

Rapid communication

# Toll-like receptor stimulation in cardiomyocytes decreases contractility and initiates an NF- $\kappa$ B dependent inflammatory response<sup>☆</sup>

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## Abstract

**Objective:** The transmembrane receptor family of Toll-like receptors (TLRs) may play a role in initiating early inflammatory and functional responses to danger signals arising from ischemia-reperfusion and inflammatory stimuli. We determined whether Toll-like receptors are expressed in cardiac tissue and whether stimulation with cognate ligands would result in a pro-inflammatory response and decreased cardiomyocyte contractility.

**Methods and results:** We observed mRNA expression of TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 in both whole heart tissue and a murine cardiomyocyte cell line (HL-1). Ligand activation of TLR2, TLR4 and TLR5, but not TLR3, TLR7 or TLR9, resulted in cardiomyocyte expression of the inflammatory cytokine IL-6, the chemokines KC and MIP-2, and the cell surface adhesion molecule ICAM-1. Activation of these Toll-like receptors was associated with decreased cardiomyocyte contractility. Using transfection of a nuclear factor kappa B (NF- $\kappa$ B)-Luciferase reporter plasmid, we found significantly increased NF- $\kappa$ B transcriptional activity in response to TLR2, TLR4 and TLR5 activation in cardiomyocytes. Further, a chemical inhibitor of NF- $\kappa$ B, pyrrolidine dithiocarbamate (PDTC), as well as transfection using a dominant negative form of IKK $\beta$ , resulted in profound reduction of the TLR-initiated pro-inflammatory response.

**Conclusions:** Cardiomyocytes express most known Toll-like receptors. Of these, TLR2, TLR4 and TLR5 signal via NF- $\kappa$ B, resulting in decreased contractility and a concerted inflammatory response.

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**Keywords:** Infection/inflammation; Contractile function; Cytokines; Myocytes

## 1. Introduction

Cardiomyocytes are specialized cells whose primary function is contraction. Increasing evidence demonstrates that cardiomyocytes have fundamental other properties, analogous in some respects to innate immune dendritic cells, so that cardiomyocytes can respond to danger signals with a complex inflammatory and functional response. For example, in response to insults as diverse as ischemia-reperfusion and systemic inflammatory stimuli, cardiomyocytes along

with neighboring cells express the pro- and anti-inflammatory cytokines IL-6 and IL-10, able to initiate and then regulate a local inflammatory response [1]. Cardiomyocytes express chemokines such as KC and MIP-2 [2,3], which recruit and activate appropriate inflammatory cell subsets necessary for response and repair, and cardiomyocytes express cell surface adhesion molecules, importantly ICAM-1 [4–10], allowing interaction with and outside-in signaling from participating inflammatory cells and the extracellular matrix. Furthermore, a cardiomyocyte inflammatory response involving cytokines [11], chemokines and the subsequently recruited leukocytes [10,12], and cell surface adhesion molecules [4,10] leads to decreased cardiomyocyte contractility thereby modulating peak systolic stress and strain, which conceivably may impact repair processes. Thus, a second key function of cardiomyocytes is

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to respond to danger signals with a directed and complex inflammatory response, including modulation of cardiomyocyte contractility.

What are the first steps that trigger the cardiomyocyte response to danger signals? Here we postulate a novel role for cardiomyocytes analogous to that of dendritic cells, which have an initial response directed by Toll-like receptors. Toll-like receptors are evolutionarily ancient molecules initially discovered as receptors for endogenous ligands such as Spatzle protein in *Drosophila*, but subsequently were found to be pattern recognition receptors (PRR) that respond to exogenous ligands for pathogen-associated molecular patterns (PAMPs) [13]. More recently, Toll-like receptors have been found to respond to endogenous ligands including heat shock proteins HSP60, HSP70, and gp96 [14–16] which are released during necrotic cell death [17,18]. Following Toll-like receptor ligation, immune cells signal through NF- $\kappa$ B to produce the diverse cytokines and chemokines required for leukocyte activation and chemotaxis [19]. Specialized tissues such as skeletal muscle [20] and synovial tissue [21], initially thought to be bystanders in the immune response to pathogens, have recently been found to be active participants in response to Toll-like receptor ligation. Cardiomyocytes express TLR2 [22] and TLR4 [23] yet the extent of Toll-like receptor expression and the role of Toll-like receptors in the cardiomyocyte inflammatory response and decreased contractility have not been fully elucidated [1,22,24,25].

Accordingly, the goal of this study was to investigate the ability of cardiomyocytes to participate in an innate immune response via Toll-like receptor signaling. We hypothesized that cardiomyocytes would express a number of Toll-like receptors and that some of them would signal through the NF- $\kappa$ B pathway to express physiologically relevant pro-inflammatory cytokines (IL-6), chemokines (KC and MIP-2) and cell surface adhesion molecules (ICAM-1). Furthermore, we postulated that Toll-like receptor ligation could have important functional consequences and lead to an associated decrease in cardiomyocyte contractility.

## 2. Materials and methods

### 2.1. Cell culture and incubations

#### 2.1.1. Cell line

HL-1 cells are an immortalized cell line with adult cardiac morphological, biochemical, and electrophysiological properties, including contraction and biochemical response to cognate ligands [26]. The cell line was kindly provided by Dr. William Claycomb. Cells were acquired at passage number 23 and subsequently used between passage 23 and 26. Cells were grown in Supplemented Claycomb media (JRH Biosciences, Lenexa, Kansas) with 10% FBS, penicillin and streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine. All transfections and stimulations were performed with the cells at confluence.

Incubations were carried out in supplemented Claycomb media+10% FBS with the following protocols. 100  $\mu$ M PDTC (Sigma, Oakville, ON) was added 1 h prior to stimulation and again with the stimulation media. Toll-like receptor ligands were added 4 h prior to harvesting for RNA and 24 h prior to harvesting conditioned media in Supplemented Claycomb media with 10% FBS, penicillin and streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine. *S. aureus* Peptidoglycan 10  $\mu$ g/mL, PolyI:C 0.25–25  $\mu$ g/mL, *E. Coli* LPS 1  $\mu$ g/mL, *Salmonella thyphimurium* Flagellin 1  $\mu$ g/mL, Loxoribine 0.01–1 mM (Invivogen, San Diego CA), and 0.1–10  $\mu$ M CpG with sequence TCCATGACGTTCCCTGACGTT and NonCpG with sequence GCTTGATGACTCAGCCGGAA phosphorothioated oligonucleotides (Sigma, Oakville, ON) were used as TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 ligands respectively.

