Original Paper

The Impact of Circulating Mitochondrial DNA on Cardiomyocyte Apoptosis and Myocardial Injury After TLR4 Activation in Experimental Autoimmune Myocarditis

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Key Words

TLR4 • EAM • Circulating mtDNA • ROS stress • TLR9

Abstract

Background/Aims: Mitochondrial DNA (mtDNA), acting as a newly found 'danger-associated molecular patterns' (DAMPs), is released into circulation upon tissue injury and performs as a considerable activator of inflammation and immune response. However, the role of circulating mtDNA in experimental autoimmune myocarditis (EAM) as well as Toll like receptor4 (TLR4) mediated cardiac inflammation and injury remains unknown. **Methods:** A model of EAM was established in BALB/c mice by immunization with porcine cardiac myosin. Lipopolysaccharide (LPS) was used to stimulate TLR4 activation in EAM mice and H9C2 cells. **Results:** LPS stimulation significantly aggravated cardiac inflammation and tissue injury in EAM, as demonstrated by increased myocardium inflammatory cell infiltration, and up-regulated inflammatory cytokines and troponin I(TnI) level in serum. Circulating mtDNA level was increased in EAM and TLR4 activation led to a greater elevation, which may be related to Reactive oxygen species (ROS) stress involved mtDNA damage characterized by reduced mtDNA copy number in myocardium tissue. In addition, the expression of Toll like receptor9 (TLR9), a ligand of mtDNA, was significantly up-regulated in the myocardium of EAM and EAM LPS group; meanwhile, TLR9 inhibition by ODN 2088 caused an inhibited apoptosis in LPS treated H9C2 cells. Moreover, in EAM and EAM LPS group, simultaneously giving ODN 2088 treatment significantly ameliorated cardiac inflammation and tissue injury compared with untreated group. **Conclusion:** Increased circulating mtDNA combined with upregulated TLR9 expression may corporately play a role in EAM as well as TLR4 activation mediated cardiac inflammation and injury.

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Introduction

Autoimmune myocarditis, a major cause of dilated cardiomyopathy (DCM), is regarded as a persistent inflammation condition generating severe and irreversible damage to the myocardium, which is triggered by endogenous alarm signals referred to as 'danger-associated molecular patterns' (DAMPs) [1, 2]. These DAMPs are recognized mainly by Toll-like receptors (TLRs) expressing on immune cells and parenchymal cells in heart [3].

TLR4 activation has been demonstrated to be indispensable for the induction of experimental autoimmune myocarditis (EAM), and the development into the phase of DCM [4, 5]. TLR4 mediated inflammatory immunity response characteristic with leucocyte activation and infiltration in myocardium leads to cytokine secretion, oxidative stress and protease release, which promotes apoptosis and necrosis of myocardial cells [1, 6]. Along with this, damaged myocardium could release more endogenous molecules termed DAMPs, including HSPs, HMGB1, crystalline uric acid, fibronectin and mitochondrial component, recognized by TLRs and forming a positive feedback loop to aggravate heart injury and promote DCM formation [2, 3]. Besides, knock-out of TLR4 or targeting downstream effectors of TLR4 such as MyD88 showed protective effects in several types of heart failure animal models, namely, myocardial ischemia, pressure overload and autoimmune myocarditis [4, 7, 8]. Taken together, TLR4 activation acts as an important mediator for autoimmune myocarditis, and to clarify the molecular basis of TLR4 functioning in a critical effector pathway is vitally needed for the therapeutic interventions in EAM [1, 5].

Recent study has clearly demonstrated that dysfunctional mitochondria are crucial sources of DAMPs [9]. Mitochondrial components including mitochondrial DNA (mtDNA), N-formyl peptides and lipids such as cardiolipin are released into cytoplasm or extracellularly performing as powerful initiators and masters of the innate immune response [9, 10]. MtDNA, consisting of an approximately 16.5 kb circular double-stranded extrachromosomal DNA, particularly with an unmethylated CpG motif that resembles bacterial DNA, as a newly found DAMP, shows an increasing role in inflammatory diseases [11, 12]. It was revealed that mtDNA may be released following cellular necrosis and apoptosis [13]; however, studies showed that TLR4 stimulation by LPS can also induce mtDNA secretion *in vivo* and *in vitro* [12, 14]. Circulating mtDNA level was significantly increased in patients with a variety of critical conditions including severe sepsis, trauma and acute ischemia in heart or kidney [15-17]. Several immune cells including monocytes and granulocytes could be activated by mtDNA and present an inflammatory phenotype [18, 19]. In isolated rat lungs, exogenous administration of mtDNA could recapitulate the clinical phenotype of acute respiratory distress syndrome, which was abrogated by the simultaneous administration of TLR9 blocker [20]. Recently, mtDNA has been discovered to play a growing role in cardiovascular diseases [11]. Damaged mtDNA mediated by oxidative stress induced a potent inflammatory response and promoted atherosclerosis development in the aortas of LDLR knockout mice [21]. McCarthy et al demonstrated that circulating mtDNA contributed to elevated arterial pressure and vascular dysfunction in spontaneously hypertensive rats [22]. Moreover, study also showed that extracellular mtDNA can directly cause mitochondrial dysfunction and death in cardiomyocytes by inducing ROS stress and NF- κ B activation [23]. However, the role of mtDNA in EAM has not been investigated.

