Periodontal induced chronic inflammation triggers macrophage secretion of Ccl12 to inhibit fibroblast mediated cardiac wound healing

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Supplemental Materials

Detailed Methods

Mice. C57BL/6J wild type mice, 3-7 months of age and equal male and female numbers, were obtained from Jackson Laboratories for this study. Mice were each separated into 4 groups: day 0 (D0) no MI negative controls (n=12), D7 post-MI positive controls (n=34), LPS infused D7 post-MI (n=27), Ccl12 infused D7 post-MI (n=4), Ccl12 blocking antibody infused D7 post-MI (n=4), or IgG D7 post-MI controls (n=4). Mice were kept in a light-controlled environment with a 12:12 h light-dark cycle and given free access to standard mice chow and water. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center in accordance with the Guide for the Care and Use of Laboratory Animals.

Experimental Design. Groups were examined simultaneously in a random experimental design, with the evaluator blinded to groups for all data acquisition and analyses. To elucidate the effects of the chronic inflammation on remodeling of the left ventricle (LV), mice were exposed to *Porphyromonas gingivalis* LPS ATCC 33277 (0.8 μg/day/g body weight; Invivo Gen) by osmotic mini-pumps (model 2004 and 1007D; Durect). After 28 days of LPS exposure, mice do not have elevated body temperature and no mortality is observed, confirming that this LPS concentration does not induce sepsis. After 28 days, MI was induced through permanent ligation of the left anterior descending coronary artery. Mice continued to receive LPS by osmotic pump until tissue collection. Day 0 no MI controls did not undergo any surgical procedure prior to sacrifice; the MI controls had MI surgery as described for the LPS+MI group. Recombinant Ccl12 (R&D 428-P5-025/CF; 180 pg/day/g body weight), Ccl12 blocking antibody (Novus AF428; 0.15 ug/day/g body weight), or an IgG anti-goat antibody (Vector PI-9500; 0.15 ug/day/g body weight) was infused by osmotic pump (model 1007D; Durect) to dissect Ccl12 mechanisms on cardiac wound healing in vivo. Surgeries for non-exposed controls, recombinant

Ccl12 infusion, and LPS+MI were performed within the same time frame and IgG controls and Ccl12i mice underwent surgery at the same time to limit day to day variability. All surgeries were performed between 8 am – 12 noon to minimize differences due to circadian rhythms. We determined by ex vivo macrophage secretome analysis of the LPS+MI mice that the Ccl12 concentration in the media was 295±22 pg/mL, which was 3 times higher than the unexposed MI macrophage amount (**Figure 2**). This concentration was used to calculate the in vivo dose. This concentration was also comparable to what was observed in our clinical plasma samples, which was 492±29 pg/mL in the high endotoxin group.

Coronary Artery Ligation. During coronary artery ligation, 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 (0.1 mg/kg) was administered. Animals were sacrificed 7 days post-MI.

Echocardiography. Transthoracic echocardiography was performed using the Visual Sonics Vevo 770 or 2100 systems 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. Cardiac strain was calculated using speckle tracking analysis on the VevoStrain analysis program.(1, 2) Strain measures the ventricular displacement during contraction. The ventricle shortens in the longitudinal and circumferential planes and thickens in the radial plane during systole with reciprocal changes in diastole. For each parameter, 3 images from consecutive cardiac cycles were measured and averaged.(3, 4) Non-exposed and IgG controls showed no significant

difference in physiological measurements; therefore, results for these two groups were combined.

Survival Analysis and Tissue Harvest. The mice were checked daily for survival. At autopsy, cardiac rupture in non-surviving mice was confirmed by the presence of coagulated blood in the thoracic cavity or observation of ruptured site on the LV. 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). For tissue collection at necropsy, mice were anesthetized with 1-2% isoflurane in an oxygen mix. Hearts were removed, and the LV and right ventricle were separated and weighed individually. The LV was sliced transversely to remove remote and infarct sections. A middle section was collected and fixed in 10% zinc formalin for histological examination. Tissue was frozen in liquid nitrogen, and stored at -80^{o}C for RT^2-PCR and immunoblotting analyses. Infarct areas were calculated as described previously.(5)

Cell Isolation. Macrophages and fibroblasts were isolated from infarcted hearts as previously described.(4, 6) The LV tissue was dissociated into single-cell suspension using 600 U/mL collagenase type II (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). For flow cytometry analysis, CD11b microbeads were used to isolate all leukocytes. The flow through contained unlabeled cells ($Ly\text{-}6G^-$ and $CD11b^-$ cells) and was plated to select for fibroblasts through differential adherence. This allowed us to asses both macrophage and fibroblast phenotypes within the same mouse.