#### 2.1.2. Primary murine cardiomyocytes

All animal experiments had institutional ethics approval and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Murine ventricular myocytes were isolated from 10 to 14 week old adult C57/BL6 mice. Animals were injected with heparin (1000 U/kg i.p.) 20 min prior to the experimental protocol and anesthetized using ketamine/xylazine (0.1 mL/100 g i.p.). Hearts were immediately placed in ice cold buffer solution (120 mM NaCl, 5.4 mM KCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.6 mM glucose, 20 mM NaHCO<sub>3</sub>, 1.6 mM MgCl<sub>2</sub> pH 7.4), then hung on a Langendorf perfusion apparatus with the same buffer (now 37 °C) running at 2 mL/min. Hearts were then perfused with buffer containing 0.125 mg/mL type B collagenase (Worthington, Lakewood, NJ) and 2.5  $\mu$ g/mL type XIV protease (Sigma, Oakville, ON) for 2 min. At this stage CaCl<sub>2</sub> was added to a concentration of 50  $\mu$ M and the enzyme digestion continued for 14 min. The heart was then removed from the perfusion rig and the atria removed. The digested heart was aspirated through a plastic transfer pipet until all tissue was disrupted, and the cells were then pelleted through centrifugation for 1 min at 500 rpm. Subsequently cells were re-suspended in buffer containing 125  $\mu$ M CaCl<sub>2</sub> and allowed to settle for 10 min at room temperature. This process was repeated for calcium concentrations of 250  $\mu$ M and 500  $\mu$ M. Cells were then re-suspended in AS media (Cellutron, Highland Park, NJ) at a density of 20,000 cells/mL and placed in a 37 °C incubator on matrigel (BD Biosciences, Franklin Lakes, NJ) coated coverslips. When cells were to be kept in culture greater than 8 h 10 mM 2,3-butanedione monoxime was added to the media. All subsequent stimulations were carried out in AS media with 10 mM 2,3-butanedione monoxime, and 30 min prior to electrical field stimulation the media was changed to AW media (Cellutron, Highland Park, NJ) with 0.2 mM CaCl<sub>2</sub> and 1 mM noradrenaline. Cells were then paced at 60 Hz, using the Grass S48 stimulator (Grass-Telefactor, Warwick, RI) with a voltage set at 120% of the threshold

capture voltage. Images were captured using a Myocam video camera (Ionoptix corp, Milton, MA) and analyzed using an Ionoptix Softedge detection package (Ionoptix corp, Milton, MA). Fractional shortening was calculated as the difference between diastolic and systolic length, divided by diastolic length.

### 2.1.3. Echocardiography

10–12 week old NF- $\kappa$ B knockout mice (*129P-Nfkb1tm1Bal/J*, Jackson Labs) and background strain C57BL/6 male mice weighing 25–30 g had baseline echocardiographic studies using the Vevo 770 cardiac ultrasound (Visualsonics Toronto Canada) while under 1% isoflurane anaesthesia. Short axis 2D views were obtained at the level of the papillary muscles and M-Mode images obtained. Left ventricular internal diameter in systole and diastole were identified and used for quantification of cardiac ejection fraction using the manufacturer's software. The following day LPS 40 mg/kg was injected intraperitoneally and 6 h later echocardiography was again performed.

### 2.1.4. Transfections

Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as per the manufacturer's instructions using 8  $\mu$ g DNA per well and OPTIMEM media (Invitrogen, Carlsbad, CA). Cells were incubated at 37 °C for 4 h with Lipofectamine 2000 DNA complexes, then supplemented Claycomb media+10% FBS for 24 h before trypsinization. One 60 mm plate was reconstituted to 12 mL in supplemented Claycomb media +10% FBS and cells were plated into one 6 well plate. A dominant negative mutant form of the I $\kappa$ B kinase, IKK $\beta$  (provided by P. Barker, McGill University, Montreal, Canada), was employed to inhibit NF- $\kappa$ B activation as previously described [27]. Transfection efficiency, as determined using a GFP containing plasmid, was 50–60% at 24 h.

### 2.1.5. RNA extraction and PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. RNA was obtained from in vivo hearts of 8–10 week old C57/BL6 mice and from HL-1 cells at confluence. 1  $\mu$ g of RNA was treated with DNase I Amplification Grade (Invitrogen, Carlsbad, CA) and the product underwent RT-PCR using M-MLV RT (Invitrogen, Carlsbad, CA) followed by PCR amplification with Taq DNA Polymerase (Qiagen, Valencia, CA). PCR was 35 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s. Primers were as follows, TLR2 480 bp Forward CACCATTTCACGGACTGTGGTACCTG; TLR2 Reverse CTGGCCTTATCCAAGGGCCACTCCAG; TLR3 Forward GACTGGGTCTGGGAACATTTCTCC; TLR3 Reverse AGCTCCAATGTGCTACTTGGCAATTTGT; TLR4 450 bp Forward ATCTACTCGAGTCAGAATGAGGACTGG; TLR4 Reverse CTCTGCTGTTTGCTCAGGATTCGAGGC; TLR5 580 bp Forward GGAATCTGTTTCCTGTGTGCTA TAAGACC; TLR5 Reverse ATGGTTGCTATGGTTTCG CAACTGGATGG; TLR7 277 bp Forward TCTATTCA

GAGGCTCCTGGATGAC; TLR7 Reverse CTTCAGGTAC CAAGGGATGTCCTA; TLR9 400 bp Forward CTA GACGTGAGAAGCAACCCTCTG; TLR9 Reverse CAGCTCGTTATACACCCAGTCGGC; HPRT 400 bp Forward GTGGATACAGGCCAGACTTTGTTG; HPRT Reverse GAGGGTAGGCTGGCCTATTGGCT. PCR products were run on a 1% agarose gel with 0.05% ethidium bromide and visualized under ultraviolet light. For each sample a negative control lacking RT was included to exclude genomic DNA contamination.

### 2.1.6. Luciferase assay

HL-1 cells were transfected as above using pNF- $\kappa$ B-Luc vector (BD Biosciences, Franklin Lakes, NJ) and a Renilla pGL4.74 [hRluc/TK] vector (Promega, Madison, WI). 7.7  $\mu$ g pNF- $\kappa$ B-Luc was used in conjunction 0.3  $\mu$ g Renilla pGL4.74. Cells were washed with ice-cold PBS and lysed using 250  $\mu$ L Passive Lysis Buffer (Promega), kept on ice 30 min then centrifuged at 13,000 rpm and the supernatant stored at –20 °C. 20  $\mu$ L of sample was added to duplicate wells in a luminometry plate, then read using the Dual-Luciferase Reporter Assay (Promega #1910) using a Fluostar Optima Luminometer (BMG Labtech, Durham NC). Relative Light Units (RLU) were obtained for both Renilla and the Firefly-Luciferase and all results expressed as the ratio of Luciferase RLU to Renilla RLU.

