Biochemical and Biophysical Research Communications 644 (2023) 70–78

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Toll-like receptor 9 signaling after myocardial infarction: Role of p66ShcA adaptor protein

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ARTICLE INFO

Article history:
Received 9 December 2022
Received in revised form
19 December 2022
Accepted 30 December 2022
Available online 30 December 2022

ABSTRACT

During myocardial infarction, cellular debris is released, causing a sterile inflammation via pattern recognition receptors. These reactions amplify damage and promotes secondary heart failure. The pattern recognition receptor, Toll-like receptor 9 (TLR9) detects immunogenic fragments of endogenous DNA, inducing inflammation by NFκB. The p66ShcA adaptor protein plays an important role in both ischemic myocardial damage and immune responses. We hypothesized that p66ShcA adaptor protein promotes DNA-sensing signaling via the TLR9 pathway after myocardial infarction.

TLR9 protein expression increased in cardiac tissue from patients with end-stage heart failure due to ischemic heart disease. Myocardial ischemia in mice *in vivo* induced gene expression of key TLR9 pathway proteins (MyD88 and Unc93b1). In this model, a functional link between TLR9 and p66ShcA was revealed as; (i) ischemia-induced upregulation of TLR9 protein was abrogated in myocardium of p66ShcA knockout mice; (ii) when p66ShcA was overexpressed in NFkB reporter cells stably expressing TLR9, NFkB-activation increased during stimulation with the TLR9 agonist CpG B; (iii) in cardiac fibroblasts, p66ShcA overexpression caused TLR9 upregulation. Co-immunoprecipitation showed that ShcA proteins and TLR9 may be found in the same protein complex, which was dissipated upon TLR9 stimulation *in vivo*. A proximity assay confirmed the co-localization of TLR9 and ShcA proteins. The systemic immune response after myocardial ischemia was dampened in p66ShcA knockout mice as interleukin-4, -17 and −22 expression in mononuclear cells isolated from spleens was reduced.

In conclusion, p66ShcA adaptor may be an interaction partner and a regulator of the TLR9 pathway post-infarction.

1. Introducion

After myocardial infarction (MI), injured and necrotic tissue releases a plethora of substances termed damage-associated molecular patterns (DAMPs) [1]. DAMPs trigger the innate immune system through pattern recognition receptors (PPRs), inducing sterile inflammation [2]. One class of PPRs, the Toll-like receptor family (TLRs), sense DAMPs and trigger inflammation. This may evoke additional tissue damage and contribute to remodelling [3].

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https://doi.org/10.1016/j.bbrc.2022.12.085

TLR9 recognizes fragments of DNA enriched in unmethylated cysteine-phosphate-guanine (CpG) motifs. These motifs are features of bacterial DNA, but are also found in mitochondrial DNA (mtDNA) [4]. mtDNA is an inflammatory mediator in cardiovascular disease [5,6] and is also released during cardiac surgery [7]. Most of our knowledge regarding TLR9 comes from immune cells [8], but we and others have shown the presence of TLR9 in cardiac cells [9,10]. TLR9 signaling pathway includes the main adaptor protein MyD88 and the Unc93b1 transporter, the latter is a key regulator of TLR9 trafficking [11]. TLR9 is constantly transported from the endoplasmatic reticulum to the endolysosomal compartment by Unc93b1 [12]. It has been shown that TLR9 signaling requires Bruton's tyrosine kinase (Btk) [13]. Upon *N*-terminal domain cleavage in the acidic endolysosomal milieu, the TLR9 receptor interacts with its ligand and activates MyD88 [8]. Two pathways exist downstream of MyD88, both leading to an inflammatory response either through NFκB activation, or via interferon-response [8]. In addition to the TLR9 pathway, cells have several other DNA sensing mechanisms like DExD/H-box helicase 9 (DHX9) [14] and receptor for advanced glycosylation end-products (RAGE) [15].

Mice stimulated with a TLR9 agonist (ODN-1668, B-type) develop myocardial inflammation [10]. Moreover, accumulation of endogenous mtDNA in pressure overloaded mouse hearts leads to TLR9 activation and adverse heart remodelling [16]. We have shown that mtDNA is released after myocardial reperfusion in patients with ST-segment elevation MI [17], and that mtDNA activates NFκB through TLR9, inducing cell death in mice cardiomyocytes [9]. mtDNA is an inflammatory mediator in cardiovascular disease [6], and the TLR9 pathway may therefore contribute to systemic and local inflammation post infarction.

p66ShcA is an adaptor protein, which regulates numerous tyrosine kinases [18] and is essential for heart development [19]. Mouse hearts lacking p66ShcA are protected from acute ischemia-reperfusion injury *ex vivo* [20] and have improved healing after MI *in vivo* [21]. However, some conflicting results about the cardioprotective role of p66ShcA have been reported [22]. p66ShcA may be involved in adaptive immunity [23], but its role in innate immunity and sterile inflammation is not well known. p66ShcA is an upstream regulator of Btk signaling in lymphocytes [24]. Btk also plays a central role in innate immunity, regulating TLR9 signaling [25]. However, a functional link between p66ShcA and TLR9 has not been previously shown.

We hypothesize that the p66ShcA adaptor protein promotes DNA-sensing signaling via the TLR9 pathway after ischemia/MI. We propose a functional link between p66ShcA and TLR9 proteins.

2. Methods

2.1. Human myocardial tissue

Explanted hearts from patients with end-stage heart failure due to either ischemic heart disease (CAD, n = 9) or non-ischemic cardiomyopathy (CMP, n = 15) undergoing cardiac transplantation were used. The studies were in agreement with the 1964 Declaration of Helsinki, and were approved by the Regional Ethics Committee. Written, informed consent was obtained from all patients or family members. Biopsies from hearts of patients dying suddenly of non-cardiac reasons were used as controls. These patients were sex- and age-matched individuals (n = 7). Their hearts could not be used for transplantation, but were used for homograft preparation (summary of material and clinical information in Supplementary Table 1). During storage, protein degradation was observed in samples from the CMP and the CAD group.