In the current study, we hypothesized that the level of circulating mtDNA is elevated in EAM, and may involve in TLR4 mediated cardiomyocyte apoptosis and myocardial injury. We observed the changes of mtDNA damage mediated by TLR4 *in vivo* and *in vitro*, and explored the possible role of ROS stress in circulating mtDNA level in EAM. Finally, we investigated the effect of TLR9 inhibition with ODN2088 in EAM as well as TLR4 mediated cardiomyocyte apoptosis and myocardial injury.

Materials and Methods

Experimental autoimmune myocarditis and experimental design

Male C57BL/6 mice aged 6 weeks were purchased from Animal Experimental Center of Fudan University (Shanghai, China). The mice were treated with a standard diet and water ad libitum at the Shanghai Medical College Animal Care Facility according to institutional guidelines. For EAM induction, mice were immunized with 200 μ L of a 1:1 emulsion of PBS with 2 mg/mL of porcine cardiac myosin (Sigma Chemical Co) in complete Freund's adjuvant (CFA) (Sigma) in the groin subcutaneously at days 0 and 7 as described previously [24]. Control mice received PBS/CFA only. The EAM animals were randomly allocated to five groups as follows: EAM group received 200 μ L normal saline; EAM LPS group received LPS (2.5mg/kg) (Invivogen, San Diego, CA, USA) (dissolved in 200 μ L normal saline); EAM+ODN Control group received ODN Control (50ug/20g) (Invivogen); EAM+ODN 2088 group received ODN 2088 (50ug/20g) (Invivogen); EAM LPS+ODN 2088 group received LPS (2.5mg/kg) and ODN 2088 (50ug/20g), which were administered intraperitoneally twice a week. The study included six experimental groups: Control, EAM, EAM LPS, EAM+ODN Control, EAM+ODN 2088 and EAM LPS+ODN 2088. All mice were sacrificed for further examinations at the inflammatory peak of EAM on Day 21 after immunization. All animal studies were approved by the Animal Care and Utilization Committee of Fudan University.

Cell culture and treatment

Myocardial myoblast H9C2 was cultured in DMEM/F12 with 10% fetal bovine serum (Gibco, Grand Island, NY). For detecting mitochondrial superoxide production, H9C2 was cultured with 10ug/mL LPS for 24 h after pre-treatment with 10nM Mito-TEMPO (Enzo Life Sciences, Farmingdale, NY) for 1 h, then incubated with 2.5 μ M MitoSOX™ Red Mitochondrial Superoxide Indicator (Molecular Probes) for 10 min, washed and mounted in warm buffer for imaging.

Immunohistochemistry analysis

The heart tissues were fixed with 4% formalin, embedded in paraffin and sectioned into 6 μ m thick slices. To evaluate the expression of TLR9 in myocardium, sections after antigen retrieval were incubated with monoclonal mouse anti-TLR9 antibodies (Abcam, Cambridge, MA, USA), followed by treatment with horseradish peroxidase-conjugated goat anti-mouse antibody. The reaction products were visualized using diaminobenzidine chromogen and counterstained with hematoxylin. Myocarditis severity was scored on hematoxylin and eosin stained sections using grades as described previously [24].

Serum Troponin Determinations

Mouse serum was collected from mice at the time of sacrifice in glass tubes and stored at -80°C until use. The levels of cardiac troponin I were measured with an ELISA kit (Mouse Cardiac TnI, Ultra Sensitive; Life Diagnostics).

Evaluation of apoptosis in tissue sections and cardiomyocytes

DNA fragmentation was detected in situ with the use of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), as described previously [25]. The hearts were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 6 μ m thick sections and treated as instructed in the In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany). The nuclear density was determined via manual counting of the 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei in five fields for each animal with $\times 40$ objective, and the number of TUNEL-positive nuclei was determined by examining the entire section with the same power objective. The cardiac caspase-3 activity was measured using a caspase colorimetric assay kit following the manufacturer's instructions (Chemicon, Temecula, CA, USA). The absorbance of the pnitroaniline cleaved by caspase was measured at 405 nm using a microplate reader (ELx800; BioTek Instruments, Winooski, VT, USA).