### 2.1.7. Ribonuclease protection assay

10  $\mu$ g of total RNA was used for each reaction using the BD RiboQuant RPA Package (BD Biosciences Cat no 556144) and a custom Mouse Template Set including GAPDH, L32, KC, MIP-2, and ICAM-1 (Cat#51-559661). Riboprobes were synthesized using T7 RNA polymerase and [32P]UTP (Amersham Cat# PB10203). The labeled Riboprobe was hybridized overnight at 56 °C and processed using the manufacturer's protocol. Protected RNA fragments were separated using a 5% acrylamide gel and analyzed by phosphorimager (Cyclone Storage Phosphor System, PerkinElmer, Downer's Grove IL). The bands representing mRNA levels were quantified using an image analysis system (ImageJ, NIH freeware), and the signals were normalized to the L32 housekeeping gene as a loading control.

### 2.1.8. Multiplex cytokine assay

HL-1 cells were cultured as above and stimulated at confluence with 500  $\mu$ L stimulation media per well in a 6 well plate. 50  $\mu$ L cell culture supernatant per microplate well was added to duplicate wells undiluted to the LUM000 mouse base kit microplate (R&D Systems, Minneapolis MN), and the protocol followed as per the manufacturer's instructions. Fluorokine Mouse IL-6 MAP (LUM406), Mouse KC Fluorokine MAP (LUM453), Mouse MIP-2 Fluorokine MAP (LUM452) beads (R&D Systems, Minneapolis MN) were used in each experiment. Microplates were analyzed using the Luminex100 with accompanying 1.7 Software (Luminex Corporation, Austin TX).

2.1.9. Statistical analysis

All values are expressed as means±SE. For each experimental condition and time point, four independent replicate analyses were performed, unless otherwise noted. Groups were using ANOVA and the *post hoc* Bonferroni test to identify specific differences between groups. The analyses were performed using Sigmastat (SPSS, Chicago, IL), and statistical significance was set at  $p < 0.05$ .

3. Results

3.1. Toll-like receptors 2, 3, 4, 5, 7 and 9 are expressed in heart muscle

Using RT-PCR we found that both in the HL-1 murine cardiomyocyte cell line and in heart tissue obtained in vivo Toll-like receptors 2, 3, 4, 5, 7 and 9 are expressed (Fig. 1).

3.2. Pro-inflammatory cytokines and chemokines are produced by stimulation of TLR2, TLR4 and TLR5

HL-1 cells were stimulated with TLR2, 3, 4, 5, 7 and 9 ligands over 24 h with the conditioned media assayed. Despite the expression of many Toll-like receptors (Fig. 1), only stimulation of TLR2, TLR4 and TLR5 resulted in generation of the cytokine IL-6 and chemokines KC and MIP-2 (Fig. 2, negative data on TLR3, 7 and 9 not shown). The pro-inflammatory cytokine IL-6 was induced only through TLR4 stimulation (Fig. 2A), while the chemokine KC was induced through stimulation of TLR2, TLR4 and TLR5 (Fig. 2B). MIP-2 was induced through stimulation of

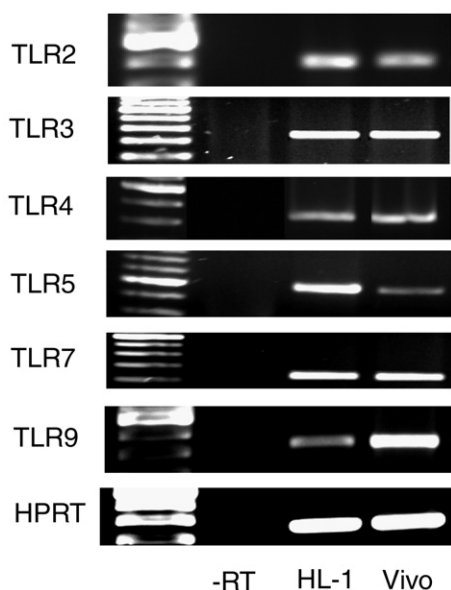


Fig. 1. Expression of Toll-like receptors by cardiac muscle. RT-PCR of RNA extracted from the HL-1 cardiac muscle cell line as well as C57/BL6 mouse heart (Vivo). TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 are expressed by all samples. Control PCR reactions (-RT) were performed without addition of reverse transcriptase to control for contaminating genomic DNA.