2.2. Animals

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals (United States National Institute of Health, NIH Publication no. 85–23, revised 1996) and ethical permission was obtained from The Norwegian Animal Research Authority. Three-month-old wild type (WT) male C57BL/6J mice were purchased from NOVA-SCB (Nittedal, Norway). Three–month-old male p66ShcA knockout (p66KO) mice of C57BL/6J background and their littermate WT siblings (p66WT) were generated by Marco Giorgio and delivered from Charles River laboratories, Milan, Italy [26]. Mice were housed under artificial lighting (12/12 h light cycle) and fed standard chow. Drinking water was available *ad libitum*.

Three series of *in vivo* experiments were performed. Detailed description of all the groups are shown in Supplementary Table 2. In brief:

Series I: MI was induced in WT mice to study gene expression of key players in DNA sensing and TLR9 pathways. Hearts were sampled 24 h or one week after permanent ligation of the left coronary artery or sham operation (four groups, seven animals in each).

Series II: Hearts were sampled three days or one week after induction of MI in p66KO. Hearts were sampled from healthy WT littermates for baseline control. Tissues from these mice are similar to our previous work [21]. In four additional groups (infarcted and healthy p66KO and infarcted and healthy WT) spleens were harvested three days post-infarction for flow cytometric analysis (nine groups in total, five animals in each) in order to assess systemic inflammation.

Series III: Ten WT mice were injected intraperitoneally (i.p) with a TLR9 agonist (CpG, Invivogen); ten controls were injected i.p with vehicle. Additional hearts were harvested from five WT mice for isolation of cardiac fibroblasts.

2.2.1. Animal model of myocardial infarction

MI was induced in mice as previously described [21]. Briefly, mice were anesthetised with a mixture of isoflurane-oxygen, intubated, and ventilated. The body temperature was kept at 37 °C during surgery by a heating plate. The thoracic cavity was opened in the fourth intercostal space followed by permanent ligation of the left coronary artery. Skin and muscle were reattached by sutures. NaCl (Sigma, Drammen, Norway) (0.9%, 0.5 mL) and Temgesic (Schering-Plough, Norway) (0.1 mg/kg) were administered subcutaneously for rehydration and analgesia after the operation. Temgesic was administered postoperatively by the veterinarian any time pain-associated behavior of the mice was observed. In sham operations all steps except coronary artery ligation were performed.

2.3. Spleen cells preparation and FACS

Interleukin expression in the mononuclear fraction of spleen cells was used as a measure of systemic sterile inflammatory response to MI. Mononuclear cells were isolated from WT and p66KO mice spleens as previously described [27]. Expression of CD4, CD8, CD25, NK1.1, INF-γ, IL-4, IL-17 and IL-22 was evaluated by flow cytometry (FACS Calibur, Becton Dickinson Biosciences,San Jose, CA, USA) [27]. In that paper the expression of IL-17 and IL-22 from WT mice and WT mice three days after myocardial infarction was shown. For comparison with the p66KO mice, these values were shown again in Supplementary Fig. 4. Antibodies are listed in the Supplemental Table 3.

2.4. Transfection of primary cardiac fibroblasts

Primary cardiac fibroblasts were isolated from mouse hearts [28] and cultured for three passages (MEM, 10% CFS, and 1% Penicillin-Streptomycin). Phoenix cells (a retroviral producer cell line based on 293T cell line, kindly provided by Marco Giorgio [29]) were transfected (Lipofectamin 2000, Invitrogen) with cloned human p66ShcA gene (pBabe construct). Retroviral supernatant was collected 24 h after transfection. Cardiac fibroblasts were infected with the supernatant and cultured for another 24 h before the experiment. We recently reported successful transfection in this cell type with p66ShcA [21]. Cells were sampled and lysed in RIPA buffer. Expression of p66ShcA was measured by Western blotting.

2.5. Hek-Blue reporter cells transfection and CpG B stimulation

Hek-Blue NFκB reporter cells overexpressing mouse TLR9 (Invivogen) were co-transfected (Lipofectamin 2000, Invitrogen) with pBabe construct containing cloned human p66ShcA gene or with empty pBabe vector. Before the experiment, p66ShcA overexpression was verified by Western blotting (data not shown). Co-transfected and non-transfected control cells were resuspended in Hek-Blue detection media and loaded on a 96-well plate. The cells were stimulated with 20 μg/mL CpG B or with vehicle (LPS-free water). Absorbance 640 nm in each well was measured each hour with FLUOstar Omega microplate reader (BMG Labtech).

2.6. In vivo TLR9 stimulation

The three main classes of synthetic CpG-rich oligonucleotides (CpG ODN) – A, B and C, induce interferon response, NFκB response or both, respectively. We used the murine-specific TLR9 agonist CpG ODN Class B (CpG-B, ODN 1668 Invivogen, 75ug per animal), which is the most potent inducer of inflammation [30]. Wild type mice were injected i.p with galactoseamin (1 mg/kg, Sigma-Aldrich), 30 min before the TLR9 agonist, to decrease clearance of the agonist by liver [30]. CpG-B or vehicle (PBS) were administrated i.p (n = 5 in each group). Hearts were sampled 30 min after agonist injection.