Dihydroethidium (DHE) detection

DHE staining was performed as described previously [26]. After being harvested, the heart tissues were embedded immediately in an optimum cutting temperature (OCT) compound and stored at -80°C . The unfixed frozen samples were cut into 6 μ m thick sections and placed on glass slides. 10 μ M/L DHE was

applied to each tissue section and the sections were covered with a coverslip. The slides were incubated in a light-protected humidified chamber at 37°C for 30 min. The ethidium fluorescence (excitation at 490nm, emission at 610nm) was examined by fluorescence microscopy.

Lipid peroxidation detection

The frozen heart tissues were homogenized in 250µl of ice-cold 20 mM Tris-HCl (pH7.4). After centrifugation, 200µl of supernatant were analyzed for lipid peroxidation products [malondialdehyde (MDA)/4-hydroxyalkenals (4-HNE)] using a lipid peroxidation assay kit (Calbiochem, Darmstadt, Germany), as described previously [26].

Mitochondrial DNA quantification

Blood was collected from mice at the time of sacrifice in EDTA tubes. Blood in EDTA tubes was immediately centrifuged at 1600 g for 10 min at 4°C. Plasma was then stored at -80°C until analysis. Circulating plasma DNA, myocardial tissue and rat H9C2 cell genomic DNA were extracted and purified separately using the QIAamp DNA Blood Mini Kit or DNeasy Blood & Tissue Kit (Qiagen). MtDNA content was assessed by using real time PCR (RT-PCR). The primers for mouse mtDNA (mMito F, mMito R), mouse nuclear B2m (mB2m F, mB2m R) and H9C2 mtDNA (rMito F, rMito R), rat nuclear B2m (rB2m F, rB2m R) were used to amplify the respective products from plasma, tissue and cells (Table 1). RT-PCR was carried out in 20 µl of total reaction volume containing 6µl H₂O, 10µl SYBR® Fast qPCR Mix (Takara Biotechnology), ROX Reference Dye 0.4µl, 0.8µl of each of the above mentioned 10 µM primers, and 2µl DNA. RT-PCR was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Branchburg, New Jersey, USA) under the following conditions: an initiation step of denaturation for 10 minutes at 95°C is followed by a further step consisting of 40 cycles of 10 seconds of denaturation at 95°C and 30 seconds of annealing and extension at 60°C, then melting at 95 °C for 5 s, 65 °C for 60 s, and 95 °C continues (melt curve analysis: 1 cycle) and a last step of cooling at 40 °C for 30s [27]. Plasma mtDNA RT-PCR results were presented as the inverse of cycle threshold (CT) for gene amplification [22]. MtDNA copy numbers in cell and tissue were calculated using the following equation: relative copy number = 2^{Dct} (Dct = Ct B2m – Ct Mito) [28]. Endpoint PCR products amplified were performed on a 1% agarose gel to confirm the specificity of the primers.

Real-time polymerase chain reaction (PCR) for gene expression

Total RNA was extracted from tissues using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology, Dalian, China), according to the manufacturer's instructions. The mRNA levels of target genes were quantified using SYBR Green Master Mix (Takara Biotechnology) with the ABI PRISM 7500 Sequence Detection System (Applied

Table 1. Primers used for RT-PCR in the study

Accession number	Primer	Oligonucleotide sequence (5'→ 3')
NC_005089.1	mMito F1	CGTACACCTCTAACCTAGAGAAGG
	mMito R1	GGTTTAAAGTCTTACGCAATTTCC
	mMito F2	CTAGAAACCCGAAACAAA
	mMito R2	CCAGCTATACCAAGCTCGT
	mMito F3	CCTTGTTCCAGAGGTTCAA
	mMito R3	ATGCCGTATGGACCAACAAT
NC_000068.7	mB2M F	GTGGTGCCAGCAGAGACTTA
	mB2M R	GGACAGTGGGTAGGGAAC TG
NC_001665.2	rMito F	CCGTCACCCCTCCTCAAATTA
	rMito R	TGTAGGCCAGATGCTTTGTG
NC_005102.4	rB2M F	CTGGCCACTGAGTTCACAGA
	rB2M R	GACGAGCATGCACTCCACTA
NM_031178.2	TLR9 F	GAGAATCCTCCATCTCCCAAC
	TLR9 R	CCAGAGTCTCAGCCAGCAC
NM_008084.2	GAPDH F	AGCTTGTCTACACGGGAAG
	GAPDH R	TTTGATGTTAGTGGGTCCTCG

Biosystems, Branchburg, New Jersey, USA). Each reaction was performed in duplicate and changes in relative gene expression normalized to GAPDH levels were determined using the relative threshold cycle method. The primer sequences are shown in Table 1.

Western blot analysis

The total lysates from heart tissue were immunoblotted and probed with primary antibodies against TLR-9 (Abcam, Cambridge, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using an adaptation of a previously described protocol [29]. Densitometry was performed using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Enzyme-linked immunosorbent assay (ELISA)

Mouse serum was collected from mice at the time of sacrifice in glass tubes and stored at -80°C until use. TNF- α , IL-1 β and IL-6 were measured using ProcartaPlex® Multiplex Immunoassays (Ebioscience) according to manufacturer's instructions.