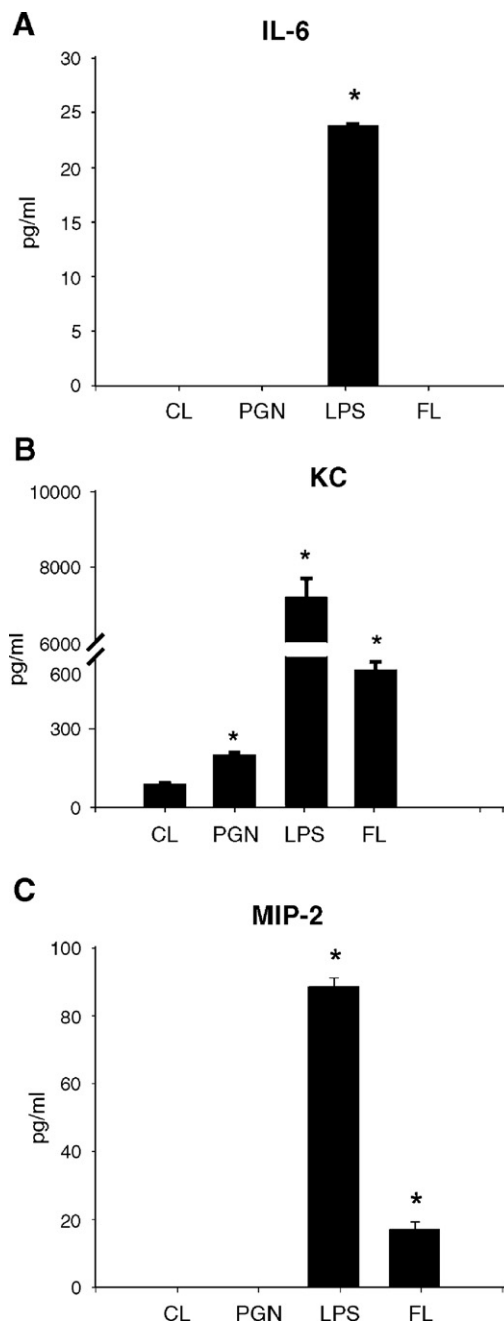


Fig. 2. Toll-like receptor-mediated cytokine and chemokine production. Cytokine and chemokine conditioned media levels were determined by bead multiplex assay 24 h after incubation of HL-1 cardiomyocytes with *S. aureus* peptidoglycan (10 µg/mL), Poly I:C (25 µg/mL), *E. coli* LPS (1 µg/mL), *S. typhimurium* flagellin (1 µg/mL), loxoribine (1 mM), and unmethylated CpG motif oligonucleotide (1 µM), representing TLR 2, 3, 4, 5, 7, and 9 ligands, respectively. Compared to non-stimulated of HL-1 cardiomyocytes (CL), only TLR2, TLR4 and TLR5 ligands induce a measurable response. Negative data not shown for TLR7 and TLR9 ligands. (A) *E. coli* LPS induces IL-6 production; (B) *S. aureus* peptidoglycan, *E. coli* LPS, *S. typhimurium* flagellin induce KC production; (C) *E. coli* LPS, *S. typhimurium* flagellin induce MIP-2 production. \* $p < 0.05$  vs Control.

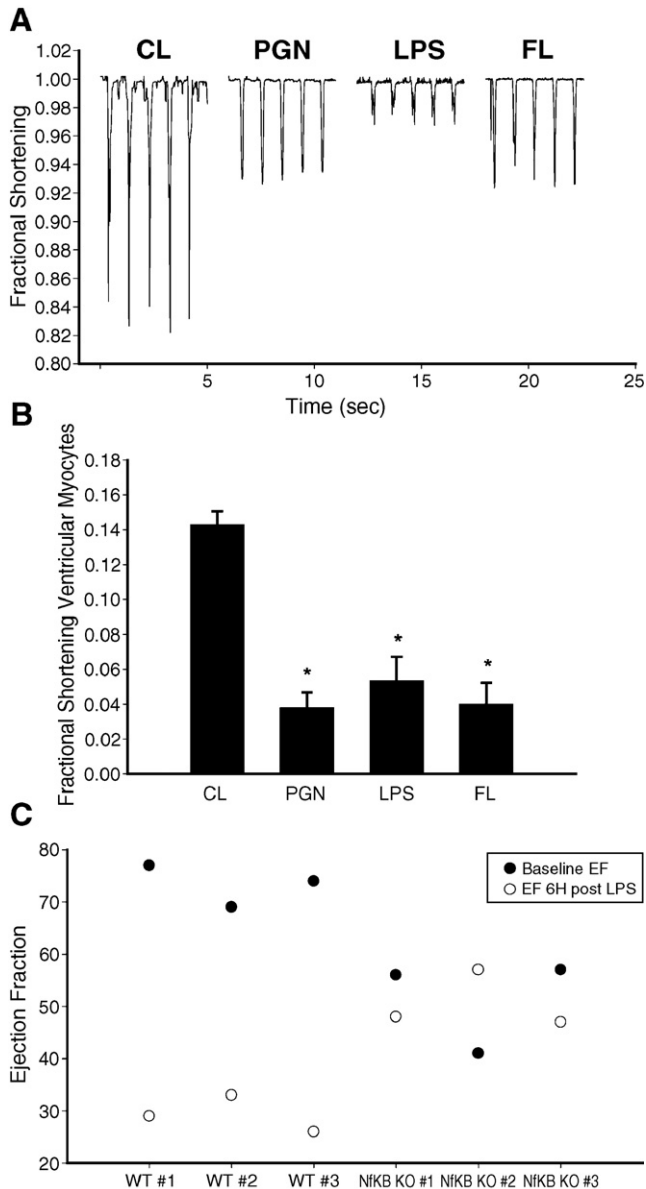


Fig. 3. Toll-like receptor stimulation impairs ventricular myocyte contraction. Primary murine ventricular myocytes were paced at 60 Hz following 24 hours incubation with TLR2 ligand *S. aureus* peptidoglycan (10  $\mu$ g/ml), TLR4 ligand *E. coli* LPS (1  $\mu$ g/ml) and TLR5 ligand, *S. typhimurium* flagellin (1  $\mu$ g/ml). (A) Representative tracings of myocyte length vs time paced at 1 bps. (B) Group mean fractional shortening of ventricular myocytes. Compared to CL treated cells, PGN, LPS and Flagellin resulted in significantly decreased contractility  $*p < 0.05$  vs Control. (C) NF- $\kappa$ B knockout mice and wildtype (WT) had echocardiographic evaluation of cardiac ejection fraction at baseline and following administration of 40 mg/kg LPS. LPS treated WT animals show dramatic reduction in ejection fraction ( $p < 0.05$  group mean data) while LPS treated NF- $\kappa$ B KO animals show no such response.

TLR4 and TLR5 (Fig. 2C). The Toll-like receptor ligands we used to stimulate TLR 3, 7 and 9, although non-functional in our study, have been confirmed by the manufacturer (Invivogen, San Diego CA) and in published studies to stimulate murine leukocytes [28], and the concentrations used in our study were above the maximal levels found to activate inflammatory cytokines and NF- $\kappa$ B activity.

### 3.3. Stimulation of TLR2, TLR4 and TLR5 results in impaired ventricular myocyte contractility through NF- $\kappa$ B dependent mechanisms

24 h following heart digestion, primary murine ventricular cardiomyocytes contract briskly when stimulated by electrical field generation, with fractional shortening of between 12 and 16%. The TLR2 ligand PGN, TLR4 ligand LPS and the TLR5 ligand flagellin significantly reduced cardiomyocyte