2.7. Co-immunoprecipitation of ShcA and TLR9

Heart tissue was lysed in co-immunoprecipitation buffer (0,15 M NaCl, 0,05 M Tris/HCl, 0,003 M EDTA, 1% Octyl-D-Glucose, 0,5% NP-40, supplemented *ex tempore* with 2x phosphatase/protease inhibitors (Halt Protease and Phosphotase inhibitor Cocktail (x100) Thermo Scientific) for isolation of transmembrane receptors and preservation of protein complexes. The extracts were cleaned by centrifugation (15000 g, 20 min, 4 °C) and protein concentration was measured with microBCA kit (Thermo Scientific). Volume of the extract corresponding to 700 μg of protein was pre-cleaned with 20 μl of A/G-Sepharose beads (Santa-Cruz) and centrifuged for 3 min at 3000 g, (4 °C) to get supernatant. Antibodies against TLR9 (TLR9 (H-100) rabbit polyclonal, sc-25468 Santa Cruz Biotechnology) and ShcA (Rabbit polyclonal ab155170 Abcam) (2 μg per sample) were pre-incubated with A/G-Sepharose beads (30μg per sample). Mixture of antibodies and the beads was added to the supernatant and gently agitated at 4 °C for 5 h. Beads were spun down (3000 g, 3 min, 4 °C), washed five times with the lysis buffer, and boiled for 5 min in 2x Laemmly buffer and run on Western blotting. Antibodies are listed in Supplementary Table 4.

2.8. Proximity ligation assay

In supplement to co-immunoprecipitation, in situ proximity ligation assay based on DuoLink technology was used (Sigma-Aldrich). This method enabled us to investigate p66ShcA-TLR9 interaction. Positive and negative technical and biological controls were used to control for unspecific signal. Detailed procedure and description are presented in supplementary methods.

2.9. Western blotting

Proteins were extracted from whole mouse heart tissue, separated by electrophoresis and transferred to PVDF membranes to detect ShcA isoforms, Btk, pBtk(Y233) and TLR9, as previously described [21]. Antibodies are listed in Supplementary Table 4. Equal loading was confirmed with either Ponceau or Coomassie staining according to the manufacturer's protocol. All Western blots were normalized to loading control. For the human samples in Fig. 1, the absolute values from the densitometry ratio is shown. Samples were randomly selected from the groups due to limitation in the gels. In Fig. 2, the expression of TLR9 proteins are normalized to the loading and to one day and one week respectively.

2.10. Real time PCR

Total cardiac RNA was extracted from heart tissue and reverse-transcribed to cDNA as previously described [21]. Target gene expression was assessed by relative quantification (ΔΔCt). Mouse Rpl32 was used as housekeeping gene. All primers were purchased as ready-to-use TaqMan gene expression assays (Applied Biosystems) for the following mouse genes: Unc93b1, Trex1, RNAseh1, DNAse2a Mavs, Tmem173, RAGE, Tbk1, Rpl32, Dhx36, Dhx9.

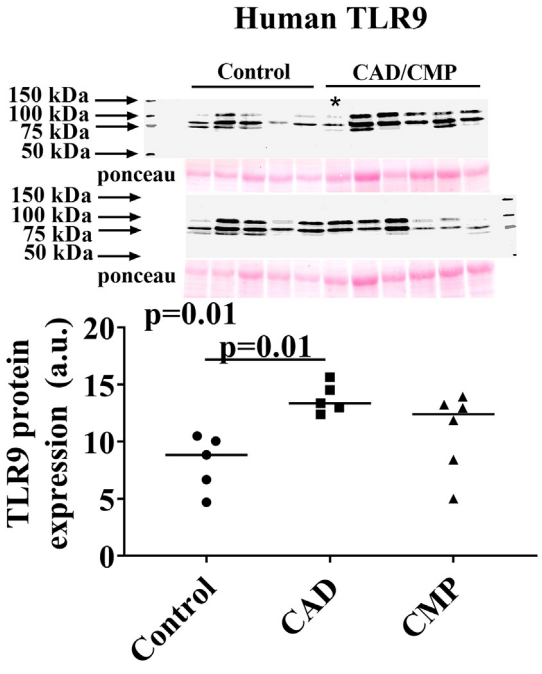

Fig. 1. Cardiac TLR9 protein expression in human explanted hearts. Control hearts from patients dying from non-cardiac causes (control), or from end stage heart failure due to ischemic heart disease (CAD), or end stage heart failure due to non-ischemic cardiomyopathy (CMP). A one way ANOVA with Dunnet post-test was used. The images above the scatter plot show the densitometry of the membranes, with the corresponding Ponceau staining. The densitometry is normalized to loading control (Ponceau). One outlier is removed from the CAD group by the Grubbs' test for outliers (* in WB images).

