Interleukin-10 activates Toll-like receptor 4 and requires MyD88 for cardiomyocyte survival

Ashim K. Bagchi, Anita Sharma, Sanjiv Dhingra, Ana R. Lehenbauer Ludke, Abd Al-Rahman Al-Shudiefat, Pawan K. Singal

Institute of Cardiovascular Sciences, Department of Physiology, St. Boniface Research Centre, Faculty of Medicine, University of Manitoba, 351 Tache Avenue, Winnipeg, MB, Canada R2H 2A6

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A B S T R A C T

Toll-like receptors (TLRs) are important in a variety of inflammatory diseases including acute cardiac disorders. TLR4 innate signaling regulates the synthesis of anti-inflammatory cytokine, interleukin-10 (IL-10) upon TLR4 agonists’ re-stimulation. Anti-apoptotic action of IL-10 in cardiac dysfunction is generally accepted but its protective mechanism through TLR4 is not yet understood. We studied the effect of IL-10 in the activation of TLR4 downstream signals leading to cardiomyocytes survival. IL-10 caused a significant increase in the expression of CD14, MyD88 and TLR4. TLR4 activation led to the translocation of the interferon regulatory factor 3 (IRF3) into the nucleus. Phosphorylation of IRF3 enhanced mRNA synthesis for IL-1β but not TNF-α and was elevated even after removal of IL-10 stimulation. Furthermore, degradation of inhibitory kappa B (IκB) kinase (Ikk) suggested that IκB was the main activating kinase for IRF3-regulated NF-κB activation and phosphorylation of p65. Phosphorylated NF-κB p65 was translocated into the nucleus. Concomitantly, an increase in Bcl-xL activity inhibited Bax and the proteolytic activity of caspase 3 as well as a decrease in PARP cleavage. An inhibition of MyD88, modulated the above listed responses to IL-10 as there was a decrease in TLR4 and IRF3 and an increase in TNF-α mRNA. This was associated with a decrease in NF-κB p65, Bcl-xL mRNA and protein levels as well as there was an activation of Bax and PARP cleavage independent of caspase 3 activation. These data in cardiomyocytes suggest that IL-10 induced anti-apoptotic signaling involves upregulation of TLR4 through MyD88 activation.

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1. Introduction

The pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α) has been shown to be upregulated in conditions of myocardial dysfunction and heart failure [1–3]. In a rodent model of myocardial infarction, we have earlier reported that there is a significant increase in TNF-α and a decrease in anti-inflammatory cytokine interleukin-10 (IL-10) [4]. Further, it has been shown in isolated cardiomyocytes that TNF-α causes a significant increase in pro-apoptotic proteins, apoptosis and membrane leakage [5,6] and that IL-10 antagonizes these TNF-α induced changes [6]. Recently, it has been reported that IL-10 mitigates the effect of TNF-α and cuts off the apoptotic signal generated by inhibitory κB kinase (Ikk) [7]. Very recently, we have also reported that when IL-10 bind to its receptor, it activates pro-survival signal via activation of Jak/Stat3 pathway [8] but details of its innate response are still unclear.