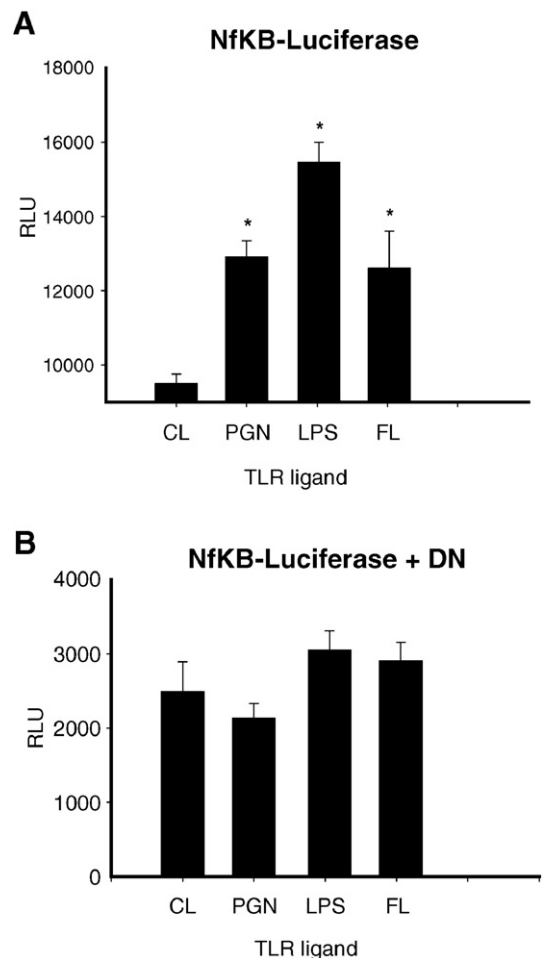


Fig. 4. (A) TLR2, TLR4 and TLR5 ligand induction of NF- $\kappa$ B-Luciferase reporter activity. HL-1 cells were transfected with a plasmid containing a NF- $\kappa$ B responsive promoter that activates transcription of firefly-Luciferase, as well as a constitutively active renilla-Luciferase plasmid to control for transfection efficiency. Following overnight incubation, cells were stimulated for 4 h with PGN (10  $\mu$ g/mL), LPS (1  $\mu$ g/mL) or FL (1  $\mu$ g/mL). All ligands induced significant elevations in NF- $\kappa$ B activity vs CL. Data are expressed as the ratio of firefly to renilla-Luciferase activity in relative light units (RLU).  $*p < 0.05$  vs Control. (B) IKK $\beta$  dominant co-transfection blocks TLR2, TLR4 and TLR5 ligand induction of NF- $\kappa$ B-Luciferase reporter activity. HL-1 cells were co-transfected with plasmids containing an IKK $\beta$  dominant negative vector, an NF- $\kappa$ B responsive promoter that activates transcription of firefly-Luciferase, as well as a constitutively active renilla-Luciferase plasmid to control for transfection efficiency. Following overnight incubation, cells were stimulated for 4 h with PGN (10  $\mu$ g/mL), LPS (1  $\mu$ g/mL) or FL (1  $\mu$ g/mL). No Toll-like receptor ligand induced NF- $\kappa$ B activity over Control, confirming the NF- $\kappa$ B pathway blocking effect of the IKK $\beta$  dominant negative construct.

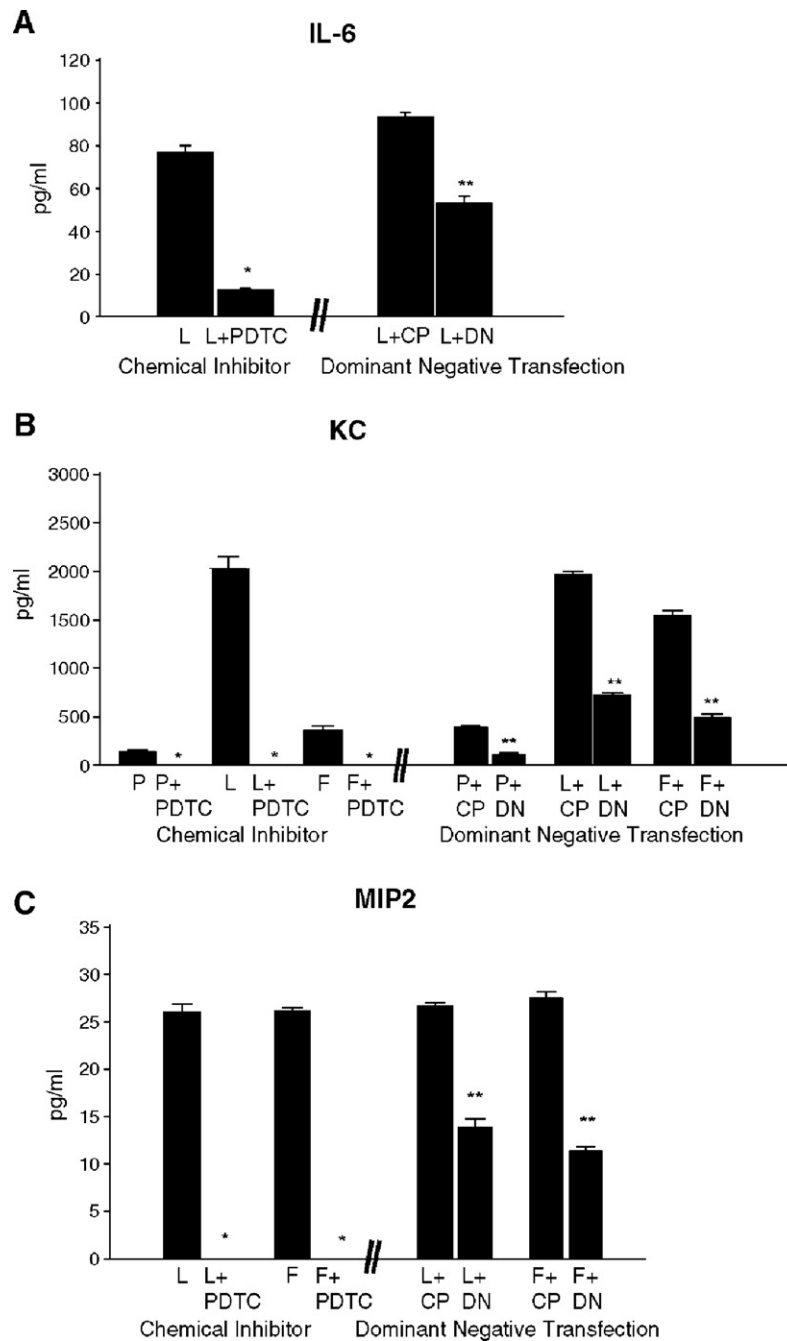


Fig. 5. Chemical and dominant negative IKK $\beta$  inhibition of NF- $\kappa$ B signaling impairs TLR-mediated cytokine and chemokine expression. HL-1 conditioned media cytokine and chemokine levels were determined by bead multiplex assay in response to *S. aureus* peptidoglycan (10  $\mu$ g/mL), *E. coli* LPS (1  $\mu$ g/mL) and *S. typhimurium* flagellin (1  $\mu$ g/mL). This was performed either with chemical inhibition of NF- $\kappa$ B in the presence or absence of the NF- $\kappa$ B inhibitor PDTC (100  $\mu$ M) or through transfection of HL-1 cells with an IKK $\beta$  dominant negative construct or an empty vector (CP). Data representing chemical inhibition is to the left of the cross-hatches on the x-axis, while dominant negative data is shown to the right. (A) LPS-mediated IL-6 production is significantly inhibited by both PDTC vs Control and IKK $\beta$  dominant negative transfection vs CP. (B) PGN, LPS and Flagellin-mediated KC production is significantly reduced by both PDTC vs Control and IKK $\beta$  dominant negative transfection vs CP. (C) LPS and Flagellin-mediated MIP-2 production is significantly reduced by both PDTC vs CL and IKK $\beta$  dominant negative transfection vs CP. \* $p$ <0.05 vs Control \*\* $p$ <0.05 vs Control Plasmid.