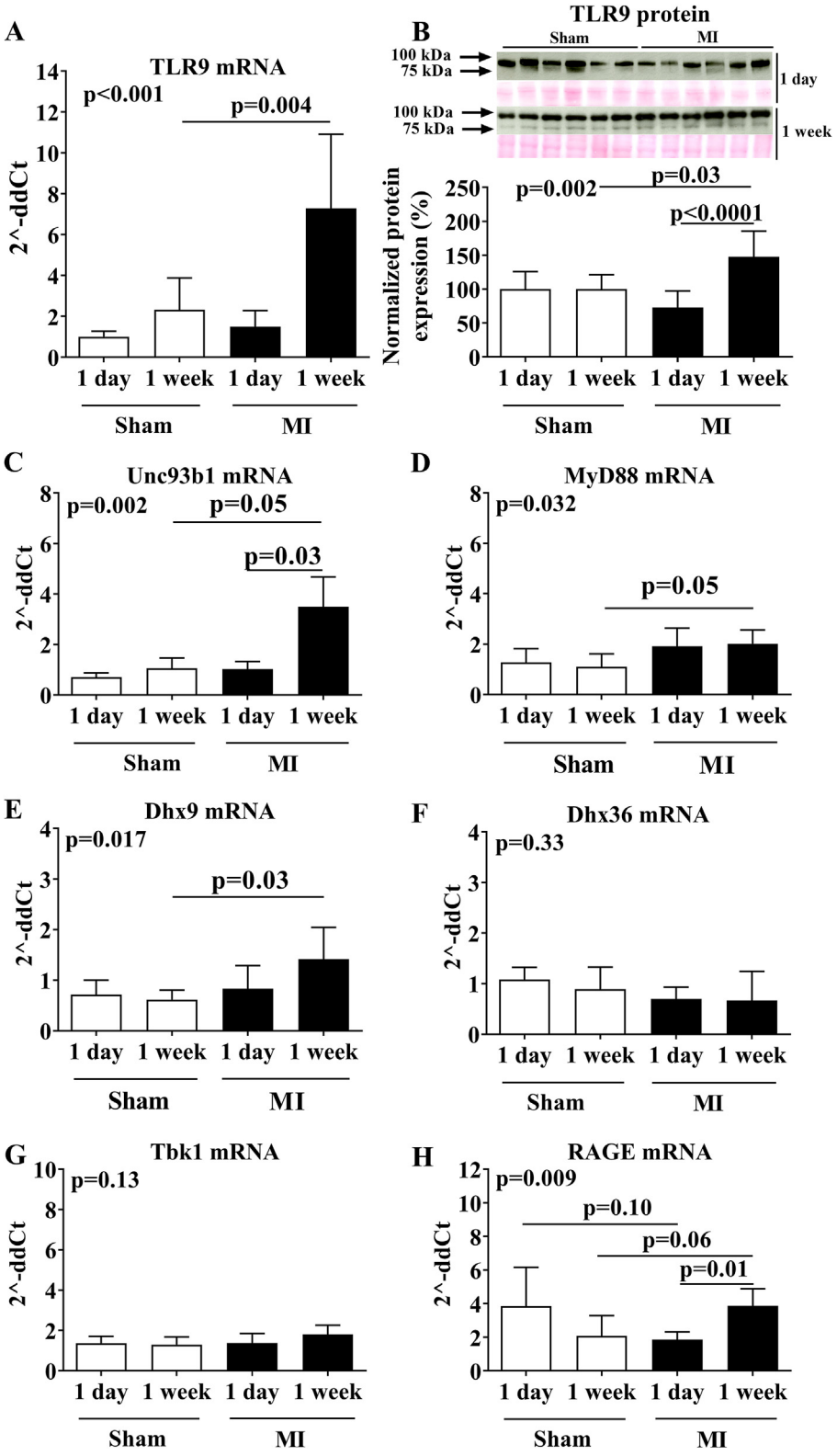

Fig. 2. Cardiac TLR9 signaling and DNA-sensing pathways were increased after myocardial infarction (MI). Wild type mouse hearts were harvested one day and one week after MI. Sham operated wild type mice (Sham) were used as controls (n = 6–8 in each group). Ct values from samples of non-operated mice were used as calibrators (arbitrary units were calculated by ΔΔCt method). Total cardiac proteins were isolated from the same tissue samples. Densitometric value of the specific signal was related to total protein load detected by Ponceau staining and expressed as per cent of sham control. A one-way ANOVA (p-value in the upper left corner of each panel) with Sidak post-test was used to test significant differences in panel B, D, E, F and G while a Kruskal-Wallis test, with a Dunn post-test was performed when SD was significantly different (A, C and H). B) above graph is the representative membranes with bands showing TLR9 banding and the corresponding loading Ponceau control. Data are shown as mean ± SD.

2.11. Statistics

Multiple comparisons were performed with ANOVA and Sidak post-hoc test. If the ANOVA displayed significant differences in SD, a non-parametric Kruskal-Wallis test with a Dunn's post-test was used. A two-way ANOVA was used to test differences in co-transfection and CpG stimulation. A Grubbs' test was used to identify outliers (human data Figs. 1 and 3B). A Student's t-test was used for continuous normally distributed data (cell culture experiments). GraphPadPrism, (San Diego, CA) was employed for the analysis. $P < 0.05$ was considered as significant and exact p-values are given for the post test in each panel if $p < 0.1$. Data are expressed as mean ± SD except data from FACS experiment which is presented as mean ± SEM.

3. Results

3.1. TLR9 protein expression was upregulated in human hearts with heart failure due to ischemic heart disease

TLR9 protein expression was increased in explanted hearts from patients with end-stage heart failure due to ischemic heart disease compared to control hearts from patients dying of non-cardiac reasons. (Fig. 1). The antibody detected bands at 100 kD, 80kD and 75kD respectively (Fig. 1).

3.2. Myocardial infarction upregulate cardiac TLR9 and other DNA-sensing pathways in wild type mice

One week after MI, TLR9 expression increased compared to sham group both at the mRNA (Fig. 2A) and protein level (Fig. 2 B). TLR9 protein increased from day one to day seven post-infarction (Fig. 2B). After one week, mRNA of Unc93b1 and MyD88, the main interacting partners of TLR9, were upregulated (Fig. 2C and D). Furthermore, the MyD88-dependent DNA receptor Dhx9 was upregulated at mRNA level after one week, while Dhx36 did not change (Fig. 2E and F). Gene expression of Tbk1 (Fig. 2G) and other MyD88-independent DNA-sensing receptors such as Mavs and Tmem173 (Supplementary Fig. S2) did not change. Gene expression of the scavenger receptor RAGE decreased one day after infarction compared to sham but increased after one week compared to one day post MI (Fig. 2H). MI had no effect on mRNA expression of the major intracellular DNAses (Trex1, DNAse2a and RNAseh1, Supplementary Fig. S2). One day post-infarction Btk expression showed a small increase (Fig. 3A), while phosphorylation was decreased, indicating decreased activation. After one week, both Btk expression and phosphorylation increased, but without any difference in Btk/pBtk ratio (Fig. 3B).

