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***P. gingivalis* lipopolysaccharide intensifies inflammation post-myocardial infarction through matrix metalloproteinase-9**

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Abstract

Rationale—Periodontal disease (PD) strongly correlates with increased mortality post-myocardial infarction (MI); however, the underlying mechanisms are unknown. Matrix metalloproteinase (MMP)-9 levels directly correlate with dysfunction and remodeling of the left ventricle (LV) post-MI. Post-MI, MMP-9 is produced by leukocytes and modulates inflammation. We have shown that exposure to *Porphyromonas gingivalis* lipopolysaccharide (PgLPS), an immunomodulatory molecule identified in PD patients, increases LV MMP-9 levels in mice and leads to cardiac inflammation and dysfunction.

Aims—To determine if circulating PgLPS exacerbates the LV inflammatory response post-MI through MMP-9 dependent mechanisms.

Methods and Results—We exposed wild type C57BL/6J and MMP-9^{-/-} mice to PgLPS (ATCC 33277) for a period of 28 days before performing MI, and continued to deliver PgLPS for up to 7 days post-MI. We found systemic levels of PgLPS 1) increased MMP-9 levels in both plasma and infarcted LV resulting in reduced wall thickness and increased incidence of LV

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DISCLOSURES

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rupture post-MI and 2) increased systemic and local macrophage chemotaxis leading to accelerated M1 macrophage infiltration post-MI and decreased LV function. MMP-9 deletion played a protective role by attenuating the inflammation induced by systemic delivery of *Pg*LPS.

Conclusion—In conclusion, MMP-9 deletion has a cardioprotective role against *Pg*LPS exposure, by attenuating macrophage mediated inflammation.

Keywords

Matrix metalloproteinase-9; myocardial infarction; periodontal disease; *Porphyromonas gingivalis*; proteomics; cardiac function

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States. Multiple epidemiological studies correlate periodontal disease (PD) with CVD, as PD patients have increased mortality and heart failure (HF) incidence post-myocardial infarction (MI) [1-3]. An estimated 75% of the US adult population has PD. Meta-analysis of over 200,000 individuals revealed that PD increases CVD risk by 35%, implicating PD as a significant public health concern [4]. While the link between oral health and CVD is strong, the cellular and molecular mechanisms that associate oral health and CVD are still unknown.

PD is a complex disease, where multiple causal factors simultaneously and interactively play a role in progression and severity of the disease. There are four main causal risk factors: 1) the subgingival microbiota, 2) individual genetic variations, 3) life style, and 4) systemic factors [5]. The link between periodontitis and CVD are thought to be caused by inflammatory mechanisms initiated by bacteria, associated with periodontal lesions [6]. *Porphyromonas gingivalis* (*Pg*) is the most frequent PD pathogen detected in the gums/circulation of patients with PD [7-9]. As the infection progresses deeper into the tissue and enters the blood stream, an innate inflammatory response is activated to combat the infection.

In order to elucidate effects of a specific periodontal pathogen in cardiac function, we previously infused mice with sub-septic concentrations (0.8 µg/g body weight/day) of *Pg*LPS ATCC 33277, a strain identified in over 25% of PD patients, and found increased systemic inflammation, evidenced by elevated plasma levels of interleukin (IL-1)α and matrix metalloproteinase (MMP)-9 [10-12]. These results were similar to those seen in PD patients [7]. We concluded that systemic inflammation due to a periodontal pathogen induced a subtle yet significant decrease in cardiac function that was MMP-9 dependent.

MMP-9 is a critical player post-MI, as it modulates the inflammatory and scar formation responses [13, 14]. In animal models, deletion of MMP-9 attenuates LV dysfunction post-MI [15]. Accordingly, we hypothesized that systemic sub-septic levels of *Pg*LPS would amplify the inflammatory response in the left ventricle (LV) post-MI, leading to a decrease in cardiac function that would be attenuated by MMP-9 deletion.