Anti-inflammatory property of IL-10 may be regulated through its innate signaling via activation of patterns recognition receptors such as Toll-like receptors (TLRs). Toll-like receptors have been shown to be the first line of host defense against microbial infection and play a central role in innate as well as adaptive immunity [9–11]. TLRs are also capable of responding to stress and modulate inflammation as well as tissue damage following non-infectious conditions such as hypoxia and ischemia in cardiac tissues [12–14]. Among 10 TLRs identified in humans, TLR2 and TLR4 have been reported during myocardial infarction [15] and ischemia reperfusion [16]. TLR2 activation by the agonist peptidoglycan-associated lipoprotein (PAL) or LTA is also reported to cause cardiac inflammation as well as dysfunction [17]. TLR4 agonist is directly...
transferred into phospholipid bilayer, and via co-receptor CD14 activates MyD88 dependent TLR4 downstream signals [18,19]. Mechanism of MyD88 dependent signaling is based on TLR4 agonist stimulation and its signal strength. TLR4 has an absolute requirement of NF-κB [20] and in this process, phosphorylation of subunit p65 occurs when the interferon-sensitive response element (ISRE) complexes with Interferon regulatory factor 3 (IRF3) [21]. Thus, MyD88 and IRF3 synergistically activate NF-κB p65 [22–24]. TLR4 ligands trigger activation of cell survival as well as some inflammatory genes via NF-κB signaling. TLR4 innate signaling [25] also induces genes to regulate the synthesis of cytokines [22,26]. Involvement of TLR4 in the activation of IL-10 has also been reported as TLR4 agonist-mediated signals led to the synthesis of endogenous IL-10 upon re-stimulation of LPS [27]. On the other hand, endogenous production of IL-10 showed a critical role in myocardial ischemia/reperfusion injury [28]. Thus there is a possible interplay between TLR4 and IL-10.

In this study, we have examined events downstream to IL-10 activation of TLR4, which requires the intracellular adaptor molecule MyD88 in the cell survival signal in isolated cardiomyocytes. LPS stimulation was used as a positive control.

2. Materials and methods

2.1. Chemicals

Anti-rabbit TLR4, anti-rabbit PARP, anti-rabbit Bax, anti-rabbit Bcl-xL, anti-mouse MyD88 and anti-mouse caspase 3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Recombinant IL-10 and anti-phospho and/or total NF-κB p65, IKK (α, β and ε), IRF3, TNF-α and IL-1β were also purchased from Cell Signaling Technology (USA). Anti-IL-10 receptor (anti-IL-10R) antibody (clone 1B1.3a) was purchased from BD Pharmingen, USA. HRP labeled anti-mouse or rabbit IgG and anti-biotinylated antibodies were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). FITC labeled CD14 antibody, and MyD88 homodimerization peptide RDVLPGT inhibitor were purchased from Calbiochem (La Jolla, California, USA). RNA isolation kit was purchased from Sigma (Sigma, USA), SYBR Green one step qRT-PCR kit from Quanta BioScience (USA) and PCR primers [29,30] for GAPDH, (sense: 5’TGC ACCACCAACT GCTTAGC 3’ and anti-sense: 5’GGCATGGACT GTGGTAGT 3’); NF-κB, (sense: 5’AAGAT CAATGGCTACACGGG3’ and anti-sense 5’ATCTTGAGCTCGGCATGG3’) TNF-α, (sense: 5’CCTCTTC

![Fig. 1. CD14 expression in isolated cardiomyocytes.](image-url)

(A) Flowcytometry: a typical single-parameter histogram selected from five independent experiments done in duplicate, for CD14 expression in cardiomyocytes after 18 h stimulation with either 10 ng/ml of IL-10 (red) or 1 μg/ml of LPS (green) or PBS (vehicle; white); (B) immunocytochemistry and (C) percent expression of IL-10 or LPS induced CD14 expression (green) in cardiomyocytes. Nuclear staining was done using Propidium Iodide (orange). *P < 0.05 vs. vehicle control and **P < 0.05 vs. LPS as well as vehicle.
CATTCCTGC TCG3’ and anti-sense: 5’GGTATGAAA TGGCAAATCG G3’; IL-1β (sense: 5’AACCTGCTG GTGTGAGTTC3’ anti-sense: 5’CAGCAGGGCTTTTGTGTT GT3’); and Bcl-xL (sense: GACGGAAG TGCTATTGGT and antisense: TCAGGCTGAAGAAG AGAT) were purchased from Sigma (Sigma Genosys, USA).