3.3. Induction of TLR9 pathway was attenuated three days after myocardial infarction in p66KO mice

Three days after infarction, we found no difference in TLR9 mRNA expression either by infarct or genotype (Fig. 4A). TLR9 protein expression decreased from one-day post infarction to three days in p66KO, but not in WT mice (Fig. 4B). In non-infarcted sham hearts, expression of TLR9 tended to be higher in p66KO compared to WT ($p = 0.1$, Fig. 4B), while mRNA expression of TLR9 increased after MI for both p66KO and WT mice (Fig. 4C). Gene expression of Unc93b1 tended to be higher in p66KO compared to WT one week after MI (Fig. 4E). One week post MI, there was no difference between p66KO and WT mice in expression and phosphorylation of Btk (Fig. 3B). For MyD88, DNASE2 (Supplementary Fig. S3) DHX9, DHX36 and Tex1 (data not shown) no difference was found between the genotypes either after three days or one week post MI.

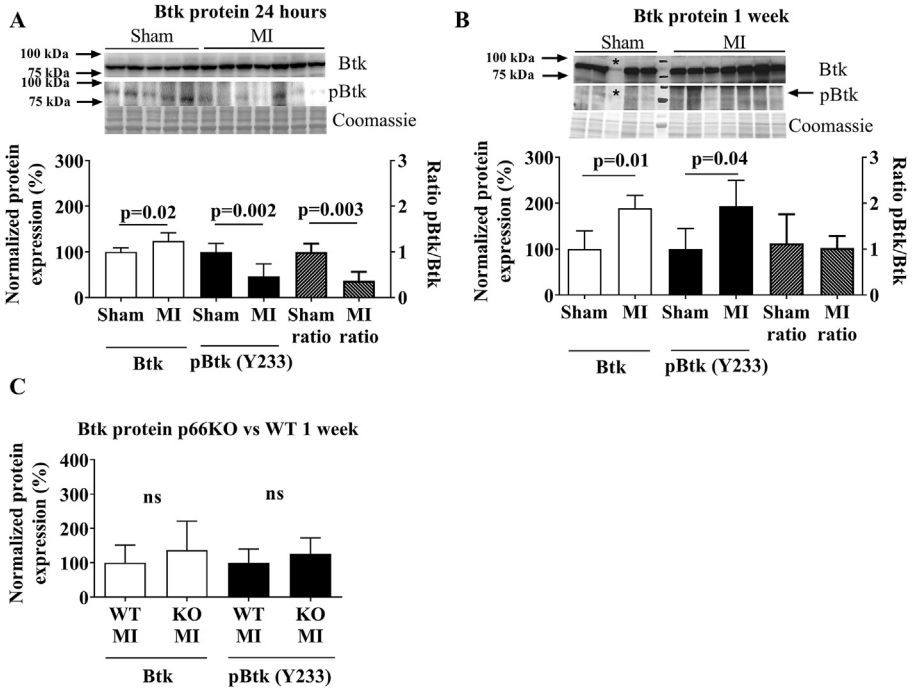

Fig. 3. Btk protein expression increased one day and one week after myocardial infarction (MI) compared to sham (Sham), while phosphorylation of Btk was decreased and increased after one day and one week respectively. A) Mouse hearts were harvested 24 h after MI (n = 7) or sham operation (n = 5) and B) one week after MI (n = 5 in sham and n = 7 in MI). C) Wild type (WT MI) and p66KO (KO MI) mouse hearts were also harvested one week post-infarction. Densitometric values are normalized to sham for Btk and pBtk. The pBtk/Btk ratio was calculated. Non-parametric *t*-test was used for statistical analysis. One outlier is removed from the one-week sham group by the Grubbs' test for outliers (* in WB images). Data are shown as mean ± SD.