2. Materials and Methods

2.1 Mice

C57BL/6J wild type (WT) and MMP-9^{-/-} male and female mice, 4-7 months of age, were used in this study (n = 6/sex/group). The MMP-9^{-/-} mice are on a C57BL/6J background. The MMP-9^{-/-} mice were generated by Zena Werb's laboratory and backcrossed by Lynn Matrisian's laboratory [16, 17]. WT and MMP-9^{-/-} mice were each separated into 3 groups: day 0 (D0) no MI controls (n = 6/sex/group), saline D1, D3, D5, and D7 post-MI (n = 6/sex/day), and PgLPS infused D1, D3, D5, and D7 post-MI (n = 6/sex/day), for a combined total of 6 groups. Mice were kept in a light-controlled environment with a 12:12 hour light-dark cycle and given free access to standard mice chow and water. WT mice were ordered from Jackson laboratories. MMP-9^{-/-} colonies were bred in-house and maintained in the same room with WT mice. Groups were examined simultaneously, with the evaluator blinded to genotype. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and the University of Mississippi Medical Center in accordance with the "Guide for the Care and Use of Laboratory Animals".

2.2 Experimental Design

To elucidate the effects of the periodontal pathogen *P. gingivalis* (Pg) on LV remodeling; mice were exposed to PgLPS ATCC 33277 (0.8 µg/g body weight/day; Invivo Gen) by osmotic mini-pumps (model 2004, 1003D, and 1007D; Durect). PgLPS ATCC 33277 is a strain commonly identified in over 25% of PD patients [10, 11]. Previously, we showed this concentration induces low-grade systemic inflammation similar to what is seen in plasma of PD patients following dental cleanings [12]. After 28 days of PgLPS exposure mice did not have elevated body temperature (p=0.6873) confirming PgLPS concentration did not induce sepsis. Except for D0 animals, mice were infused with either saline or PgLPS for 28 days before MI. After 28 days, coronary artery ligation as well as removal and replacement of pump were performed. No mortality was observed from PgLPS exposure alone, and the saline treated group served as the negative technical control for this procedure. MI was induced through permanent ligation of the left anterior descending coronary artery using a well-established method [18]. In summary, mice were anesthetized with 1-2% isoflurane in oxygen, intubated, and put on a standard rodent ventilator. An incision was made between the 3rd and 4th rib and a rib retractor was used to allow visualization of the heart. An 8-0 suture was used to ligate the left coronary artery at a location approximately 1-2 mm distal to the left atrium, and MI was confirmed by LV blanching and ECG changes showing ST segment elevation. Prior to the surgery, buprenorphine was administered to ease pain. Animals were sacrificed at 1, 3, 5, or 7 days post-MI. Day 0 controls did not undergo surgery prior to sacrifice.

2.3 Echocardiography

Transthoracic echocardiography was performed using the Visual Sonics Vevo 770 system (VisualSonics) with a 30-MHz image transducer at baseline (before MI) and at termination. Mice were anesthetized with 1-2% isoflurane in an oxygen mix, and electrocardiograms and heart rates were monitored throughout the imaging procedure. All images were acquired at

heart rates >400 bpm to ensure physiological relevance. Measurements were taken from the parasternal long axis B- and M-mode views. For each parameter, 3 images from consecutive cardiac cycles were measured and averaged [16, 19]. Echocardiography data were analyzed, and no sex or age differences were found between groups (for example, $p=0.60$ for the fractional shortening comparison).

2.4 Survival Analysis and Tissue Harvest

The mice were checked daily for the survival analysis. At necropsy, cardiac rupture was confirmed by the presence of coagulated blood in the thoracic cavity or observation of ruptured site on the LV. For tissue collection, mice were anesthetized with 1-2% isoflurane in an oxygen mix. Five minutes after heparin administration (4 U/g body weight, i.p.), blood was collected from the common carotid artery and immediately centrifuged for collection of plasma. A 1x proteinase inhibitor cocktail (Roche, 50-720-4060) was added to the plasma, which was stored at -80°C . Plasma samples (100 μL) were sent to Rules Based Medicine (Austin, TX) for multi-analyte proteomic profiling [20]. The coronary vasculature was flushed with cardioplegic solution (69 mM NaCl; 12 mM NaHCO_3 ; 11 mM glucose; 30 mM 2,3-butanedione monoxime; 10 mM EGTA; 0.001 mM Nifedipine; 50 mM KCl; and 100 U Heparin in 0.9% saline, pH 7.4). Hearts were removed, and the LV and right ventricle (RV) separated and weighed individually. The LV was sliced transversely into apex, middle, and base sections. Infarct areas were calculated as described previously [21]. The infarct region (LVI) was dissected from the apex and base sections, frozen in liquid nitrogen, and stored at -80°C for RT²-PCR, immunoblotting, or bioplex analyses. The middle section was fixed in 10% zinc formalin for histological examination.