Apoptosis assay by flow cytometry

The amount of apoptosis in the cardiomyocytes was determined using the annexin V-EGFP/PI Apoptosis Detection Kit (Vazyme, Nanjing, China) according to the manufacturer's instructions. Briefly, H9c2 cell was treated with 10 $\mu\text{g/mL}$ LPS for 24 h with or without pretreatment of 1 $\mu\text{g/mL}$ ODN 2088 or ODN control for 5 hours, then stained with EGFP-conjugated annexin V and PI for 10min at room temperature. The percentage of positive cells collected from over 10,000 events was analyzed by a FACSCalibur system (BD BioSciences, San Jose, CA, USA).

Statistics

The data are presented as the means $\pm$ SD unless indicated otherwise. The differences were evaluated using unpaired Student's t-tests between two groups when the data were normally distributed and group variances were equal. The Mann-Whitney rank sum test was used when the data were not normally distributed or if group variances were unequal. A one-way analysis of variance (anova) was used for multiple comparisons followed by a post-hoc Tukey's procedure for multiple range tests when the data were normally distributed and the group variances were equal. The Kruskal-Wallis test, followed by Dunn's test, was used when the group data were not normally distributed or if the group variances were unequal. GraphPad Prism version 6.0 software was used for statistical analyses, and the statistical significance was set at $P < 0.05$.

Results

TLR4 activation aggravated cardiac inflammation and injury in EAM

To investigate the effect of TLR4 activation on inflammatory severity after EAM induction, firstly, protein expression in the myocardium of mice was performed. Induction of EAM resulted in an increased expression of TLR4, and simultaneously given LPS treatment at a dose of 2.5 mg/kg further added the trend compared with EAM alone (Fig. 1A and B). Stained by hematoxylin and eosin of hearts, EAM group presented an evident inflammatory cell infiltration compared with control group as showed in Fig. 1C. However, LPS treatment more significantly amplified the response in EAM, and the gross severity scores were also obviously higher than that in EAM group (Fig. 1D).

Induction of EAM results in an increased expression of adhesion molecules and pro-inflammatory cytokines which are collectively involved in the initiation and progression of EAM [1]. Compared with control mice, EAM mice showed an increased expression of TNF- α , IL-6 and IL-1 β in serum, which was further augmented by TLR4 activation (Fig. 1E-G). Obvious inflammation condition mediated by inflammatory cells and cytokines within the heart leads to increased tissue injury [1]. Next we detected the cardiac specific serum Troponin I (TnI) in serum and found that TLR4 activation more significantly raised the level of TnI induced in EAM (Fig. 1H). These results indicate that TLR4 activation aggravated cardiac inflammation and injury in EAM.

Fig. 1. TLR4 activation aggravated cardiac inflammation and injury in EAM. Representative western blot (A) and quantitative analysis (B) of TLR4 expression in myocardium (n=6). (C) Representative illustrations of inflammatory cell infiltration in cardiac sections taken on day 21 by H&E staining (40 $\times$). (D) Myocarditis severity scores in the heart sections (n = 6). Serum levels of TNF- α (E), IL-1 β (F), IL-6(G) were measured by Multiplex Immunoassays(n=6-8). (H) Troponin I serum levels are indicated (n=5-6). *p<0.05 versus Control, # p<0.05 versus EAM.

TLR4 activation induced cardiomyocytes apoptosis and oxidative stress in EAM

Apoptosis has been proposed to be an important mechanism for myocardial injury in EAM [1]. As shown in Fig. 2A and B, the representative TUNEL-stained sections demonstrated drastically more apoptotic cells in EAM LPS group compared with EAM group. Furthermore, TLR4 activation also greatly increased the activation of caspase-3 (Fig. 2C), as determined by a caspase colorimetric assay of myocardium tissue in EAM mice. As an excessive ROS is a hallmark of cardiomyocyte apoptosis mediated by oxidative stress, we investigated whether the increased apoptosis upon TLR4 activation was also associated with a higher level of ROS *in vivo*. As shown in Fig. 3 A and B, ROS stress in EAM mice significantly increased compared with control mice, and LPS treatment further intensified ROS production in myocardial tissue of EAM mice. Consistent with the above result, the ROS-producing activity of cardiac homogenates in EAM mice, as evaluated with lipid peroxides (malondialdehyde and 4-hydroxyalkenal), was also significantly enhanced after LPS stimulation (Fig. 3C). These results suggest that TLR4 activation may promote cardiomyocyte apoptosis by inducing ROS production in EAM.