2.2. Isolation of adult rat ventricular cardiomyocytes

Cardiomyocytes were isolated from normal adult male Sprague Dawley rats (200–250 g) using a previously described procedure [4]. Hearts were excised and mounted on a modified Langendorff perfusion apparatus. The Ca²⁺ free perfusate (modified Krebs buffer) contained: 110 mM NaCl; 2.6 mM KCl; 1.2 mM KH₂PO₄; 1.2 mM MgSO₄; 25 mM NaHCO₃ and 11 mM Glucose (pH 7.4). The Ca²⁺ free perfusion was then switched to re-circulating mode with 0.1% w/v collagenase buffer supplemented with 25 μM calcium and final isolated cardiomyocytes preparations were obtained [4]. The viability of adult cardiomyocytes was checked using 0.4% trypan blue (1:1). More than 90% of cardiomyocytes were viable and cells were re-suspended in serum-free M199 media supplemented with 12 mM HEPES and 100 μg/ml of penicillin and streptomycin. 1 × 10⁶ cells/ml were seeded on laminin (20 μg/ml) coated 6-well tissue culture plates with or without glass coverslips (22 mm) and incubated at 37 °C under 5% CO₂. After 2 h, cells were washed twice with complete media without fetal bovine serum (FBS), and the adherent cardiomyocytes were provided with fresh complete media and incubated overnight under same culture conditions.

Fig. 2. Expression of TLR4 (A and B) and MyD88 (C) in the presence or absence of anti-IL-10R antibody or MyD88 inhibitor. Cardiomyocytes pre-incubated for 18 h with or without anti-IL-10R antibody (10 μg/ml) or MyD88 inhibitor (50 μM), were stimulated with either IL-10 (10 ng/ml) or LPS (1 μg/ml) or PBS as vehicle. Upper panels in each figure are western blots for TLR4 and MyD88 protein expression while β-actin or GAPDH were used as loading control. Lower panels i.e. histogram in each figure, represent TLR4 expression (fold control) or MyD88/β-actin expression. Data are Mean ± SE from five independent experiments done in duplicate. *P < 0.05 and **P < 0.002 vs. respective vehicle control and #P < 0.05 vs. MyD88 respective control without inhibitor. †P < 0.05 vs. IL-10 without antibody.
2.3. Treatment of cardiomyocytes

After overnight incubation, viable cardiomyocytes (average 90%) were treated in triplicate with either 10 ng/ml of IL-10 or 1 μg/ml of LPS (positive control) for 4 or 18 h. In order to study the role of MyD88 adaptor molecule in TLR4 activation and downstream NF-κB signaling, cells were pre-incubated with MyD88 homodimerization peptide RDVLPGT (50 μM) inhibitor for 18 h. Cardiomyocytes were also incubated with PBS/DMSO alone as control for the same time points. Dose and time of exposure to IL-10 were standardized for a maximum response and are listed in results and figure legends. We found that in the presence of IL-10 (10 ng/ml), cardiomyocyte viability was 75% at 18 h of incubation, which was comparable to the control (85%) whereas significantly higher than that seen in the presence of LPS (48%). Whether IL-10 activates TLR4 via activation of IL-10R on cardiomyocytes, a competitive inhibition study using 10 μg/ml of clone 1B1.3a, an IL-10R antibody [31] was also carried out to understand the mutual interaction between IL-10 and TLR4.

2.4. Fluorescence microscopy

Cardiomyocytes were allowed to adhere onto laminin (20 μg/ml) coated 22 mm glass coverslips placed in 6-well tissue culture plates and maintained as previously described. Coverslips were washed once with M199 and also with PBS containing 0.1% Tween-20 (PBS-T) and 1% BSA to remove the non-adherent cells, then fixed with 4% paraformaldehyde and stored at −20°C until used. The cells were rehydrated for 15 min at room temperature (RT) using PBS containing 0.1% sodium citrate and 0.1% Triton-X100 for membrane permeabilization. Fc-receptor sites were blocked using bovine immunoglobulin (Ig) at a concentration of

![A. K. Bagchi et al. / Cytokine 61 (2013) 304–314](image)

