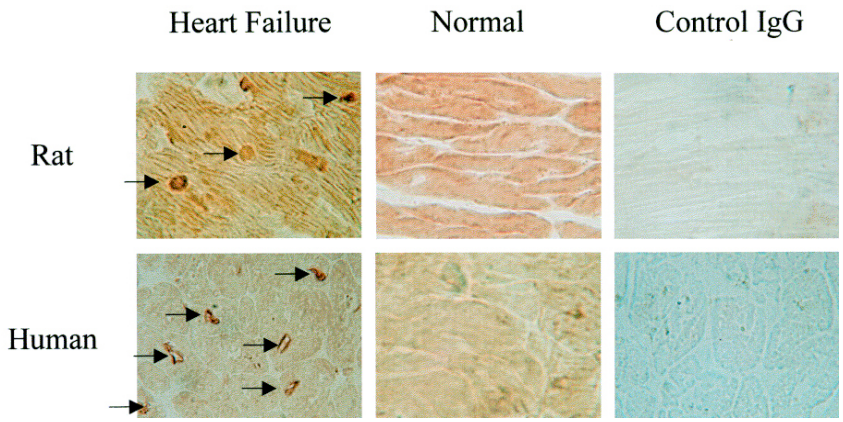

Figure 4. Immunohistochemical analysis of NF-κB in rat and human cardiac muscle. Photomicrographs (x400) are shown from rat hearts 10 weeks post myocardial infarction and human failing hearts collected at the time of transplantation. Activated p65, visualized by nuclear translocation of p65, was mainly immunolocalized to cardiac myocytes in experimental and human heart failure (representative nuclei are marked). (Figure reprinted with permission of (50)).

cardiomyocyte-restricted expression of a NF-κB super-repressor had impaired angiotensin II-induced cardiac hypertrophy (49). These results conclusively establish TLR as an important pro-hypertrophic pathway in addition to mTOR signaling.

5. CONCLUSION

Toll-like receptors are an important family of innate pattern recognition receptors that both trigger innate immune effector proteins and provide essential co-stimulatory signals for adaptive immunity. TLRs are readily detectable in the cardiovascular system and upregulated in ischemic cardiac diseases. There is good experimental evidence that inhibition of TLR signaling could reduce cardiac damage after ischemic injury. However, a transfer of these results in the clinic is hampered by the fact that an intact immune system is necessary for many protective pathways. On the other side, prolonged immune activation may also activate unfavourable signal cascades that drive disease progression. Thus, further research in this field is necessary to find a consensus as to the specific role played by the innate immune system in these diseases. Finally, a growing understanding of the innate immune system in the heart could lead to novel therapeutic strategies.

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