Fig. 2. TLR4 activation induced cardiomyocytes apoptosis in EAM. (A) Apoptotic cell nuclei in cardiac sections were evaluated by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining (green), and total nuclei by 4', 6-diamidino-2-phenylindole (DAPI) staining (blue). Bar, 50 μ m. (B) Quantitative analysis of percentage of cardiomyocytes undergoing apoptosis *in vivo* (n=5-6). (C) Caspase-3 activity in myocardium was assessed (n=6). *p<0.05 versus Control, # p<0.05 versus EAM.

Fig. 3. TLR4 activation induced cardiac oxidative stress in EAM. (A) Reactive oxygen species (ROS) production in the cardiac sections was evaluated with dihydroethidium (DHE) staining. Bar, 20 μ m. (B) Quantitative analysis of ROS, as normalized to Control (n=5-6). (C) Malonaldehyde+4hydroxy-alenals (MDA+4HAE) contents in myocardium were evaluated with the lipid peroxidation detection (n = 6). *p<0.05 versus Control, # p<0.05 versus EAM.

Circulating mtDNA level was increased in EAM and aggravated by TLR4 stimulation

TLR4 activation has been showed to contribute to inflammation response and tissue injury by inducing mitochondrial dysfunction, which often accompanies the release of mitochondrial components including mtDNA [14, 30]. As an important newly found PAMP, circulating mtDNA level was investigated in EAM as well as after TLR4 activation. Relative mtDNA level in plasma of each group was quantified by RT-PCR. Firstly, to design a primer which does not co-amplify nuclear DNA, we searched for similar sequences between the mouse nuclear genome reference sequence and mitochondrial genome sequence (accession

Fig. 4. TLR4 activation promoted cardiomyocytes mtDNA damage and increased circulating mtDNA level. (A) The first red line representing the mitochondrial genome sequence accession number NC_005089.1, was blasted against the reference sequence of the mouse genome using Nucleotide Blast, and below fragments of varying length are regions of the nuclear genome matched with mitochondrial genome. (B) The result showed no statistic difference for RT-PCR of the same genomic DNA from HL-1 or MEF with three pairs of designed primers. HL-1, mouse atrial cardiac muscle cells; MEF, mouse embryonic fibroblast. (C) Quantification of circulating plasma mtDNA of EAM mice, as measured by RT-PCR. 1/Ct denotes the reciprocal of the count where the sequence is detected, as a direct indication of mtDNA level (n=6-8). (D) Quantification of circulating plasma mtDNA, as measured

by RT-PCR from plasma of mice treated with a low dose of LPS-EB (n=6). (E) MtDNA content in myocardium of EAM mice was calculated relative to nuclear genome (n=6). (F) MtDNA content in myocardium of mice treated with a low dose of LPS-EB was calculated relative to nuclear genome (n=6). (G) Representative illustrations of inflammatory cell infiltration in cardiac sections of mice treated with PBS or with a low dose of LPS-EB by H&E staining (20X). *p<0.05 versus Control, # p<0.05 versus EAM, & p>0.05 versus Normal mice.

number NC_005089.1) using blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), showing that many regions of the mouse mitochondrial genome have high sequence identity with regions situated in the nuclear genome (Fig. 4A). Using this approach, we found that a special mitochondrial sequence between position 200–3000 of the mouse mitochondrial genome, the frequency and length of similar nuclear sequence were few, so we used this mitochondrial sequence to design primers mMitoF and mMitoR, and three pairs of primers were designed (Table 1). Furthermore, with the same samples, the results of RT-PCR for mtDNA showed consistent with each other (Fig. 4B), confirming the specificity of the primers, and we chose mMitoF1 and mMitoR1 for the following experiment. Fig. 4C showed that mtDNA copy number in circulation was significantly increased in EAM, and TLR4 activation resulted in a higher elevation after that. To confirm whether the higher elevation of circulating mtDNA in EAM LPS group was caused by the TLR4 activation directly, we further observed the circulating mtDNA level in normal mice treated with the same dose of LPS-EB alone, and found that circulating mtDNA level was just slightly increased with no statistical difference (Fig. 4D), showing that elevated circulating mtDNA of EAM LPS group was mainly owing to the more severe lesion mediated by TLR4 activation. To ascertain where this elevated circulation mtDNA comes from in EAM, we measured the mtDNA content in myocardium, which often indicates mtDNA damage [30]. As showed in Fig. 4E, mtDNA copy number normalized to nuclear DNA holds an inverse relationship with circulation in EAM and EAM LPS group compared with control group. Interesting, we also found that a low dose of LPS treatment alone did not cause a reduce of mtDNA copy number in myocardium as compared with control group (Fig. 4F), which was consistent with the result that the inflammatory cells

Fig. 5. ROS was involved mtDNA damage in H9C2 cell treated with LPS. (A) Representative images of mtROS measured by MitoSox Red fluorescence in H9C2 cells. (B) Quantitative analysis of mtROS from H9C2 induced by LPS with or without TEMPO, as normalized to Control (n=4-5). (C) MtDNA content in H9C2 was calculated relative to nuclear genome (n=4-5). & p>0.05 versus Control, *p<0.05 versus Control, # p<0.05 versus LPS group.