**Fig. 3.** IL-10 induced activation of IRF3. Cardiomyocytes in the presence or absence of MyD88 inhibitor were stimulated with IL-10 (10 ng/ml). (A) Representative immunoblots of phospho-IRF3, total-IRF3 and β-actin in the absence (I) or presence (II) of MYD88 inhibitor (50 μM) at 4 h. Histogram represents Mean ± SEM IRF3 expression (phospho-IRF3/total IRF3) in five independent experiments (n = 5) done in duplicate. *P < 0.05 vs. respective control. **P < 0.002 vs. no MyD88 inhibitor. (B) Immunofluorescence of intracellular localization and dimerization of IRF3 in isolated cardiomyocytes under the same experimental conditions as mentioned in A. IRF3 localization at 4 h was in cytoplasm and at 18 h, a nuclear translocation (arrows) was noted. α-Actinin was used as control and DAPI was used to detect nuclei (Blue). IRF3 dimerization and localization in the nucleus was seen after merging two images; red fluorescence represents positive expression for IRF3 dimerization in the stimulated cardiomyocytes. (magnification 20×).
5 mg/ml for 30 min at RT. After two washes with PBS-T, the cells were incubated with primary antibodies to CD14 and phospho-IRF3 (Imgenex Corp., USA) at 1:200 dilution along with α-actinin antibody (Sigma, USA) diluted at 1:500 in PBS-T containing 5% BSA (Ig free) for 1 h at RT. Finally, the cells were washed twice with PBS-T containing 1% BSA and stained with either FITC or AlexaFluor 498 and/or 594 conjugated to goat anti-rabbit or anti-mouse F(ab)2 antibody (Invitrogen, USA) respectively. Counterstaining for nuclei was done using 1 μl of propidium iodide (PI) and 4,6-diamidino2-phenylindole (DAPI). Expressions of CD14 and phospho-IRF3 in cardiomyocytes were analyzed and quantified by microscopic analysis (Olympus Optical Co., Ltd., Tokyo, Japan) in 100 μm² area.

2.5. Flowcytometry

Quantitative analysis of in vitro stimulated cardiomyocytes cells was done using FACSorter (Becton–Dickinson, Mountain View, CA, USA) using FITC conjugated-specific monoclonal antibody for the target population. Briefly, 1 x 10⁶ cells were washed with cold PBS-T containing 1% BSA and stained with CD14-FITC labeled specific antibody for 1 h at 4 °C after incubating with 2% BSA for 30 min at 37 °C. Then, the cells were washed twice with PBS-T and suspended in PBS containing 4% paraformaldehyde [32]. The cells were counted and fluorescence intensity was detected, optimized and read through database-Lysys II analysis software (Becton–Dickinson, USA) against specific negative as well as experimental controls. For each set of experiments, single parameter histogram (specific stained cell number vs. fluorescence intensity) was plotted for 20,000 events per sample to measure cell surface expression [33].

2.6. Analysis of RNA

Total RNA was isolated using a GenElute Mammalian Total RNA Miniprep kit (Sigma) as per the manufacturer’s instructions. Total RNA (25 ng) was used for quantitative realtime PCR reactions performed using B-R One-Step SYBR Green qRT-PCR Kit (Quanta BioSciences, USA) on an iQ5 multicolor real-time PCR detection system (Biorad, USA). mRNA abundance for TNF-α, IL-1β, Bcl2 and NF-κB p65 was determined using the 2⁻ΔΔCT method and was normalized to GAPDH.