2.5 Leukocyte Isolation

Neutrophils and macrophages were isolated from infarcted hearts as previously described [19]. The LV tissue was dissociated into single-cell suspension using 600 U/mL collagenase type 2 (Worthington Biochemicals, CLS-2) and 60 U/mL DNase1 (AppliChem, A3778.0500). Cells were washed and resuspended in cold PBS supplemented with 0.5% BSA and 2 mM EDTA. Cells were sequentially incubated with Ly-6G microbeads (Miltenyi Biotec, 130-092-332) to remove neutrophils, and with CD11b microbeads (Miltenyi Biotec, 130-049-601) to isolate macrophages. Positive cells were isolated using magnetic MS columns (Miltenyi Biotec, 130-042-201). RNA extraction was performed on the isolated neutrophils and macrophages using PureLink RNA Mini Kit (Invitrogen, 12183-018A). RNA levels were quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Reverse transcription of equal RNA content (400 ng) was performed using the High Capacity RNA-to-cDNA Kit (Life Technologies 4837406). Quantitative RT-PCR was performed using taqman gene expression assays (Applied Biosystems) to evaluate mRNA expression of pro-inflammatory M1 and anti-inflammatory M2 macrophage markers as shown in Table 1. Isolated cells were also analyzed for MMP-9 expression (Mm00442991_m1). MMP-9 mRNA levels were normalized against cell number to determine MMP-9 levels/cell. Macrophages were analyzed for the inflammatory analytes listed in Table 1. All values were normalized to hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) (Mm01545399_m1) housekeeping gene. Analysis of PCR data was done using the MIQE guidelines.

2.6 Real Time RT²-PCR

RNA was extracted from the infarcted LV (LVI) using TRIzol® Reagent and Purelink® RNA (Invitrogen) according to the manufacturer's instructions. RNA levels were quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Reverse transcription of equal RNA content (400 ng) was performed using the RT² First Strand Kit (Qiagen 330401). Quantitative Real Time RT²-PCR gene array for inflammatory cytokines and receptors (Qiagen PAMM-011A) and MMPs and tissue inhibitors of metalloproteinase (TIMPs) (Qiagen PAMM-013A) were used to quantify mRNA levels in the LVI. All values were normalized to the *Hprt1* housekeeping gene, since the expression of this gene does not change following injury. Analysis of PCR data was done using the MIQE guidelines.

2.7 Protein Extraction and Analysis

Protein was extracted by homogenizing the samples sequentially in phosphate buffered saline (PBS) with 1× protease inhibitor cocktail (16 µL per mg tissue, soluble protein fraction), and in protein extraction reagent type 4 (Sigma; 7 M urea, 2 M thiourea, 40 mM Trizma® base and the detergent 1% C7BzO, 15 µL per mg tissue) with 1× protease inhibitor cocktail (insoluble protein fraction). Protein concentrations were determined by the Quick Start™ Bradford Protein Assay (Bio-Rad). Total protein (10 µg) was separated on 4-12% Criterion™ XT Bis-Tris gels (Bio-Rad), transferred to a nitrocellulose membrane (Bio-Rad), and stained with MemCode™ Reversible Protein Stain Kit (Thermo Scientific) to verify protein concentration and loading accuracy. After blocking with 5% nonfat milk (Bio-Rad), the membrane was incubated with an antibody against galectin-3 (R&D, AF1197, 1:500), secondary antibody (Santa Cruz, SC2020, 1:5000), and detected with ECL Prime Western Blotting Detection Substrate (Amersham). Galectin-3 levels were quantified by densitometry using the IQ-TL image analysis software (GE Healthcare, Waukesha, WI). The relative expression for each immunoblot was calculated as the densitometry of the protein of interest divided by the densitometry of the entire lane of the total protein stained membrane. Measurement of cytokines in the LVI were determined by fluorescent bead-based Luminex technology (BioRad; Bio-plex Pro Mouse Cytokine 23-plex panel MD0-009RDPD and 9-plex panel MD0-00000EL), in accordance with the manufacturer instructions. Samples (10 µg of homogenized lysates) were incubated for 30 min in a 96-well plate. Detection antibodies were added, and the plate was incubated for 30 min with shaking. After incubation, Streptavidin was added to each well, and the plate was incubated for 10 min. Samples were resuspended in assay buffer, and the plate was measured for fluorescence emission on the Bio-plex system. Concentrations were calculated from standard curves for each of the analytes.