Protein Extraction and Analysis. Protein was extracted from LV tissue by homogenizing the samples sequentially in phosphate buffered saline (PBS) with 1x 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 1x protease inhibitor cocktail (insoluble protein fraction). Protein concentrations were determined by the Quick Start™ Bradford Protein Assay (Bio-Rad). Total protein (10 µg for tissue and 1:20 dilution of media) 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 antibodies against primary antibody [Collagen I (Cedarlane cl50141ap; 1:2000), Collagen III (Cedarlane cl50341ap-1; 1:1000), lysyl oxidase (LOX; Novus nb110-41568; 1:2000), fibronectin (Millipore AB1954; 1:5000), matrix metalloproteinase (MMP)-2 (Cell Signaling 13132s; 1:1000), Ccl12 (Abcam ab84272; 1:500)], secondary antibody (Santa Cruz, SC2020, 1:5000), and detected with ECL Prime Western Blotting Detection Substrate (Amersham). For evaluation of ECM proteins secreted by fibroblasts, media was collected and volume reduced in a Speed Vac Concentrator (Thermo Fisher). 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. Protein levels were quantified by densitometry using the IQ-TL image analysis software (GE Healthcare, Waukesha, WI).

Fibroblast Phenotyping. For the *in vitro* BrdU assay, culture medium was removed and replace with BrdU labeling solution. Cells were incubate at 37°C for 2 hours. Cells were fixed with 4% paraformaldehyde for 10 min. Paraformaldehyde was removed and cells were incubated with 0.25% Triton X in PBS for 10 minutes on a rocker at room temperature to permeabalize cells. Cells were stained for actin filaments by incubating cells in the dark for 30 min in 200 µL of 100 nM rhodamine phalloidin (Thermo Fischer; R415). Cells were washed three times in PBS and anti-BrdU primary antibody was added and incubated overnight at 4°C.

After incubation, Alexa Fluor 488 conjugate (Invitrogen, S32354) was applied and cells were incubated for 30 min at room temperature.

Immunofluorescence. For histological analysis, the middle section (mid-papillary region) from day 7 post-MI LVs were embedded in paraffin and sectioned at 5 μm. Immunofluorescence was performed using antibodies specific for leukocytes (CD11b, Novus Bio NB220-89474; 1:200), macrophages (Mac-3, Cedarlane CL8943AP; 1:100) and α-smooth muscle actin (αSMA, abcam ab32575; 1:100). Blocking was performed in goat serum (Vector Laboratories, PK-6101) and secondary antibodies used were Alexa Fluor 488 conjugate (Invitrogen, S32354) and Alexa Fluor 546 conjugate (Invitrogen, S11225). To quantify staining, five random images were captured 40x magnification. Quantification was measured by Image-Pro Plus version 6.2. Representative images are shown at 60x magnification.

Flow cytometry. $CD11b^+$ cells were isolated and concentrations were adjusted to 0.5×10^6 cells/100 uL with blocking buffer (PBS with 0.5% BSA + 5% heat inactivated mouse serum) and incubated for 5 min at 4°C. Primary antibodies (PE-Vio770 Anti-F4/80; Alexa Fluor 488 rat anti mouse CD206; or mix) were added and cells were incubated for 20 min at 4°C in the dark. Cells were then washed in PEB and centrifuged at 300xg for 8 min. Cells were then filtered with 5 mL tube with cell-strainer cap and analyzed on the MACS Quant Analyzer (Miltenyi Biotec).

Gene expression. RNA extraction was performed on LVI tissue and the isolated macrophages and fibroblasts 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 (10 ng) was performed using the High Capacity RNA-to-cDNA Kit (Life Technologies 4837406). Real Time RT^2-PCR gene array for inflammatory cytokines and receptors (Qiagen PAMM-011A) and ECM (Qiagen PAMM-013A) was performed to quantify mRNA levels in the LV infarct. Inflammatory and ECM gene arrays were performed with the LV infarct. Macrophages were evaluated for M1 and M2 markers (**Table S2**). The experiments were performed according to the Minimum Information for Publication of

Quantitative Real-Time PCR Experiments (MIQE) guidelines with one exception hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*), was the only reference gene used, as GusB, Hsp90ab1, Actb, and Gapdh have all been shown to significantly change post-MI.(7)

Ccl12 ELISA. Ccl12 concentration in the macrophage secretome was measured by Mouse Ccl12/MCP-5 Quantikine ELISA (R&D; MCC120) as described by the manufacturer. Cells were isolated from the infarct and plated overnight (0.5 x 10^6 cells/well). Media was collected for Ccl12 measurements.