Fig. 6. TLR9 expression was increased in EAM and promoted TLR4 stimulation mediated cell apoptosis *in vitro*. (A) Immunohistochemistry (20×) of cardiac sections showed increased expression of TLR9 in EAM and EAM LPS groups. (B-D) TLR9 expression in heart was examined by western blot and RT-PCR analyses (n = 6). (E and F) TLR9 inhibition decreased LPS induced H9C2 cell apoptosis with flow cytometry (n=4-5). *p<0.05 versus Control, # p<0.05 versus EAM or LPS group, & p>0.05 versus LPS group.

infiltration was not increased when directly giving a low dose of LPS to normal mice (Fig. 4G). These results imply that increased circulating mtDNA may be released from injured cardiac tissue.

Fig. 7. TLR9 antagonist ODN2088 ameliorates TLR4 mediated cardiac inflammation and injury in EAM. (A) Representative illustrations of inflammatory cell infiltration in cardiac sections taken on day 21 by H&E staining (40 $\times$). (B) Myocarditis severity scores in the heart sections (n = 6). Serum levels of TNF- α (C), IL-1 β (D), IL-6(E) were measured by Multiplex Immunoassays(n=6-8). (F) Troponin I serum levels are indicated (n=5-6). ↑ p>0.05 versus EAM, # p<0.05 versus EAM, *p<0.05 versus Control,↗p<0.05 versus EAM LPS.

The above results showed an obviously increased ROS stress in EAM as well as EAM LPS group, which had been reported to damage mtDNA and promote mtDNA secretion [21, 31]. Next, we further explored the role of mitochondrial ROS stress in LPS mediated mtDNA damage with H9C2 cell. Consistent with previous study, LPS stimulation significantly increased mitochondrial ROS produce, which could be blocked by TEMPO (Fig. 5A and B). Compared with control, TLR4 activation led to mtDNA damage, as demonstrated by an obvious mtDNA copy number reduction in H9C2. However, the damage was inhibited after mitochondrial ROS produce suppressed by TEMPO (Fig. 5C). These results altogether suggest that TLR4 activation may promote cardiomyocytes mtDNA damage and increase circulating mtDNA level by inducing ROS stress.

TLR9 expression was increased in EAM and promoted TLR4 activation mediated cell apoptosis

Studies have showed that mtDNA is released into circulation upon tissue injury and is able to induce systemic inflammation and cell apoptosis through TLR9, a key ligand of mtDNA [14, 22]. So we detected the TLR9 expression of myocardial tissue to validate the role of mtDNA mediated inflammation and tissue injury in EAM. Compared with control mice, EAM heart showed an elevated expression of TLR9 (Fig. 6A), which was further increased when TLR4 was activated by LPS. This result was also confirmed by western blotting and RT-PCR (Fig. 6B-D). To investigate if TLR9 was involved in cardiomyocytes apoptosis mediated by TLR4 activation, we used a TLR9 special inhibitor (ODN 2088) to block TLR9 function before LPS stimulation in H9C2. As showed in Fig. 6E and F, compared to control group, ODN

Fig. 8. A proposed model for increased circulating mtDNA promoting TLR4 mediated cardiac inflammation and injury in EAM. See discussion for details. Mito, mitochondrial.

2088 reduced the percent of apoptosis induced by LPS stimulation. From these results, it can be concluded that the circulating mtDNA may mediate cardiac inflammation and tissue injury in EAM via TLR9.

TLR9 antagonist ODN2088 ameliorated EAM as well as TLR4 activation mediated cardiac inflammation and injury

Based on above findings, we further blocked mtDNA ligand TLR9 using ODN 2088 to observe if increased circulation mtDNA played a role in EAM as well as TLR4 activation mediated cardiac inflammation and lesion. Compared with ODN control group, we found that ODN 2088 significantly alleviated inflammatory cell infiltration and decreased gross severity scores in EAM. Moreover, simultaneously giving ODN 2088 in EAM LPS group also showed a mitigatory inflammation response (Fig. 7A and B).

TNF- α , IL-6 and IL-1 β in serum were distinctly increased in EAM and EAM LPS groups, which can be lowered after ODN 2088 treatment in both groups (Fig. 7C-E). Finally, as showed in Fig. 7F, the cardiac specific serum Troponin I (TnI) in serum was also obviously ameliorated with ODN 2088 treatment groups. Taken together, increased circulating mtDNA combined with upregulated TLR9 expression may corporately contribute to EAM as well as TLR4 activation mediated cardiac inflammation and injury.