2.8 Histology

The middle section of the LV (mid-papillary region) was embedded in paraffin and sectioned at 5 µm. Immunohistochemistry was conducted using the Vectastain ABC Kit (Vector Laboratories). Antibodies specific for neutrophils (Cedarlane; CL8993AP; 1:100) and macrophages (Mac-3, Cedarlane CL8943AP; 1:100) were used. HistoMark Black (KPL 54-75-00) was used to visualize positive staining, with eosin as a counterstain. Negative controls were incubated with no primary antibody. For each LV section, five 60×

magnification images were captured. The percentage of positive staining per field of view (macrophage levels) was measured by Image-Pro Plus version 6.2. Results are shown as percent stained area.

2.9 Statistical Analyses

Data are presented as mean $\pm$ SEM. Multiple group comparisons were analyzed by one-way ANOVA, followed by the Student Newman-Keuls when the Bartlett's variation test passed, or by the nonparametric Kruskal-Wallis ANOVA test, followed by Dunn post-hoc test when the Bartlett's variation test did not pass. The survival rate was analyzed by Kaplan-Meier survival analysis and compared by the log-rank test. Rupture rates were analyzed by Fisher's exact test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 *Pg*LPS exposure increased rupture rates and kinetics post-MI in WT mice but not in MMP-9^{-/-}

Survival numbers post-MI were not affected by *Pg*LPS (Figure 1A). As reported previously, MMP-9 deletion reduced mortality post-MI in the unexposed mice ($p < 0.05$) [22]. MMP-9 deletion also reduced mortality post-MI in the *Pg*LPS exposed mice ($p < 0.05$). *Pg*LPS exposure increased deaths due to rupture, compared to non-exposed MI controls, as 8/8 (100%) had rupture in WT+LPS vs. 11/23 (47%) in non-exposed MI mice ($p < 0.05$). This increase in rupture was attenuated by MMP-9 deletion, where only 2/3 (66%) deaths were due to rupture in MMP-9^{-/-}+LPS (Figure 1B; $p < 0.05$). In the murine permanent occlusion model, cardiac rupture normally occurs between days 3 to 7 post-MI. Interestingly, *Pg*LPS exposure promoted earlier ruptures at D1 post-MI in WT mice but not in MMP-9^{-/-}. These results indicate that systemic *Pg*LPS infusion accelerates the occurrence and timing of post-MI rupture. Based on this result, we determined what mechanisms occurred at D1 post-MI to cause this increased rupture and what cell populations modulated this effect.

3.2 MMP-9 levels increased in plasma and LVI after *Pg*LPS exposure in WT mice

MMP-9 in the early phase of MI has been shown to impair infarct healing and aggravate early remodeling, which in turn causes cardiac rupture [15, 23]. We reported that chronic *Pg*LPS exposure increased MMP-9 in plasma and LV [12]. In order to determine if *Pg*LPS presence can modulate MMP-9 production post-MI, proteomic profiling and gene array analysis were performed in plasma and LVI up to 7 days post-MI. MMP-9 levels increased significantly post-MI in both plasma and LVI gene levels in WT animals (both $p < 0.05$). This increase was intensified by *Pg*LPS exposure (Figure 2). By D7 post-MI, plasma MMP-9 levels were returning to levels comparable to unexposed controls. This suggests that the intensified inflammatory response has subsided by D7 post-MI in the *Pg*LPS exposed WT mice. No sex differences were identified from MMP-9 levels ($p = 0.23$). These results indicate that MMP-9 increases post-MI, and this effect was amplified with *Pg*LPS exposure.

3.3 *Pg*LPS accelerated M1 macrophage infiltration in WT mice and MMP-9 deletion attenuated this effect

To determine if *Pg*LPS exposure amplified leukocyte infiltration into the infarcted tissue, LV sections were stained for neutrophils and macrophages. Neutrophil levels were elevated similarly in both unexposed and *Pg*LPS exposed mice at D1 post-MI (Figure 3A-B). Unexpectedly, macrophages were present at D1 post-MI in WT+LPS (Figure 3C-E). Significant numbers of macrophages do not normally infiltrate the LV post-MI until D3, and peak infiltration occurs at D5 [16].