Dot blot immunoassay. Samples were diluted in sterile water to a final dilution of 1 ng/ µL, and 200 µL of sample (200 ng total protein) was loaded and filtered onto a nitrocellulose membrane. Total membrane staining was performed as described above using MemCode™ Reversible Protein Stain Kit (Thermo Scientific). The nitrocellulose membrane was placed in blocking solution (5% bovine serum albumin, 0.05% Tween-20, in PBS) and incubated with gentle agitation for 1 h at room temperature. After blocking, MMP-12 antibody (Epitomics 1906; 1:500) was added and incubated overnight at 4°C. The nitrocellulose membrane was washed 3 times for 10 min followed by incubation with an anti-rabbit secondary antibody (Vector PI-1000; 1:500) for 1 h at room temperature. Quantification of ECL signal was performed using the Image Quant LAS4000. Concentrations were calculated using MMP-12 recombinants as reference and were normalized to total membrane staining. MMP-12 protein levels were normalized to white blood cell (WBC) count to assess leukocyte MMP-12 contribution.

***In vitro* stimulation**. For macrophage secretome stimulations, macrophages were isolated from the infarct area and plated overnight (0.5 x 10^6 cells/well). Conditioned media was collected and used to stimulate naïve cardiac fibroblasts in the presence or absence of a Ccl12 blocking antibody (Novus #AF428; 10 µg/mL; 24 h). Naïve cardiac fibroblasts were also stimulated with recombinant Ccl12 (R&D 428-P5-025/CF; 300 pg/mL; 24 h) in the presence or absence of a Ccr2 inhibitor (Calbiochem 227016; 5uM; 24 h). Cardiac fibroblasts stimulated with 10% fetal bovine serum (FBS) served as positive controls to assess the ability of a fibroblast culture to

respond to stimulation, and fibroblasts incubated in 0.1% FBS alone were the negative controls to subtract out potential autocrine effects. Conditioned media was collected and stored at -80^{0}C. Fibroblast activation and ECM synthesis was assessed by TaqMan ECM gene expression (**Table S3**).

Electric Cell-substrate Impedance Sensing (ECIS). Cell migration and proliferation were analyzed using electric cell-substrate impedance sensing (ECIS®, Applied Biophysics) as described previously.(7) Cells at passage 3 were plated in an ECIS-wound 96-well plate (4.0x10^4 cells/mL, duplicates/condition). Cells were allowed to proliferate until stable impedance values were observed (~48h), at this point the instrument was paused and the plate removed. For macrophage conditioned media stimulation, the fibroblast media was removed and replaced with the following conditions: 1) 0.1% FBS media; 2) 10% FBS media; 3) conditioned media from MI mice; 4) conditioned media from LPS+MI mice; 5) conditioned media from MI mice + Ccl12 blocking antibody (10 µg/mL); or 6) conditioned media from LPS+MI mice + Ccl12 blocking antibody (10 µg/mL). For the Ccl12 stimulation, the fibroblast media was removed and replaced with the following conditions: 1) 0.1% FBS media; 2) 10% FBS media; 3) Ccl12 (300 pg/mL); 4) Ccl12 (300 pg/mL) + Ccr2 inhibitor (5uM); or 5) Ccr2 inhibitor (5uM). Wells with media only were used as a negative control. The plate was replaced in the ECIS instrument and wounded for 10 seconds at 1200 uA, 40'000 Htz. After wounding, impedance values were recorded for 48 hours.

Integrated Pathway Analysis. Functional analysis of identified genes were assessed using Ingenuity Pathways Analysis (IPA; QIAGEN Redwood City; www.qiagen.com/ingenuity).

Patient Selection Criteria. Patient selection was based on two criteria: 1) Typical increase and gradual decrease of biochemical markers of myocardial necrosis (CK-MB and troponin) with either ischemic symptoms, development of pathological Q-waves on the ECG, or ECG changes indicative of ischemia, and 2) ST-segment changes consisting of elevation (STEMI) in two or more contiguous leads with the cut-off points >0.2 mV in leads V1, V2, or V3 and >0.1 mV in

other leads (continuity in the frontal plane is defined by the lead sequence aVL, I, inverted aVR, II aVF, III) or ST-segment depression in 2 or more contiguous leads (NSTEMI).