contractility vs no treatment (CL) after 24 h of incubation (Fig. 3). Stimulation of TLR3, TLR7 and TLR9 did not result in significant impairment of contractility. In vivo, LPS resulted in a dramatic decrease in cardiac ejection fraction in wildtype mice while NF- $\kappa$ B knockout mice did not demonstrate a significant myocardial depressant effect.

3.4. NF- $\kappa$ B transcriptional activity was stimulated through TLR2, TLR4 and TLR5, and is blocked by blocked by IKK $\beta$  DN

NF- $\kappa$ B activity, as measured using an NF- $\kappa$ B-Luciferase reporter assay was significantly increased over baseline upon

stimulation of TLR2, TLR4 and TLR5 (Fig. 4A). Prior to nuclear translocation of the major active subunit of NF- $\kappa$ B, IKK $\beta$  must phosphorylate the inhibitory complex subunit I $\kappa$ B causing disassociation of the complex and degradation of I $\kappa$ B. To assess the efficacy of our IKK $\beta$  DN construct we co-transfected the DN along with the NF- $\kappa$ B-Luciferase

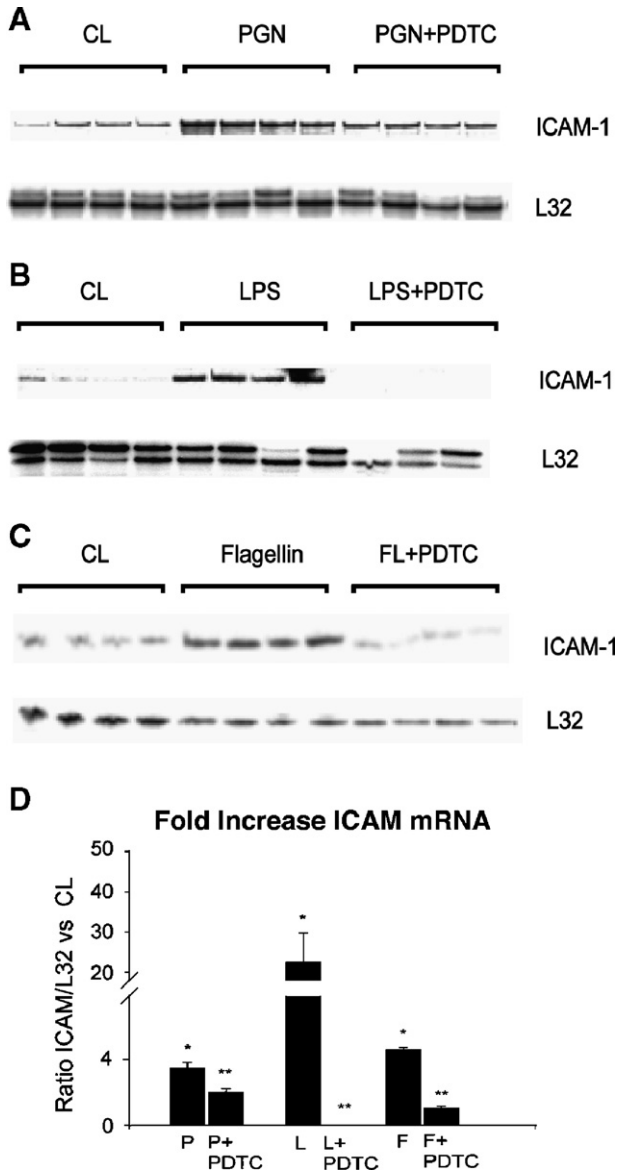


Fig. 6. Chemical inhibition of NF- $\kappa$ B signaling impairs TLR-mediated ICAM-1 expression. ICAM-1 mRNA levels were determined by ribonuclease protection assay 4 h after incubation of HL-1 cells with (A) *S. aureus* peptidoglycan (10  $\mu$ g/mL), (B) *E. coli* LPS (1  $\mu$ g/mL), (C) *S. typhimurium* flagellin (1  $\mu$ g/mL). Compared to non-stimulated HL-1 cells (CL), PGN, LPS and flagellin stimulation induce a measurable ICAM-1 response, while pre-incubation PDTC (100  $\mu$ M) for 1 h greatly attenuates the response to all three ligands. (D) Graphical representation of ICAM-1 band densitometry, confirming a significant increase in Toll-like receptor stimulated ICAM-1 expression vs CL. Pre-incubation with PDTC (100  $\mu$ M) for 1 h greatly attenuates the response to all three ligands. Data is normalized to the L32 control gene and expressed in arbitrary units (a.u.); each condition represents the group mean $\pm$ SEM ( $n=4$ ). \* $p<0.05$  vs Control \*\* $p<0.05$  vs Ligand alone.