In the post-MI setting, MMP-9 is expressed predominantly by the neutrophils and macrophages. At D1 post-MI, MMP-9 is expressed 2600-fold higher in neutrophils compared to the rest of the cells combined ($p < 0.05$). To determine if the acceleration in macrophage recruitment is responsible for the increase in MMP-9, RT²-PCR was performed on neutrophils and macrophages isolated from the LVI at D1 post-MI. As expected, neutrophils expressed higher levels of MMP-9 per cell compared to macrophages in the control mice (Figure 4). Of note, after *Pg*LPS exposure neutrophils and macrophages were equally responsible for the increase in MMP-9, suggesting the acceleration in macrophage recruitment increased overall MMP-9 levels. This result also suggested that the macrophage phenotype might be altered.

To determine the macrophage phenotype, macrophages isolated from the LVI at D1 were analyzed for M1 (pro-inflammatory) and M2 (anti-inflammatory) markers. M1 markers, *Ccl3*, *IL1 β* , and *TNF α* , mRNA levels were increased in WT+LPS compared to unexposed and MMP-9^{-/-}+LPS MI groups (Figure 5). *Il6* was significantly higher in the MMP-9^{-/-}+LPS MI group, and this cytokine has been reported to be produced by both M1 and M2b macrophages [24-26]. The M2 marker arginase (*Arg1*) was significantly higher in the WT+LPS MI group, whereas *Fizz1* was significantly higher in the MMP-9^{-/-}+LPS MI group.

Combined, these results indicated that *Pg*LPS induced an earlier and more pro-inflammatory influx of macrophages into the ischemic myocardium. However in the absence of MMP-9, *Pg*LPS induced both M1 and M2 polarization, suggesting that *Pg*LPS modulation of macrophage-driven inflammation occurs in an MMP-9 dependent manner through several mechanisms. Macrophage polarization is dynamic and flexible, with classically activated pro-inflammatory M1 cells initiating and sustaining inflammation and anti-inflammatory M2 cells resolving chronic inflammation [27, 28]. The accelerated influx of M1 macrophages in the WT after *Pg*LPS exposure may explain the increased rupture rates [29, 30].

3.4 *Pg*LPS increased macrophage mediated inflammation in WT mice at D1 post-MI is attenuated by MMP-9 deletion

MMP-9 processes cytokines affecting the inflammatory response post-MI [31]. To determine which signaling molecules were responsible for the earlier recruitment of macrophages, we performed multi-analyte proteomic profiling of plasma and LVI, as well as RT²-PCR cytokine and ECM gene arrays on the LVI tissue (Figures 6-7 and Table 2). Compared to D0 samples, D1 post-MI plasma showed elevations in 12 of the 40 analytes

examined, including IL-6 that served as a positive control [32]. Eotaxin, granulocyte macrophage colony-stimulating factor (GM-CSF), Cxcl1, MCP-1, and tumor necrosis factor (TNF) α increased in the LVI of all groups post-MI, indicating responses that were independent of *Pg*LPS or MMP-9.

Compared to D1 post-MI WT samples, plasma and LVI tissue levels of the signaling molecules interferon (IFN) γ , interleukins (IL)-1 β , -6 and -18, macrophage inflammatory proteins (MIPs), monocyte chemotactic proteins (MCPs) and integrins were intensified by *Pg*LPS (Figures 6-7 and Table 2). This effect was attenuated by MMP-9 deletion. MIP-1 β , a macrophage chemotactic protein, was the only inflammatory molecule exacerbated in both plasma and LVI by *Pg*LPS exposure. Of note, IFN γ , IL-1 β , IL-6, and IL-18 are known to promote macrophage M1 polarization [33]. This increase in macrophage chemoattractants explains the unexpected presence of macrophages at D1 in the LVI of WT+LPS animals [34, 35].

MMP-9 also processes extracellular matrix proteins and proangiogenic factors such as collagen, vascular endothelial growth factor (VEGF), and fibroblast growth factor (bFGF) to influence post-MI remodeling [31]. Despite the accelerated rupture rate seen in the WT MI mice exposed to *Pg*LPS, the ECM gene array showed no changes in collagen types I and III at D1 post-MI, compared to the D1 saline WT MI control. This was not unexpected, since infarct scar formation does not begin until around day 3 post-MI [36]. Vascular endothelial growth factor (VEGF) was increased in the WT groups only. VEGF stimulates angiogenesis and is associated with increased MMP activity [37, 38]. *Pg*LPS also increased bFGF and platelet derived growth factor (PDGF) in the LVI at D1 post-MI (Figure 7). This effect was attenuated by MMP-9 deletion.