Supplemental References

1. Kailin JA, Miyamoto SD, Younoszai AK, and Landeck BF. Longitudinal myocardial deformation is selectively decreased after pediatric cardiac transplantation: a comparison of children 1 year after transplantation with normal subjects using velocity vector imaging. *Pediatric cardiology.* 2012;33:749-56.

2. Shah AM, and Solomon SD. Myocardial deformation imaging: current status and future directions. *Circulation.* 2012;125:e244-8.

3. Lindsey ML, Escobar GP, Dobrucki LW, Goshorn DK, Bouges S, Mingoia JT, McClister DM, Jr., Su H, Gannon J, MacGillivray C, et al. Matrix metalloproteinase-9 gene deletion facilitates angiogenesis after myocardial infarction. *Am J Physiol Heart Circ Physiol.* 2006;290:H232-H9.

4. Zamilpa R, Kanakia R, Cigarroa Jt, Dai Q, Escobar GP, Martinez H, Jimenez F, Ahuja SS, and Lindsey ML. CC chemokine receptor 5 deletion impairs macrophage activation and induces adverse remodeling following myocardial infarction. *Am J Physiol Heart Circ Physiol.* 2011;300:H1418-H26.

5. Ma Y, Halade GV, Zhang J, Ramirez TA, Levin D, Voorhees A, Jin YF, Han HC, Manicone AM, and Lindsey ML. Matrix metalloproteinase-28 deletion exacerbates cardiac dysfunction and rupture after myocardial infarction in mice by inhibiting M2 macrophage activation. *Circ Res.* 2013;112:675-88.

6. DeLeon-Pennell KY, de Castro Bras LE, Iyer RP, Bratton DR, Jin YF, Ripplinger CM, and Lindsey ML. P. gingivalis lipopolysaccharide intensifies inflammation post-myocardial infarction through matrix metalloproteinase-9. *J Mol Cell Cardiol.* 2014;76C:218-26.

7. Lindsey ML, Iyer RP, Zamilpa R, Yabluchanskiy A, DeLeon-Pennell KY, Hall ME, Kaplan A, Zouein FA, Bratton D, Flynn ER, et al. A Novel Collagen Matricryptin Reduces Left

Ventricular Dilation Post-Myocardial Infarction by Promoting Scar Formation and Angiogenesis.

J Am Coll Cardiol. 2015;66:1364-74.

Supplemental Tables

Table S1 (Related to Figure 2). Expression values of the 165 inflammatory and ECM genes measured. All genes were used to generate the heat map. Genes are ranked highest to lowest, according to p-value and fold-change (LPS+MI D7/ MI D7 ratio).

Gene name	MI D7	LPS+MI D7	Fold-Change	p-value
Ccl12	**0.164 ± 0.028**	**0.334 ± 0.018**	**2.1**	**0.001***
Il1r1	0.271 ± 0.015	0.452 ± 0.025	1.7	0.001*
C3	0.907 ± 0.056	1.778 ± 0.230	2.0	0.004*
Ccl19	0.116 ± 0.010	0.197 ± 0.021	1.7	0.006*
Tnfrsf1a	0.349 ± 0.036	0.485 ± 0.024	1.4	0.010*
Vcam1	0.207 ± 0.013	0.294 ± 0.025	1.4	0.011*
Icam1	0.140 ± 0.015	0.229 ± 0.025	1.6	0.012*
Itga3	0.035 ± 0.003	0.051 ± 0.004	1.5	0.012*
Il18	0.044 ± 0.006	0.066 ± 0.005	1.5	0.013*
Itga5	0.577 ± 0.049	0.777 ± 0.044	1.3	0.013*
Ecm1	1.916 ± 0.148	2.950 ± 0.316	1.5	0.014*
Il6st	1.295 ± 0.099	1.763 ± 0.129	1.4	0.016*
Timp3	0.363 ± 0.079	0.116 ± 0.040	0.3	0.019*
Ccr6	0.006 ± 0.001	0.003 ± 0.001	0.5	0.020*
Itgam	0.365 ± 0.034	0.492 ± 0.050	1.3	0.020*
Entpd1	0.128 ± 0.020	0.193 ± 0.013	1.5	0.021*
Pf4	0.387 ± 0.078	0.768 ± 0.127	2.0	0.029*
Ccl6	0.046 ± 0.008	0.126 ± 0.030	2.7	0.030*
Cdh4	Undetermined	0.003 ± 0.001	∞	0.036*
Vcan	0.351 ± 0.019	0.456 ± 0.039	1.3	0.036*
Ctnna1	1.582 ± 0.174	2.066 ± 0.106	1.3	0.039*
Mmp2	1.825 ± 0.239	2.829 ± 0.359	1.6	0.042*
Selp	0.039 ± 0.006	0.084 ± 0.019	2.2	0.046*
Lamc1	0.985 ± 0.132	1.455 ± 0.160	1.5	0.047*
Ccl8	0.189 ± 0.046	0.425 ± 0.097	2.2	0.049*
Scye1	0.586 ± 0.037	0.726 ± 0.053	1.2	0.054
Ccl9	0.091 ± 0.011	0.158 ± 0.030	1.7	0.059
Fbln1	0.262 ± 0.063	0.529 ± 0.109	2.0	0.060
Lama3	0.011 ± 0.001	0.018 ± 0.003	1.6	0.060
Sparc	17.351 ± 1.978	26.080 ± 3.698	1.5	0.064
Bcl6	0.144 ± 0.013	0.212 ± 0.030	1.5	0.065
Thbs3	0.231 ± 0.015	0.301 ± 0.031	1.3	0.066
Tnfrsf1b	0.336 ± 0.037	0.429 ± 0.027	1.3	0.066
Tollip	0.188 ± 0.011	0.216 ± 0.008	1.1	0.068
Col3a1	42.514 ± 6.530	63.184 ± 7.912	1.5	0.072
Il13ra1	0.282 ± 0.017	0.344 ± 0.026	1.2	0.072
Itga2	0.012 ± 0.001	0.016 ± 0.002	1.3	0.072
Sele	0.010 ± 0.002	0.023 ± 0.006	2.3	0.075
Ccl2	0.155 ± 0.030	0.243 ± 0.033	1.6	0.076
Tgfbi	1.443 ± 0.176	1.980 ± 0.207	1.4	0.076
Itgal	0.016 ± 0.004	0.029 ± 0.006	1.8	0.077
Pecam1	0.506 ± 0.043	0.672 ± 0.076	1.3	0.086