Fig. 4. TNF-α (A) and IL-1β (B and C) responses to IL-10 stimulation of cardiomyocytes. (A and B) After 4 h of incubation with IL-10 (10 ng/ml) in the presence or absence of MyD88 inhibitor (50 μM) medium was replaced and at 18 h cardiomyocytes were analyzed for mRNA for TNF-α and IL-1β. (C) Protein activity for IL-1β was measured in the presence or absence of anti-IL-10R antibody (10 μg/ml). Data represent Mean ± SEM from three independent experiments (n = 3) done in triplicate. *P < 0.05 vs. respective vehicle, #P < 0.05 vs. no MyD88 inhibitor and δP < 0.05 vs. IL-10 without antibody.
2.7. Immunoprecipitation

Immunoprecipitation analysis was performed as previously described [34] with slight modifications. After 18 h of culture, the cells were washed once with ice-cold PBS. Cells were scraped and centrifuged at 1200 rpm at 4 °C for 10 min. The pellet was re-suspended in 500 μl of lysis buffer (0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.5% sodium deoxycholate, 1% SDS and 2 mM Na₂VO₄) supplemented with 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml each of leupeptin and aprotinin. Cell lysates were incubated for 15 min on ice to ensure lysis. Lysates were then centrifuged at 12,000 rpm for 15 min at 4 °C, and supernatants were collected. Lysates (200–500 μg; in a volume of 500 μl) were pre-cleared with 10 μl of protein A/G-conjugated Sepharose beads (Cell Signaling Technology, USA) for 1 h at 4 °C. Separately, 20 μl of the gel beads was preloaded with 1–2.5 μl of appropriate antibodies (IkBα/β/ε or phospho-p65) for at least 1 h. Preloaded beads were washed once with lysis buffer and mixed with pre-cleared lysates and incubated overnight at 4 °C at a constant speed 200 rpm. Next day, the incubation mixtures were subjected to centrifugation at 12,000g for 15 min at 4 °C, and the resulting immunoprecipitates were washed three times in lysis buffer. Finally, immunoprecipitates were resuspended in 20 μl of SDS–sample buffer. The samples were boiled for 5 min and resolved on 10% SDS–polyacrylamide gel electrophoresis (PAGE).

2.8. Western immunoblot

After electrophoresis, the proteins from the gel were transferred onto PVDF membrane for 1 h at 300 mA at 4 °C. Blotted PVDF membrane was washed twice in TBS-T (tris-buffered saline Tween 20) and non-specific binding sites on the membrane were blocked by incubating with 5% fat free skimmed milk for 1 h at RT. After

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Fig. 5. MyD88 dependence of IL-10 induced activation of NF-κB in cardiomyocytes. Cardiomyocytes pre-incubated with or without MyD88 inhibitor for 18 h and were stimulated for further 18 h with IL-10 (10 ng/ml). (A) Represents phospho-NF-κB p65 (p-p65) and total NF-κB (p65) expression in the absence (I) or presence (II) of MyD88 (50 μM) inhibitor. Representative immunoblot of three independent experiments (n = 3) are shown in upper panels. Histogram shown is Mean ± SEM (lower panel) of phospho-NF-κB p65 from three independent experiments (n = 3) each done in duplicate. *P < 0.05 vs. vehicle and #P < 0.05 vs. MyD88 control. (B) Phosphorylation of p65 by IkBα/β/ε degradation. After 18 h of treatment, whole cell lysates were immunoprecipitated (IP) and analyzed by western blotting (WB) using specific anti-IkBα, IkBβ and IkBε and NF-κB phospho-p65 antibodies. GAPDH was used as a loading control. (C) qRT-PCR results are shown as fold (Mean ± SEM) changes in NF-κB p65 mRNA expression levels from three independent experiments (n = 3) done in triplicate. **P < 0.001 vs. respective vehicle and *P < 0.05 vs. MyD88 control.
incubation, it was rinsed twice with washing buffer (TBS-T with 1% skimmed milk) and the membrane was probed by incubating at RT for overnight with anti-TLR4, anti-MyD88, anti-phospho-IRF3 or anti-total p65 and anti-phospho-p65. For apoptosis studies, anti-caspase 3 or anti-PARP, anti-Bax and Bcl-xl antibodies were diluted in TBS and incubated overnight. The membranes were washed three times and then incubated for 1 h with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were visualized using ECL kit (Thermo Scientific, USA).