The expression of 11 MMPs and 3 TIMPs were evaluated by gene array. MMP-1, MMP-2, MMP-7, MMP-10, MMP-11, MMP-13, TIMP-1, and TIMP-3 were not changed at D1 post-MI over respective day 0 values. Without MI, MMP-9 deletion resulted in a compensatory increase in MMP-13 that has been reported previously and was not further changed with MI [39, 40]. At D1 post-MI, MMP-8, and MMP-14 increased and MMP-15 and TIMP-2 decreased in all groups compared to the D0 control, indicating an MI effect that was not altered by *Pg*LPS or MMP-9 deletion (Table 2). MMP-3 expression was intensified by *Pg*LPS exposure in WT mice, and MMP-9 deletion attenuated this effect. MMP-12 increased in *Pg*LPS exposed MMP-9^{-/-} mice at D1 post-MI.

*Pg*LPS exposure stimulated genes and protein analytes associated with leukocyte chemotaxis. The majority of these genes and analytes are linked to macrophage recruitment and infiltration. MMP-9 deletion attenuated the increase of these analytes, demonstrating that MMP-9 deletion has a cardioprotective role against *Pg*LPS exposure, by attenuating macrophage mediated inflammation.

3.5 *Pg*LPS intensified LV dysfunction post-MI and this was attenuated by MMP-9 deletion

Our data showed that *Pg*LPS accelerated early M1 macrophage infiltration post-MI in the WT, which would increase damage to the myocardium eventually affecting LV function [29, 30]. In order to determine how these early changes affected cardiac function after 1 week of

MI, we measured LV function at D7 using 2-dimensional echocardiography (Table 3). Interestingly, both infarct wall thinning and reduced FS were amplified in WT+LPS mice, and this effect was attenuated by MMP-9 deletion ($p < 0.05$ for all). These data demonstrate that *Pg*LPS exposure intensified LV dysfunction in the WT mice post-MI but not in MMP-9^{-/-}, indicating a role for MMP-9 in *Pg*LPS-induced cardiac dysfunction.

4. Discussion

The goal of this study was to investigate the mechanisms that underlie the connection between periodontal disease (PD) and post-MI remodeling of the left ventricle (LV). Our results show that *Pg*LPS exposure: 1) increased MMP-9 levels in both plasma and LV infarct, resulting in augmented infarct wall thinning and decreased LV function; 2) increased systemic and local macrophage chemotaxis, resulting in accelerated M1 macrophage infiltration post-MI and increased deaths due to rupture post-MI; and 3) MMP-9 deletion played a protective role by attenuating the exacerbated inflammation caused by systemic delivery of *Pg*LPS. These data reveal that PD plays key roles in MI by inducing a strong macrophage mediated inflammatory response regulated by MMP-9.

The occurrence of rupture post-MI is an unpredictable event [39]. Excessive inflammation post-MI has been shown to impair infarct healing, which in turn causes cardiac rupture [23, 40]. Our data revealed that *Pg*LPS increased and accelerated ruptures post-MI in WT mice and this was due to an increase in the amount of post-MI LV wall thinning. This was accompanied by an increase in MMP-9 and M1 pro-inflammatory macrophages, illustrating that *Pg*LPS exposure can accelerate rupture through an acceleration of MMP-9 mediated macrophage infiltration. In addition to increased levels of MMP-9, *Pg*LPS also increased MMP-3 levels in WT mice at D1 post-MI. Studies in a rabbit permanent occlusion model showed increased MMP-3 levels post-MI led to LV dysfunction by causing myocyte disruption and misalignment [41].

Circulating periodontal pathogens stimulates inflammation and is believed to be the source of CVD risk in PD patients [12, 42, 43]. Our results showed that *Pg*LPS exposure enhanced systemic and local inflammation post-MI in WT mice, through increased levels of inflammatory markers associated with macrophage chemotaxis. This effect was attenuated by MMP-9 deletion. Immunohistochemistry showed *Pg*LPS accelerated macrophage infiltration post-MI in the LV of WT mice, and these macrophages were pro-inflammatory (M1 phenotype). This finding indicates that *Pg*LPS induced increased myocardial damage through MMP-9 regulation of macrophage mediated inflammation to decrease LV function.