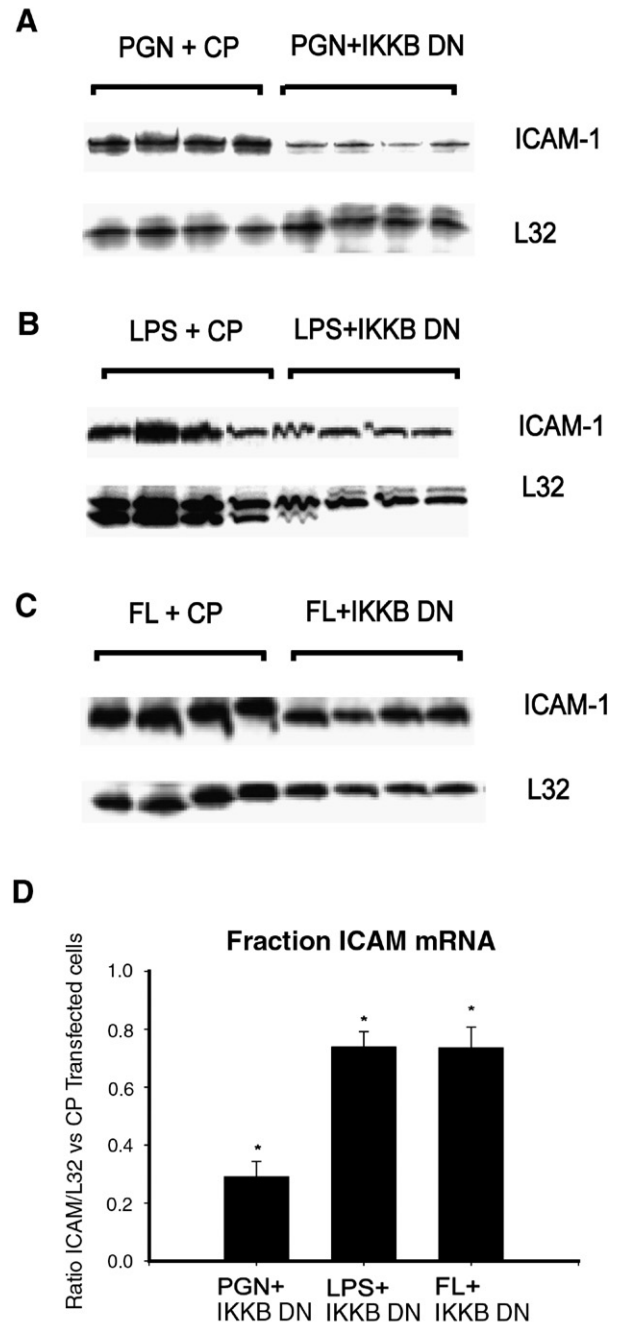


Fig. 7. Dominant negative IKK $\beta$  inhibits TLR-mediated ICAM-1 expression. ICAM-1 mRNA levels were determined by ribonuclease protection assay 4 h after incubation of HL-1 cells with (A) *S. aureus* peptidoglycan (10  $\mu$ g/mL), (B) *E. coli* LPS (1  $\mu$ g/mL), (C) *S. typhimurium* flagellin (1  $\mu$ g/mL). Transfection of HL-1 with an IKK $\beta$  dominant negative construct vs empty vector (CP) prior to Toll-like receptor stimulation attenuates the ICAM-1 response to all three ligands. (D) Graphical representation of ICAM-1 band densitometry, confirming a significant decrease in Toll-like receptor stimulated ICAM-1 expression vs CP for PGN, LPS and flagellin. Data is normalized to the L32 control gene and expressed in arbitrary units (a.u.); each condition represents the group mean $\pm$ SEM ( $n=4$ ). \* $p<0.05$  vs Control Plasmid.

reporter vector. Co-transfection of the DN resulted in no significant increase in NF- $\kappa$ B transcriptional activity vs control (Fig. 4B), confirming that the IKK $\beta$  DN vector effectively blocks signaling via the NF- $\kappa$ B pathway.

### 3.5. Toll-like receptor-mediated cytokine and chemokine production is inhibited by chemical and IKK $\beta$ dominant negative inhibition of NF- $\kappa$ B signaling

TLR4-mediated IL-6 production was significantly inhibited by both 100  $\mu$ M PDTC and the IKK $\beta$  DN construct (Fig. 5A). KC production induced by TLR2, TLR4 and TLR5 was significantly inhibited by both 100  $\mu$ M PDTC and the IKK $\beta$  DN construct (Fig. 5B). MIP-2 production induced by TLR4 and TLR5 was significantly inhibited by both 100  $\mu$ M PDTC and the IKK $\beta$  DN construct (Fig. 5C).

### 3.6. The cell surface adhesion molecule ICAM-1 is produced by stimulation of TLR2, TLR4 and TLR5; chemical inhibition of NF- $\kappa$ B signaling attenuates this response

A ribonuclease protection assay was used to determine whether Toll-like receptor stimulation resulted in ICAM-1 production, and further whether this response occurs via NF- $\kappa$ B signaling. PGN (Fig. 6A), LPS (Fig. 6B) and flagellin (Fig. 6C) treated cells show marked increases in ICAM-1 when compared to their respective controls. HL-1 cells pre-incubated with 100  $\mu$ M PDTC demonstrate a highly blunted response to all three Toll-like receptor ligands (Fig. 6A, B and C). Activation of TLR4 causes the greatest increase in ICAM-1 transcription (>20 fold) as well as the most significant inhibition by PDTC (Fig. 6D). Both TLR2 and TLR5 induce ICAM-1 transcription approximately four-fold, and show significant blunting of this induction in response to pre-incubation with 100  $\mu$ M PDTC.

### 3.7. Dominant-negative IKK $\beta$ decreases ICAM-1 response to TLR2, TLR4 and TLR5

Inhibiting the phosphorylation of IKK $\alpha$  through the use of a non-functional IKK $\beta$  dominant negative (Fig. 7A, B and C) resulted in a significant reduction in ICAM-1 production in response to TLR2, TLR4 and TLR5 stimulation compared to control plasmid. Relative inhibition in ICAM-1 transcript level (Fig. 7D) reveals a significant decrease in response to all Toll-like receptor ligands in cells transfected with the IKK $\beta$  DN construct.

## 4. Discussion

The central discovery of this study is that cardiomyocytes express a wide array of Toll-like receptors including TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9. By signaling via NF- $\kappa$ B, these Toll-like receptors initiate expression of pro-inflammatory cytokines (e.g. IL-6), chemokines (e.g. KC and

MIP-2), and cell surface adhesion molecules (e.g. ICAM-1). Thus, a pleiotropic immunologic response to danger signals, either endogenous molecules such as heat shock proteins or exogenous molecules such as PAMPs, is initiated. Importantly, this is associated with an NF- $\kappa$ B dependent rapid down regulation of cardiomyocyte contractility.

We demonstrate that TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 are expressed both *in-vivo* and in HL-1 cardiomyocytes. The Toll-like receptor family includes more than ten members [13] with differential expression among immune cell subtypes, resulting in a variety of responses to pathogenic stimuli [29]. Toll-like receptors are type I transmembrane receptors consisting of a variable number of extracellular leucine rich repeats linked to a cytoplasmic Toll/Interleukin-1 receptor (TIR) homology domain. Activation of most Toll-like receptors leads to the recruitment of the IL-1R-associated kinase and nuclear translocation of the latent cytoplasmic transcriptional regulator nuclear factor-kappa B (NF- $\kappa$ B) [30,31]. Underscoring its central role in regulating the immune response, NF- $\kappa$ B is a critical mediator of several inflammatory pathways in addition to the Toll-like receptors [32]. TLR2 and TLR4 bind bacterial cell wall components. TLR2 and TLR4 also respond to the endogenous ligands heat shock protein 60 (HSP60), HSP70 and Gp96, which may be released during necrotic cell death. TLR3 recognizes polyI:C and double-stranded viral RNA, TLR5 binds gram negative bacterial flagellin, TLR7 recognizes viral single-stranded RNA while TLR9 recognizes unmethylated CpG bacterial DNA [13]. Thus, the full complement of Toll-like receptors involved in viral and bacterial defence are expressed, at least at the mRNA level.