2.9. Statistical analysis

The Mean ± SEM were calculated and Student’s unpaired or paired T-test was applied to compare the values. Results were accepted to be statistically significant at a value of $P < 0.05$.

3. Results

3.1. Expression of CD14 during IL-10 stimulation

We examined the effects of IL-10 or LPS on the CD14 expression in cardiomyocytes by flow cytometry and immunocytochemistry by fluorescent microscopy. The cell surface expression of CD14 was measured up to 18 h of stimulation. After 4 h of incubation, both IL-10 (10 ng/ml) and LPS (1 μg/ml) caused an insignificant increase in CD14 (data not shown). However, CD14 expression was significantly upregulated at 18 h by IL-10 stimulation. This effect was significantly higher with IL-10 as compared to LPS as seen by flow cytometry as well as fluorescent microscopy (Fig. 1A and C).

3.2. TLR4 expression in IL-10 treated cardiomyocytes

The TLR4 innate immune system is known to respond to LPS in the pathogen-associated molecular patterns. Therefore, as a positive control, we determined whether TLR4 is regulated by LPS in isolated cardiomyocytes. LPS-treated cardiomyocytes exhibited significant increase in TLR4 levels after 18 h (Fig. 2A). IL-10 exposed cardiomyocytes also showed a significant increase in TLR4 expression as compared to control (Fig. 2A). Furthermore, this effect of IL-10 on TLR4 expression was much higher than the LPS effect (Fig. 2A).

In order to examine direct mutual action between IL-10 and TLR4 on cardiomyocyte survival, IL-10 receptor sites on the cardiomyocytes were blocked using 10 μg/ml of clone 1B1.3a (anti-IL-10R antibody). There was a complete attenuation of TLR4 response to IL-10 (Fig. 2B). Anti-IL-10R antibody by itself had a marginally depressant effect on the baseline TLR4 expression (Fig. 2B).

In order to find out, whether TLR4 requires an adaptor molecule to activate the downstream signaling pathway, we also assessed MyD88 expression in the IL-10 and LPS stimulated cardiomyocytes (Fig. 2C). In both IL-10 and LPS-induced cardiomyocytes, the expression levels of MyD88 were significantly higher than the vehicle control levels (Fig. 2C). The increase in TLR4 and MyD88 was blunted in the presence of MyD88 inhibitor (Fig. 2A and C).

3.3. IRF3. dimerization, phosphorylation and localization in IL-10 treated cardiomyocytes

Since IRF3 is suggested to be a transcription factor downstream to TLR4 activation, its induction and activation was assessed in IL-10 treated cardiomyocytes at 4 h and 18 h. At 4 h, IL-10 caused a three fold increase in IRF3 and this effect was blocked in the presence of MyD88 inhibitor (Fig. 3A). To assess activation of IRF3 signaling at a single-cell level, paraformaldehyde-fixed cardiomyocytes were stained for the intracellular localization of IRF3 (Fig. 3B).

3.4. Changes in the expressions of TNF-α and IL1β

We tested the delayed effects (at 18 h) of the incubation of cardiomyocytes to a 4 h pre-exposure to IL-10 by measuring the mRNA expression for TNF-α and IL1β at 18 h. This exposure of exogenous IL-10 inhibited the cardiomyocyte transcriptional activity for TNF-α. However, MyD88 inhibition, not only blocked this effect of IL-10 but levels of TNF-α mRNA were even higher than the
respective controls (Fig. 4A). On the other hand, the IL-β mRNA expression was significantly increased by IL-10 and MyD88 inhibition completely blocked this effect (Fig. 4B). Furthermore, when IL-10R was blocked by its antibody, the production of IL-1β in cardiomyocytes was inhibited (Fig. 4C).