In conclusion, PD induces increased risk for heart failure post-MI by altering the inflammatory response in a MMP-9 dependent manner. The data suggest that an increase in peripheral inflammation by *Pg*LPS exposure accelerated macrophage infiltration into the LV. This in turn increased the amount of tissue damage as demonstrated by the increase in LV rupture post-MI. The early changes observed at D1 led to a decrease in D7 LV function, which was attenuated by MMP-9 deletion. These results highlight the importance of future studies evaluating the use of MMP-9 inhibitors in high risk patients with periodontal disease to prevent cardiovascular events. Periodontal disease is associated with increased risk for

heart failure post-MI. Previously, we reported that chronic *Pg*LPS exposure induced a subtle yet significant decrease in LV function [12]. It is expected that *Pg*LPS would decrease LV function further at later points post-MI. Future studies focused on longer time points post-MI would be necessary to identify changes that lead to heart failure. These findings give us insight into the molecular events that increase the risk of heart failure in periodontal disease patients who have MI.

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Highlights

- Mice were given *Pg*LPS for 28 days before and through 7 days of myocardial infarction (MI).
- The post-MI increase in MMP-9 in both the plasma and infarct was amplified by *Pg*LPS.
- *Pg*LPS increased wall thinning post-MI in WT mice, resulting in increased LV rupture.
- M1 macrophage infiltration was accelerated in WT mice exposed to *Pg*LPS post-MI.
- MMP-9 deletion attenuated *Pg*LPS effects post-MI.

Figure 1.

*Pg*LPS increased rupture rates and accelerated time of death in WT mice. (A) *Pg*LPS exposure did not affect final day 7 survival rate post-MI; however, MMP-9 deletion attenuated mortality post-MI. (B) Rupture rate was accelerated by *Pg*LPS in WT mice which was attenuated by MMP-9 deletion. Kaplan-Meier survival analysis and log-rank test; #p<0.05 vs respective MI; †p<0.05 vs WT MI+LPS.

Figure 2.

Exposure to *Pg*LPS exacerbated MMP-9 levels in both plasma and LVI. MMP-9 levels increased post-MI in (A) plasma protein levels, as determined by proteomic profiling (n=12/group) and (B) infarcted LV mRNA levels, as determined by qRT-PCR in WT mice (n = 6/group); ANOVA with Student Newman-Keuls post-test; *p<0.05 vs respective D0 (Day 0); #p<0.05 vs respective MI.

Figure 3.

*Pg*LPS induced accelerated macrophage infiltration at D1 post-MI. (A, B) Staining for neutrophils showed *Pg*LPS had no effect on cell number at D1 post-MI. (C, D) Staining for Mac3 showed higher macrophage numbers in the LV post-MI at D1 in the *Pg*LPS exposed groups. (E) Immunoblot of galectin-3, a marker for macrophages, confirmed increased macrophages at D1 post-MI in WT mice. This effect was attenuated by MMP-9 deletion; n=6/group; ANOVA with Student Newman-Keuls post-test; *p<0.05 vs respective d0; #p<0.05 vs respective MI; †p<0.05 vs WT MI+LPS. Scale bars are 100 μ m.

Figure 4.

*Pg*LPS stimulated macrophages to increase expression of MMP-9. Isolated neutrophils and macrophages were analyzed for MMP-9 expression; n=6/group; ANOVA with Student Newman-Keuls post-test; ^p<0.05 vs MI D1 neutrophils; ‡p<0.05 vs MI D1 macrophages.

Figure 5.

*Pg*LPS induces M1 macrophage polarization in WT but not MMP-9^{-/-}. Isolated macrophages were analyzed for M1 and M2 macrophage markers; n=6/group; ANOVA with Student Newman-Keuls post-test; *p<0.05 vs respective d0; #p<0.05 vs respective MI; †p<0.05 vs WT MI+LPS.

Figure 6.

Plasma proteomic profiling revealed increased macrophage associated chemokines, (A) macrophage inhibitory protein (MIP)-1 β , (B) monocyte chemotactic protein (MCP)-1, and (C) MCP-3 in *Pg*LPS infused WT mice post-MI compared to unexposed controls. MMP-9 deletion attenuated this effect; n=12/group; ANOVA with Student Newman-Keuls post-test; *p<0.05 vs respective D0; #p<0.05 vs respective MI; †p<0.05 vs WT MI+LPS.

Figure 7.

Proteomic profiling of LV tissue revealed *Pg*LPS increased the inflammatory response post-MI in WT mice and this effect was attenuated by MMP-9 deletion. Majority of the increased analytes are associated with macrophages demonstrating *Pg*LPS induced macrophage immunity post-MI; n=6/group; ANOVA with Student Newman-Keuls post-test; *p<0.05 vs respective d0; #p<0.05 vs respective MI; †p<0.05 vs WT MI+LPS.