Systemic administration of LPS, not infectious itself but rather a PAMP, triggers an inflammatory cascade which results in myocardial dysfunction [1,5,33–35]. Another PAMP, peptidoglycan (PGN) from the gram-positive organism *Staphylococcus aureus* also induces myocardial dysfunction both from live infection [25] and injected alone as a PAMP [22]. Cardiomyocytes themselves have been found to express both cytokines [23] and surface immunologic molecules [4,5,10] in response to systemic pro-inflammatory stimuli. However, it is difficult *in vivo* to distinguish the systemic effects of leukocyte derived circulating cytokines from direct effects of PAMPs on the cardiomyocyte. We chose to study Toll-like receptor induced IL-6 and ICAM-1 expression since both influence contractility in the myocardium [36–38], reflecting important biological roles. We further investigated Toll-like receptor induction of chemokines as they are not only key in linking innate and adaptive immunity, but have recently been shown to be critical in muscle repair and re-generation [39].

When we assessed the functionality of the expressed Toll-like receptors only TLR2, TLR4 and TLR5 were significantly active. PGN stimulation of TLR2 resulted in production the CXC chemokine KC as well as ICAM-1, while LPS stimulation of TLR4 induced production of IL-6, KC, MIP-2 and ICAM-1. Flagellin, through stimulation of TLR5,



induced production of KC, MIP-2 and ICAM-1. Interestingly, while TLR3, TLR7 and TLR9 were expressed at the mRNA level, we were unable to demonstrate production of pro-inflammatory mediators in response to their ligands. This held true even after priming the cells overnight with 200 U/mL IFN- $\gamma$ , known to upregulate Toll-like receptors and their signaling [21]. Since TLR3, TLR7, and TLR9 are expressed in the intracellular compartment it is possible that their cognate ligands were unable to enter HL-1 cardiomyocytes so that the lack of response merely indicates an inability to access these intracellular receptors. In a variety of published data, however, simply incubating cells of different lineages with polyI:C, loxoribine and CpG is enough to initiate a strong immune response [40,41].

We then show for the first time that the activation of TLRs on primary myocyte preparations, rather than via leukocyte-mediated systemic inflammation, results in decreased contractility. We feel that it is likely that previous studies were unable to show this effect due to experimental factors. The prolonged survival time and contractility of our primary myocyte preparation allowed longer incubation with the ligands. Other studies have been limited to either pre-treating animals in vivo with post-digestion contractility measurements or to brief post-digestion incubations [23,42]. It is probable that any in vivo pre-treatment effect on isolated myocyte contractility is in effect masked by the stimulus of enzymatic digestion and physical disruption, while a short incubation with ligand would not allow adequate time for contractile changes.

We went on to look at signaling following Toll-like receptor activation. The TLR/IL-1R superfamily are type I integral membrane glycoproteins which upon ligand binding change conformation and recruit a variety of downstream signaling molecules [43,44]. The major signaling pathway results in activation of the complex, liberation of NF- $\kappa$ B from its inhibitor I $\kappa$ B $\alpha$  and subsequent translocation to the nucleus where it induces pro-inflammatory cytokines [30]. It has previously been shown in cardiomyocytes that TLR2 is necessary for NF- $\kappa$ B activation following oxidative stress. Here we demonstrate that TLR2, TLR4 and TLR5 signal via NF- $\kappa$ B in cardiac muscle. Through the use of a firefly-luciferase assay we find highly upregulated NF- $\kappa$ B transcriptional activity 24 h after ligand induction. Interestingly, at maximal doses LPS derived from *E. coli* induces transcriptional activity to a greater extent than does PGN from *S. aureus* or Flagellin from *S. Typhi*. This corresponds to our observation that LPS is the strongest of the three Toll-like receptor ligands with respect to induction of pro-inflammatory mediators. Using LPS in an in vivo model we found rapid and dramatic depression of cardiac contractility, an effect which was completely abolished through knocking out NF- $\kappa$ B.

NF- $\kappa$ B is known to be a transcriptional regulator of IL-6 [45], KC [46], MIP-2 [46,47] and ICAM-1 [48], and in this study we block their Toll-like receptor induced production through inhibition of NF- $\kappa$ B. PDTC is a chemical which prevents phosphorylation and subsequent degradation of the inhibitory protein I $\kappa$ B $\alpha$ . Pre-incubation of HL-1 cells with

PDTC greatly attenuates the production of IL-6, KC, MIP-2 and ICAM-1 in response to PGN, LPS and Flagellin. To verify that the non-specific antioxidant properties of PDTC are not responsible for the observed effect we show went on to use plasmid-based inhibition of NF- $\kappa$ B. We show similar inhibition of Toll-like receptor induced pro-inflammatory mediator production by inhibiting the phosphorylation of I $\kappa$ B $\alpha$  through the use of a non-functional IKK $\beta$  mutant. We confirmed that the IKK $\beta$  mutant targets NF- $\kappa$ B activity by means of a co-transfection with an NF- $\kappa$ B-Luciferase reporter, with effective blockade of Toll-like receptor-mediated NF- $\kappa$ B transcriptional activity.

Thus we have shown that TLR2, TLR4 and TLR5 are expressed in cardiac muscle and respond to their known ligands through activation of NF- $\kappa$ B. The subsequent inflammatory response is strikingly similar to that seen with PAMP activation of professional immune cells, with production of pro-inflammatory chemokines, cytokines and the surface immunologic molecule ICAM-1. While it is thought that the primary purpose behind Toll-like receptor-mediated production of cytokines, chemokines and cell surface adhesion molecules such as ICAM-1 is the activation and recruitment of leukocytes [49,50], it is also known that these molecules have important effects on cardiac contractility. Whether our observed decreased contractility is a direct consequence of TLR activation; subsequent outside-in signaling (e.g. from ICAM-1) or a combination of both is of great interest, and is the subject of ongoing studies.

While the exact mechanisms underlying this decreased contractility remain to be clarified, it is clear that the cardiac myocyte is capable of recognizing pathogens and altering its physiology accordingly.

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