3.5. P65 mediated NF-κB signaling in IL-10 induced cardiomyocytes

Since NF-κB signaling is known to be involved in IL-10 induced downstream changes, we examined phosphorylation of NF-κB subunit p65 at serine 536 subsequent to IL-10 treatment. Phosphorylation of p65 protein and activation of mRNA was seen at 18 h of IL-10 stimulation (Fig. 5A–C). IL-10 induced upregulation of p65 activation was modulated by MyD88 inhibitor at both protein and mRNA levels (Fig. 5A and C). MyD88 inhibition of NF-κB p65 was also seen in immunoprecipitates (Fig. 5B). The degradation of IκB into IκBα, IκBβ and IκBε was also examined in immunoprecipitates after IL-10 treatment. IL-10 caused a significant upregulation of IκBβ as compared to control, while IκBα and IκBε were also stimulated by IL-10 but the effect was not as pronounced (Fig. 5B). IL-10 induced increase in the p65 mRNA expression was significantly blocked by MyD88 inhibition (Fig. 5C).

3.6. Bcl-xl mRNA and Bax/Bcl-xl activity

Bcl2 is a pro-survival protein and has an inhibitory effect on apoptosis. Effects of IL-10 on the mRNA and protein expression of Bcl-xl were measured with and without MyD88 inhibitor (Fig. 6). Baseline expression of Bcl-xl mRNA was very low and was not affected by MyD88 inhibition. IL-10 stimulation caused a
significant increase in Bcl-xL while in the presence of MyD88 inhibition, Bcl-xL expression (Fig. 6A) and its activity was significantly reduced and there was an increased expression of pro-apoptotic protein Bax (Fig. 6B). Furthermore, there was a marked elevation in Bax to Bcl-xL ratio which was significantly higher than IL-10 stimulation (Fig. 6B).

3.7. Effect of IL-10 on caspase 3 activation and PARP cleavage

IL-10 stimulation of cardiomyocytes in the presence or absence of MyD88 inhibitor had no effect on caspase 3 activity (Fig. 7A). IL-10 had an inhibitory effect on the baseline PARP cleavage (89 kDa). However, in the presence of MyD88 inhibitor, IL-10 caused a significant increase in PARP cleavage even above the baseline activity (Fig. 7B).

4. Discussion

TNF-α is one of the most studied cytokines and it is produced by many cells including cardiomyocytes [4,35]. The role of TNF-α in cardiac contractility is generally correlated with cardiac dysfunction [1,36,37]. TNF-α provokes cardiomyocyte apoptosis and cardiac remodeling through activation of multiple cell death pathways [38,39]. IL-10 is a potent antagonist of TNF-α [2] which prevents inhibitory nuclear factor kappa (IkB) breakdown into inhibitory κB kinase (IκB) [19,40]. We have reported that IL-10 reduces the effect of TNF-α-induced changes in MAPK activity, prevented phosphorylation and activation of p65 subunit of NF-κB following IκB degradation [7]. Pre-exposure of cardiomyocytes to an IKK inhibitor (PS-1145) prevented TNF-α-induced caspase and PARP cleavage. In the same study, it was also reported that inhibition of ERK1/2 MAPK with PD98059 attenuated the protective role of IL-10 against TNF-α-induced activation of IκB and NF-κB as well as cardiomyocyte apoptosis [7]. Protective effect of IL-10 may also be regulated through the activation of TLR4 in innate signaling. It is known that TLR4 activation promotes IL-10 synthesis upon secondary stimulation of LPS [27]. Very recently, we have also reported that IL-10 results in the upregulation of STAT3 [1,36,37] which is mediating anti-apoptotic effect of IL-10 [8]. It is likely that STAT3 upregulation may also be involved in the IL-10 induced activation of TLR4. However this point needs to be examined. Thus, the interplay between IL-10 and TLR4 activation appears to be complex. Our study provides newer information about the role of IL-10 induced TLR4 signaling in the survival of adult cardiomyocyte (Fig. 8).