Table 1

RT²-PCR primers (Applied Biosystems) used for M1 and M2 macrophage phenotyping.

M1	M2
Ccl3 (Mm00441259_g1)	arginase-1 (Mm00443258_m1)
IL-1 β (Mm01336189_m1)	Fizz (Mm00445109_m1)
IL-6 (Mm00446190_m1)	mannose receptor 1 (Mm00485148_m1)
TNF- α (Mm00443258_m1)	Ym1 (Mm00474091_m1)

Table 2

Summary of changes in inflammatory mRNA levels. Arrows indicate differences in D1 post-MI samples compared to D0 controls (↑- increased and ↓- decreased). Bolded analytes are intensified by *Pg*LPS and underlined analytes are attenuated by MMP-9 deletion.

	WT MI D1	MMP-9 ^{-/-} MI D1	WT <i>Pg</i> LPS+MI D1	MMP-9 ^{-/-} <i>Pg</i> LPS+MI D1
↑	Ccl2, Ccl6, Ccl9, Ccl17, Ccr1, Ccr2, Cxcl1, Cxcl4, Cxcl5, IL1r1, IL1r2, IL8rb, Itgam, Itgb2, MMP- 3, MMP-8, MMP-14, MIPiβ, Tnfrsf1a	Ccl9, Ccl17, Ccr1, Ccr2, Ccr3, IL1r1, Itgam, Itgb2, MMP-3, MMP-8, MMP-14, Spp1, Tgfb1	Ccl6, Ccl7, Ccl9, Ccl12, Ccl17, Ccr1, Ccr2, Ccr3, Ccr5, Cxcl1, Cxcl4, Cxcl5, Cxcl10, IL1p, IL1r1, IL1r2, IL8rb, IL11, Itga3, Itgal, Itgam, tgav, Itgb2, MIP1β , MMP-3 , MMP-8, MMP-14, Spp1, Tgfb1, Tnfrsf1a	Ccl6, Ccl7, Ccl8, Ccl9, Ccl12, Ccl17, Ccl22, Ccr1, Ccr2, Ccr3, Ccr5, Ccr7, Cxcl1, Cxcl4, Cxcl5, Cxcl10, IL1a, IL1r1, IL1r2, IL6ra, IL8rb, IL11, Itgam, Itgb2, Ltb, Mif, <u>MIP1β</u> , <u>MMP-3</u> , MMP-8, MMP-14, Spp1, Tgfb1, Tnf, Tnfrsf1a
↓	Ccr10, Cxcl11, IL10rb, IL15, MMP- 15, TIMP-2	IL10rb, IL15, MMP- 15, TIMP-2,	Ccr10, IL10rb, IL15, MMP-15, TIMP-2	Ccr10, IL10rb, IL15, MMP-15, TIMP-2

n=6/group; All p<0.05 by Kruskal-Wallis ANOVA with Dunn post-hoc test

Table 3

*Pg*LPS exacerbates LV dysfunction post-MI in WT, but not MMP-9^{-/-} mice.

	D0		MI D7		LPS+MI D7	
	WT	MMP-9 ^{-/-}	WT	MMP-9 ^{-/-}	WT	MMP-9 ^{-/-}
Heart Rate (bpm)	469±9	448±12	475±15	485±10	465±8	479±14
Infarct area (%)	N/A	N/A	58±3	56±2	55±5	50±5
Wall Thickness (mm)	1.23±0.04	1.19±0.03	0.59±0.05 [*]	0.57±0.05 [*]	0.39±0.01 ^{*#}	0.49±0.04 ^{*†}
EDD (mm)	3.50±0.11	3.22±0.16	5.60±0.22 [*]	5.36±0.34 [*]	5.53±0.17 [*]	5.28±0.20 [*]
ESD (mm)	2.28±0.08	2.05±0.15	5.21±0.26 [*]	4.98±0.36 [*]	5.27±0.17 [*]	4.94±0.20 [*]
FS %	35±1	37±1	7±1 [*]	7±1 [*]	5±1 ^{*#}	7±1 ^{*†}

Values are mean ± SEM; EDD-End diastolic dimension; ESD-End systolic dimension; FS-Fractional shortening; n=12/group (6M/6F); ANOVA with Student Newman-Keuls post-hoc test

* p<0.05 vs respective D0

p<0.05 vs WT MI

† p<0.05 vs WT MI+LPS