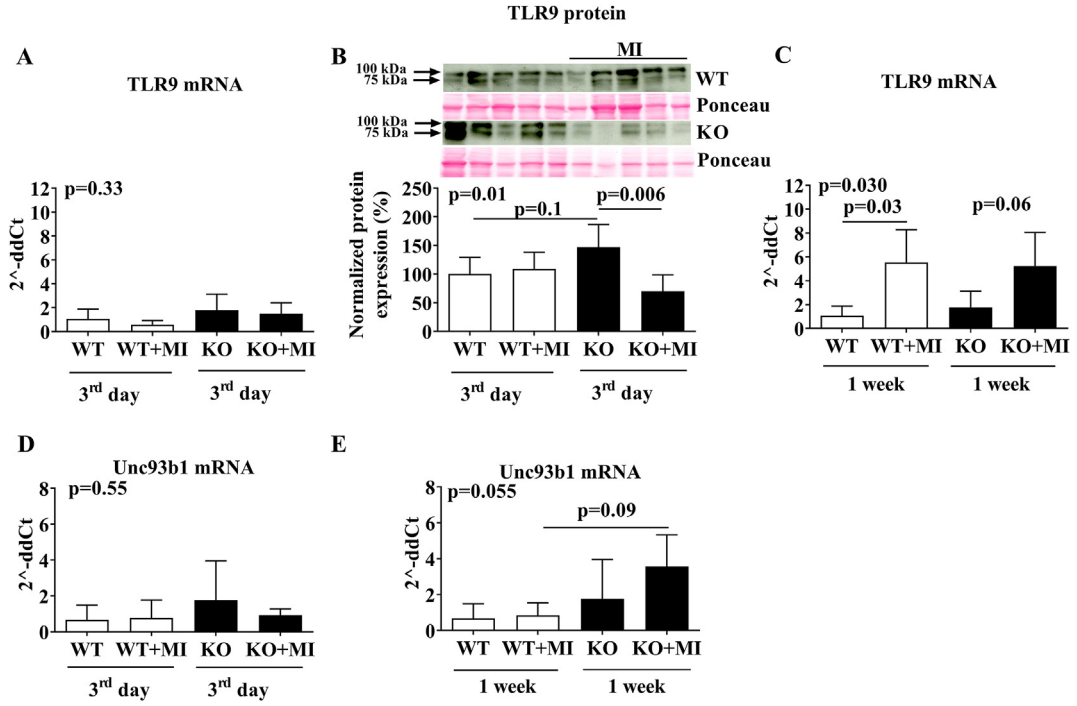

Fig. 4. Expression of TLR9, Unc93b1 in hearts of p66ShcA knockout (KO) and wild type mice (WT) mice before as well as three days (A, B, D) and one week (C, E) after myocardial infarction (MI) (n = 5 in each group). Additional hearts were sampled from non-operated animals (n = 5 in each group) and used for calibration of gene expression. (B) Densitometric value of the specific TLR9 signal was normalized to total protein load detected by Ponceau staining and expressed in per cent of WT mice. All groups were run on the same gel but divided in image to fit frame. (B–E) Ct values from samples of non-operated mice were used as calibrators (arbitrary units were calculated by ΔΔCt method). A one-way ANOVA with Sidak post-test was used to test significant differences. Data are shown as mean ± SD.

3.4. Targeted deletion of p66ShcA modulate the systemic inflammatory response after myocardial infarction

Baseline expression of IL-4 was higher in activated mononuclear cells isolated from p66KO spleens compared with WT littermates (Supplementary Fig. S5B). After myocardial infarction expression of IL-4 increased in cells from WT mice, while IL-4 expression decreased in p66KO (Supplementary Fig. S5B). Moreover, deletion of p66ShcA reduced expression of interleukin-17 in activated mononuclear cells isolated from spleen three days after MI as compared to WT (Supplementary Fig. 4B). MI decreased interleukin-22 expression in activated mononuclear cells from p66KO spleen cells after MI (Supplementary Fig. S4D). Neither MI nor targeted deletion of p66ShcA influenced the percentage of CD4, CD8, CD25 and NK1.1 positive cells (Supplementary Fig. S6). IFN-γ from monocytes changed neither before nor after activation *in vitro* (Supplementary Fig. S6).

3.5. Overexpression of p66ShcA in cardiac fibroblasts increased TLR9 expression and NFκB activation upon ligand stimulation

In primary cardiac fibroblasts transfected with p66ShcA-containing plasmid, TLR9 expression increased three-fold compared to cells transfected with empty plasmid (Fig. 5A). Transfection of Hek-Blue NFκB reporter cells overexpressing TLR9 with p66ShcA plasmid increased NFκB activation upon stimulation with the TLR9 agonist CpG B compared to cells co-transfected with empty vector or in non-transfected cells (Fig. 5B).

3.6. TLR9 and ShcA protein interaction dissociates upon TLR9 stimulation

The TLR9 agonist, CpG ODN class B, increased TLR9 protein expression in myocardial tissue 30 min after administration to WT mice (Fig. 6A). At the same time phosphorylation of ShcA isoforms on tyrosine residue Y317 was reduced (Fig. 6B). Phosphorylation at Y239/240 and Ser 36 was not significantly reduced (data not shown). Anti-TLR9 and Anti-ShcA antibodies were used to pull down ShcA and TLR9 proteins with associated protein complexes. Less TLR9 and ShcA proteins were co-immunoprecipitated 30 min after i.p injection of the TLR9 agonist compared to vehicle control (Fig. 6C and D).

Proximity ligation assay demonstrated that cardiac fibroblasts treated with ODN CpG Class B for 30 min had reduced number of TLR9-ShcA complexes compared to untreated cells (Supplemental Fig. S7). Treatment with CpG Class B reduced the average amount of spots per cell by 45% (2.7 ± 0.1 to 1.3 ± 0.1). Hek-Blue cells overexpressing murine TLR9 and human p66ShcA simultaneously were used as positive controls and had higher number of complexes than Hek-Blue cells overexpressing murine TLR9 only (4.7 ± 0.8 and 1.3 ± 0.4 respectively) (Supplementary Fig. 7). Low positive signal was detected in negative technical controls (0.8 ± 01) (Supplementary Fig. S7).

4. Discussion

The present work demonstrated that: 1. TLR9 protein was upregulated in patients with end stage heart failure after ischemic heart disease; 2. TLR9 and key regulators of the TLR9 pathway were upregulated in the mouse myocardium after MI; 3. The MI induced upregulation of TLR9 pathway were partly abolished by p66ShcA deletion *in vivo*; 4. p66ShcA overexpression *in vitro* upregulated TLR9 mRNA expression and sensitized the TLR9 pathway to its ligand, CpGB; 5. Cardiac TLR9 and ShcA proteins may exist as a protein complex during baseline conditions, which dissipates upon TLR9 stimulation.