Discussion

In this study, we report for the first time the elevated circulating mtDNA was involved in EAM as well as TLR4 activation mediated cardiac inflammation and injury (Fig. 8). Our data demonstrated that TLR4 activation damaged mtDNA, increased cardiomyocytes apoptosis and ROS produce in myocardium, which were accompanied with elevated mtDNA level in circulation and increased expression of TLR9 in heart. Mechanistically, we showed that LPS stimulation induced mtDNA damage depended on ROS stress and promoted cardiomyocytes apoptosis by TLR9 *in vitro*. Furthermore, EAM and TLR4 mediated cardiac inflammation and injury were obviously ameliorated by TLR9 inhibition with ODN2088.

Current evidence has demonstrated that EAM is mainly mediated by the host inflammation and immune response, where TLR4 signaling is considered to be a major contributor by influencing the interaction between cardiac cells and immune cells [1, 5]. Apart from inducing a pro-inflammatory phenotype on cardiac cells, TLR4 promotes dendritic cells (DCs) activation, which is critical for the expansion of autoreactive CD4⁺T cells in EAM [32]. TLR4 activation induces two complex signaling pathways: MyD88-dependent and MyD88 independent pathways, activating many transcription factors including NF- κ B and interferon-regulatory factors (IRFs) [5]. Studies have also indicated that some other signaling pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt and HIF-1 dependent non-canonical TLR4 signaling pathway, may serve as an endogenous negative feedback regulator of TLR4 signaling in myocardial inflammation, which possibly explains the inconsistency of the role

of TLR4 in EAM [5, 33]. In this study, we found that LPS mediated TLR4 activation increased circulating mtDNA level in EAM, which was associated with cardiac inflammation and injury. Increasing evidence proved that impaired mitochondrial function and mtDNA damage significantly led to inflammation and oxidative stress mediated diseases [10, 13]. MtDNA released from dysfunctional mitochondria appeared to function as an important mediator in inflammatory diseases [13, 17]. In LPS stimulation induced sepsis model, mitochondrial dysfunction and increased circulating mtDNA tightly related to organ inflammation and injury severity [16]. Studies showed that inflammatory cytokines, especially TNF- α , could undermine mitochondrial function, and contribute to the secretion of mitochondrial content when inducing cells necrosis [34, 35]. So the upregulated inflammatory cytokines in our EAM model may also aggravate the myocardium mtDNA damage and elevate circulating mtDNA level. In this study, TLR4 activation may significantly promote mitochondrial dysfunction, as showed by decreased mtDNA copy number in myocardium, which then led to the increased circulating mtDNA level. Acting as a new important inflammatory mediator, mtDNA may also play a role in EAM transition to DCM, which deserves further investigation [36].

Mounting evidence has revealed that mtDNA plays a crucial role during sterile inflammation in cardiovascular diseases [11]. MtDNA was showed to skew endotheliocytes and cardiomyocytes to an inflammatory feature and involved in atherosclerosis, hypertension, myocarditis and dilated cardiomyopathy induced by pressure overload [21, 22, 36]. MtDNA can provoke the immune response directly via the activation of TLR9, and the following NF- κ B signaling increases the expression of other pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-1 β [12, 20, 22]. Nakahira et al showed that increased mitochondria reactive oxygen species (mROS) and NLRP3 activation in ATP or LPS stimulated macrophages promoted the release of mtDNA into the cytosol, which in turn activated NLRP3 inflammasome and mediated the secretion of IL-1 β and IL-18, ultimately participated in the induction of inflammatory disease [12]. Our study presented that lowering ROS level could inhibit LPS mediated mtDNA damage in cardiomyocytes, which was consistent with the study that peroxyauraptenol(PXT) significantly inhibited inflammation and NLRP3 inflammasome in LPS activated macrophages by preventing ROS generation and the release of mtDNA [37]. MtDNA can also directly induce cell apoptosis by targeting TLR9, as showed in podocyte, HUVECs and cardiomyocytes [21, 23, 38]. In our EAM model, TLR9 expression in myocardium was obviously increased. Moreover, the level was further upregulated by TLR4 activation, which is consistent with the study showing that cardiac TLR9 expression was significantly increased in acute phase of CVB3-induced myocarditis [39]. Interesting, we showed that TLR9 was involved in TLR4 mediated cardiomyocytes apoptosis *in vitro*. Furthermore, with ODN2088, an antagonist of TLR9, cardiac inflammation and injury was obviously suppressed in EAM and EAM LPS group. Altogether, our study indicated that LPS mediated TLR4 activation increased circulating mtDNA level via inducing ROS stress, which with upregulated TLR9 corporately contributed to cardiac inflammation and injury in EAM.