It has been suggested earlier that TLR stimulation in response to LPS is either dependent or independent of CD14 and MyD88 [19,41–44]. Here, we are reporting for the first time that besides LPS, anti-inflammatory cytokine IL-10 can also activate the TLR4 innate signaling in rat cardiomyocytes. In IL-10 stimulated cardiomyocytes, TLR4 expression followed the upregulation of CD14 and MyD88. Furthermore, we did not see any activation of TLR4 response upon blocking of IL-10 receptors suggesting that IL-10 does not activate TLR4 directly rather it requires activation of IL-10 receptor to trigger downstream pathway to upregulates TLR4. This finding supports a previous report showing that IL-10R activation enhanced cell survival activity via STAT3/AKT pathway [8]. Present study also shows that there is IL-10 and TLR4 interaction in the cellular innate signaling upon specific ligand stimulation. Such a linkage between IL-10 and TLR4 has also been reported [27]. It is known that TLR4 stimulation activates a distinct transcription factor, IRF-3 [45]. We also observed an activation of IRF3 in response to IL-10 stimulation. Activated IRF3 generally binds with ISRE and induces genes for the synthesis of different cytokines including IL-1β [20,46,47]. Upregulation of IL-1β subsequent to TLR4 activation was also seen in the present study. Simultaneously, there was a reduction in TNF-α mRNA suggesting a reduction in the levels of TNF-α activity. It is important to note that, the pathway leading to production of IL-1β in the present study was active even after the removal of IL-10 from the medium. More interestingly, such IL-1β synthesis in cardiomyocytes was inhibited upon blocking of IL-10 receptors or MyD88 inhibition. It appears that TLR4 activation in response to IL-10 requires a co-receptor CD14 and a signaling adapter molecule MyD88 to upregulate a downstream transcription factor IRF-3 leading to IL-1β synthesis.

NF-κB, an important transcription factor, is reported to bind to a specific promoter site and turns on further downstream signaling [40]. In our present study, in the IL-10 stimulated cardiomyocytes, a degradation of IκB suggests that IκBα is an activating kinase for IRF3-regulated NF-κB activation by p65 phosphorylation resulting in the translocation of activated NF-κB p65 from cytoplasm to the nucleus. This in turn resulted in the activation of Bcl-xL and down-regulation of caspase 3 as well as PARP activity. Such an activation of NF-κB and its translocation into the nucleus [21,48] as well as a similar chain of events for cell survival signal have been reported [49–51].
MyD88 is a major adaptor molecule for TLR innate signaling, and blocking of this adaptor molecule may prevent further normal downstream signal transduction through TLRs (Fig 8). MyD88 recruitment during TLR4 activation has been reported in various cell types stimulated either by LPS or LBP [43,52,53]. In our study, inhibition of MyD88 generated very novel information about a significant change in some of these events. Use of the MyD88 inhibition of MyD88 generated very novel information about a decrease in IRF3 expression; significant change in some of these events. Use of the MyD88 inactivation of MyD88 requires MyD88 adaptor molecule in the execution of downstream signals. Blockage of MyD88 adaptor molecule, inhibited IL-10 induced IRF3-dependent, IL-1β production as well as NFκB p65 phosphorylation suggesting that MyD88 adaptor molecule is a key molecule and when activated it may prolong the cardiomyocyte survival. Since we did not see any elevation in caspase 3 after MyD88 inhibition and since there was a decrease in Bcl-xl with an increase in the activity of pro-apoptotic, Bax followed by PARP cleavage, it appears that such a cell death signal is executed by an alternative pathway, in which another proteolytic enzyme such as caspase 1 or caspase 9 may be involved.

In conclusion, the anti-apoptotic function of IL-10 in TLR4 activation requires MyD88 adaptor molecule for cardiomyocyte survival and an inhibition of MyD88 has a converse effect (Fig. 8). Thus, MyD88 is a key signaling molecule in the TLR4 innate signaling pathway which determines the ultimate effect of IL-10 in the induction of cardiomyocyte survival or apoptosis.

Disclosures

None.

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