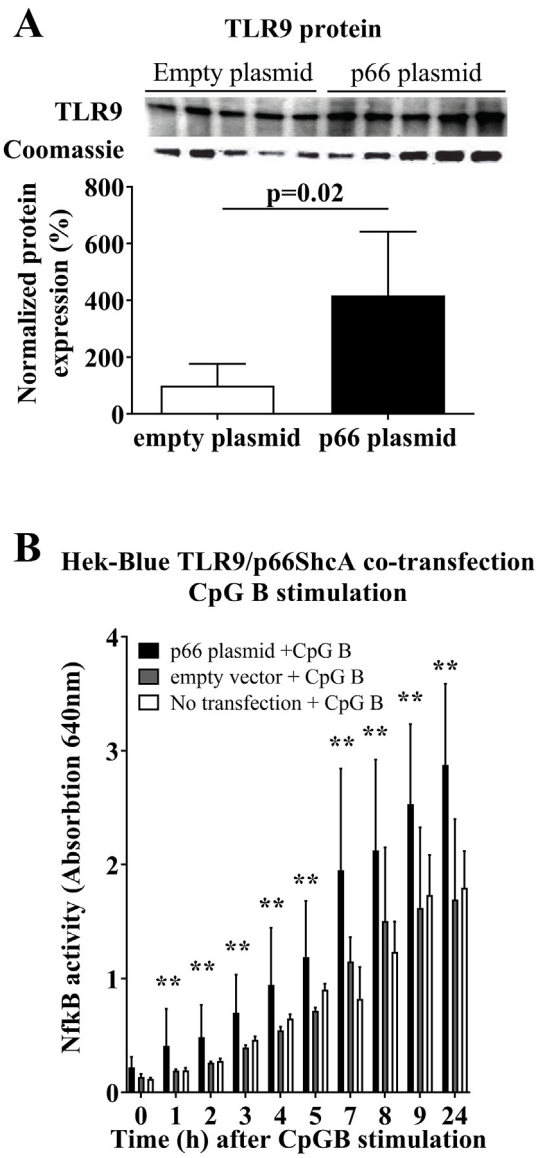

Fig. 5. p66ShcA overexpression was performed by retroviral infection with pBabe plasmid containing p66ShcA gene in primary cardiac fibroblasts from mouse hearts. (A) TLR9 expression was evaluated by Western blotting (loading controls by Coomassie) in fibroblasts with p66ShcA overexpression (p66 plasmid) or controls transfected with empty plasmid (empty plasmid) (mean ± SD). (B) Hek-Blue NFκB reporter cells overexpressing TLR9 and transfected with p66ShcA or empty plasmid were stimulated with CpG B (20μg/μL) and NFκB activity was measured for 12 h. Non-transfected stimulated by CpG B served as controls. Two-way ANOVA was used for statistical analysis. Data are shown as mean ± SD.

The activated TLR9 pathway in cardiac tissue after MI may come from two sources, either infiltration of professional immune cells or activation in cardiac specific cells. The time dependent infiltration profile has previously been carefully evaluated in our model of permanent coronary ligation in mice [31]. The major infiltration is seen during the first day (neutrophils and monocytes), and at day seven (macrophages and dendritic cells) [31]. Less infiltration is expected in the present model compared to an ischemia-reperfusion model. There was no increase in TLR9 protein in mice at 24 h and three days post infarction. We therefore speculate that the increased expression of TLR9 after seven days is a combination of cardiac specific upregulation and infiltration of immune cells in the border zone of the ischemic area.

TLR9 signaling is mostly known from specialized immune cells, which cannot directly be extrapolated to cardiac cells. TLR9 expression has been demonstrated in heart tissue [10], but few reports focus on TLR9 signaling in cardiomyocytes and cardiac fibroblasts. Activation of the TLR9 pathway in cardiac fibroblasts culminates in a classic inflammatory response [32] and decreased proliferation activity [33]. It has been suggested that TLR9 activation in cardiomyocytes, may lead to energy depletion followed by AMPK stimulation [32]. However, the main evidence points towards a role for TLR9 as an agonist of mtDNA in sterile inflammation and development of heart failure [16]. Interestingly, inhibiting TLR9 in a mouse model reduced development and progression of heart failure [34]. Due to the abundance of mtDNA in heart tissue, DNA binding pathways may be particularly important during myocardial infarction.

4.1. Activation of TLR9 pathways after myocardial infarction

TLR9 was upregulated in human hearts with end stage heart failure due to ischemic disease and in our model of permanent ligation in mouse hearts. Gene expression of key TLR9 pathway elements increased. In addition, we show that the Unc93b1transporter and the main adaptor protein MyD88 were upregulated together with TLR9 one week after myocardial ischemia in mice.

To our knowledge, our study is the first to measure Btk expression and phosphorylation in cardiac tissue. Btk is activated by phosphorylation, and we observed a reduced phosphorylation 24 h after MI in mice, concomitant with a low TLR9 expression. Both Btk protein and phosphorylation as well as TLR9 expression increased after one week. Btk is required for TLR9-induced NFκB activation [35].

TLR9 is one of many DNA-sensing receptors in innate immunity. We found upregulation of the DNA-sensing receptor DHX9, a MyD88-dependent receptor, which is able to sense the same ligand as TLR9 [14]. RAGE mediates TLR9-dependent DNA detection via transporting self-DNA into the cell as a part of an immune complex [36]. The upregulation of RAGE gene expression in the present study may thus be part of general upregulation of DNA sensing pathways. There was no evidence for transcriptional upregulation of DNAses during the first days after MI. All taken together we find evidence of a general upregulation of DNA sensing pathways in cardiac tissue after MI.

4.2. Modified TLR9 expression in p66SchA knockout mice

p66KO mice had increased baseline TLR9 protein expression compared to WT. Moreover, p66ShcA overexpression in two different *in vitro* systems led to TLR9 upregulation and increased sensitivity of TLR9 pathway to its ligand. This indicate a possible link between TLR9 and p66ShcA. Although the protein expression of TLR9 in the p66ShcA KO was lower than WT three days after MI, the gene expression was similar one week post MI. p66ShcA KO leads to less superoxide production in mice macrophages [23], indicating reduced innate immune response. We have previously showed that p66KO mice are protected against adverse cardiac remodelling and oxidative stress post infarction [20,21]. Our findings indicate that p66ShcA serve as a regulator of the TLR9 pathway.