It is reasonable that elevated circulating mtDNA could further aggravate the inflammatory response and cause cardiac injury in EAM, but the source of which remains largely unknown [11, 36]. In our EAM model, elevated circulating mtDNA may result from decreased mtDNA copy number in myocardium [13], as demonstrated by the results of cardiomyocytes treated with LPS *in vitro*. Stress stimulus or DNase II knockdown can stimulate mtDNA release and activate TLR9 to mediate inflammatory response in HUVECs and cardiomyocytes [21, 36]. Besides, studies showed that the mtDNA level in circulating monocytes was decreased in patients with obstructive sleep apnea, peritoneal dialysis and CKD [40-42]. Furthermore, activated neutrophils, macrophages and platelets have been proved to release mtDNA and may also contribute to the elevated mtDNA in circulation [12, 19, 43]. We also showed that circulating mtDNA was slightly increased when giving LPS-EB alone to normal mice, as existing an obvious inflammatory response in EAM mice, so it should not be ignored that other cells, especially immune cells, may also involve in elevated circulating mtDNA in EAM. Under various stress conditions, noxious stimuli damaged mitochondria and resulted

in mitochondrial fragmentation; studies have showed that LPS, TNF- α and ROS stimulation promoted cell apoptosis simultaneously with fragmented mitochondrial accumulation [44]. In TNF- α treated c-Flip $-/-$ MEFs, fragmented mitochondria engulfed by vacuoles originating from the plasma membrane were released into the extracellular spaces not in an autophagy, but an actin polymerization dependent manner [45]. However, in LPS stimulated hepatocytes and MEFs, the pharmacological inhibition of autophagy by 3MA or atg5 deletion blocked the secretion of mitochondrial constituents [46]. These findings indicate that fragmented mitochondria containing damaged mtDNA can be secreted in a different mechanism, which is consistent with the observation that mtDNA release can be induced by mitochondrial permeability transition (MPT), as followed by mitochondrial fragmentation, osmotic swelling and rupture [47]. A recent study also demonstrated that secreted phospholipase A2 IIA (sPLA2-IIA) mediated hydrolysis of the mitochondrial membrane contributed to mtDNA excretion which promoted leukocyte activation and inflammatory response [48]. Furthermore, acetylcholine significantly attenuated calcium or ROS stress induced mitochondrial damage and mtDNA release [49]. Together, mtDNA may be released extracellularly following cellular stress and damage in a manner dependent on cell and stimulus type, and elucidating the detailed molecular mechanism by which mtDNA release may provide underlying targets to treat various inflammatory diseases caused by mitochondrial malfunction [11, 44].

MtDNA functions an important role in the development of different kinds of inflammatory diseases, such as RA, sterile SIRS, diabetes mellitus, Parkinson's disease and heart diseases [11, 13, 17, 50, 51]. Mitochondrial damage characteristic with membrane permeability transition, ROS production and insufficient mitophagy corporately leads to mtDNA secretion, which exerts potent activation effect on inflammation response [12, 47, 52]. The increased mtDNA in circulation shows the extent of tissue injury, triggering a vicious cycle that inflammation induces mtDNA damage and secretion, then increased mtDNA in turn amplifies inflammation response and tissue injury [17, 53]. Our study indicated that direct suppression of inflammation response or alleviation of mitochondrial ROS stress would diminish mtDNA damage and the secretion of mtDNA, which can significantly ameliorate the cardiac inflammation and injury in EAM. Furthermore, other studies targeting in mtDNA excretion or its ligand also acquired obviously beneficial results in reversing mtDNA mediated adverse effect [36, 54]. Pretreatment or transfection with DNase I decreased the cytosolic mtDNA and impaired IL-1 β , IL-18 production in macrophages treated with LPS and ATP [12]. As an important ligand of mtDNA, TLR9 inhibition by chloroquine, ODN2088 or gene ablation also significantly alleviated the mtDNA mediated sterile inflammation response and tissue injury in blood vessel, lung and heart [22, 31, 36], which was further demonstrated in our EAM model. These studies indicate that mtDNA presents a promising therapeutic target in inflammatory diseases.

The present study acknowledges a few limitations that should be stated. A major limitation is the absence of TLR4 inhibition or TLR4 deficient mice to further clearly observe the role of TLR4 activation in circulating mtDNA level in EAM. Although our results indicated ROS may be involved in mtDNA damage and release into circulation, the exact molecular mechanism still remains unclear, which impedes the targeted intervention of mtDNA in diseases and needs a further investigation. Our study did not include the clinical detect of circulating mtDNA in patients with myocarditis, the result of which may facilitate the understanding of pathophysiology and show diagnostic and therapeutic potential. In summary, the present study showed that the circulating mtDNA was involved in TLR4 mediated cardiac inflammation and injury in EAM. TLR4 activation induced mtDNA damage in myocardium, elevated mtDNA in circulation, moreover, upregulated cardiac TLR9 expression, which corporately contributed to TLR4 mediated cardiac inflammation and injury in EAM. These data imply mtDNA a potential therapeutic target for EAM.

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Disclosure Statement

The authors declare no conflict of interest.

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