4.3. Targeted deletion of p66ShcA may reduce systemic inflammation after myocardial infarction

Acute MI is associated with systemic inflammation and increased levels of circulating cytokines, increased production of reactive oxygen species, and increased *ex vivo* activation of

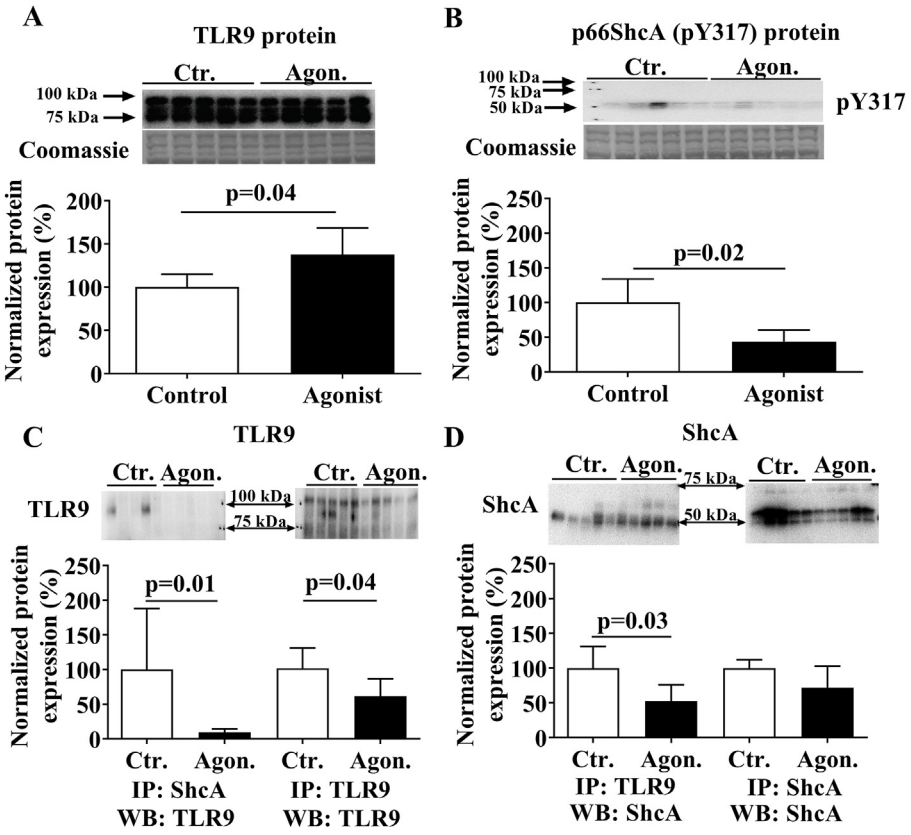

Fig. 6. TLR9 and ShcA proteins co-exist in the same protein complex, which is dissociated upon TLR9 stimulation. (A, B) Hearts of wild type, non-infarcted mice were sampled 30 min after treatment with TLR9 agonist or vehicle control. Protein extracts from ventricular tissue were used to measure accumulation of truncated TLR9 (A) and p66ShcA phosphorylation on Y317 residue (B). The same extract has been used for co-immunoprecipitation, when ShcA proteins or TLR9 was pulled down with beads loaded with corresponding antibodies. The bounded proteins were washed from beads and probed with the same antibodies on Western blotting (C–D). Densitometric value of the specific TLR9 or ShcA signal was normalized to loading and expressed as per cent of vehicle-treated control. Data are shown as mean ± SD.

monocytes and polymorphonuclear granulocytes [2]. Our observations that the p66KO mice were associated with a decrease in interleukin-4, -17 and −22 induction after MI is in the line with known pro-inflammatory effects of p66ShcA protein. Our observations may suggest that splenocytes were less activated by MI in p66KO mice.

4.4. TLR9 and ShcA interaction

The difference in ischemia-induced TLR9 pathway response between WT and p66KO mice *in vivo* and our *in vitro* data suggests a possible link between p66ShcA adaptor protein and the TLR9 pathway. We therefore performed co-immunoprecipitation and found indications of a protein-protein interaction as ShcA proteins was pulled down with TLR9 antibodies and vice versa. Moreover, we found less cardiac TLR9 and ShcA protein expression after treating mice with the TLR9 agonist CpG-B. Using pulldown with TLR9 only the p46 and the p52 was visible, indicating that TLR9 protein interacts with these two isoforms of the ShcA proteins. To further test the protein link, we performed a proximity assay, also supporting a direct interaction of TLR9 and Shc proteins. In HEK cells, TLR9 stimulation together with p66ShcA transfection increased NFκB activity, which indicates a synergistic effect of TLR9 and p66ShcA.

ShcA is localized at the endoplasmic reticulum and is recruited to the plasmalemma upon dephosphorylation of the Y317 residue [37], which we found after stimulation with the TLR9 agonist, CpGB. Btk may be another link between TLR9 and ShcA proteins as phosphorylation of Btk kinase on Y233 tyrosine residue regulates its binding activity to the interaction partners [38]. However, Btk expression or phosphorylation was not influenced by p66ShcA deletion. We suggest that Btk may function upstream to p66ShcA because no effect on its expression and phosphorylation was observed in our gain-of-function experiment.

5. Conclusion

TLR9 signaling and DNA-sensing partners are increased in heart tissue after MI. We show both *in vivo* and in *in vitro* that the ShcA adaptor protein may be a partner and a regulator of the TLR9 pathway.

Sources of funding

Financial support was provided by the Norwegian Research Council, Gjensidigestiftelsen, the Norwegian Health Association, the Anders Jahre Foundation, Odd Fellow and the University of Oslo. Telethon-Italy (GGP11224) to Marco Mongillo and University of Padova (Progetto Giovani Studiosi 2010, contract: GRIC101133) to Tania Zaglia.

Declaration of competing interest

No conflict of interest.

Acknowledgements

We greatly acknowledge Torun Flatebø for technical support with Western blotting and qPCR and Dr. Arkady Rutkovskiy for graphical design.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.12.085.

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