Adamts5	0.0456 ± 0.006	0.071 ± 0.012	1.6	0.089
Il10rb	0.694 ± 0.040	0.867 ± 0.086	1.2	0.098
Cxcl12	1.259 ± 0.184	1.679 ± 0.143	1.3	0.102
Mmp13	0.006 ± 0.001	0.010 ± 0.002	1.7	0.112
Mif	1.671 ± 0.078	2.068 ± 0.215	1.2	0.113
Mmp15	0.081 ± 0.005	0.132 ± 0.029	1.6	0.113
Ccl7	0.136 ± 0.024	0.253 ± 0.064	1.9	0.119
Itgb3	0.077 ± 0.005	0.096 ± 0.010	1.2	0.125
Mmp3	0.018 ± 0.003	0.077 ± 0.035	4.3	0.128
Cxcl5	0.011 ± 0.003	0.091 ± 0.050	8.3	0.142
Adamts8	0.033 ± 0.003	0.026 ± 0.004	0.8	0.148
Sell	0.017 ± 0.003	0.031 ± 0.008	1.8	0.148
Il2rg	0.191 ± 0.023	0.240 ± 0.023	1.3	0.156
Col6a1	2.657 ± 0.210	3.221 ± 0.305	1.2	0.158
Itga4	0.036 ± 0.007	0.051 ± 0.007	1.4	0.163
Il1b	0.036 ± 0.005	0.124 ± 0.059	3.5	0.166
Il15	0.132 ± 0.013	0.157 ± 0.012	1.2	0.173
Il8rb	0.006 ± 0.002	0.023 ± 0.011	3.8	0.174
Col5a1	3.830 ± 0.563	5.134 ± 0.695	1.3	0.176
Itgb2	0.509 ± 0.064	0.610 ± 0.029	1.2	0.178
Il16	0.047 ± 0.008	0.059 ± 0.004	1.3	0.186
Ccr1	0.074 ± 0.013	0.137 ± 0.046	1.9	0.214
Ccl25	0.007 ± 0.001	0.005 ± 0.001	0.7	0.22
Mmp8	0.012 ± 0.001	0.045 ± 0.026	3.8	0.223
Il1r2	0.021 ± 0.005	0.109 ± 0.068	5.2	0.224
Il2rb	0.008 ± 0.002	0.013 ± 0.003	1.6	0.226
Itgb4	0.003 ± 0.001	0.004 ± 0.001	1.3	0.243
Abcf1	0.551 ± 0.051	0.638 ± 0.048	1.2	0.246
Cxcl9	0.024 ± 0.004	0.093 ± 0.058	3.9	0.255
Emilin1	1.475 ± 0.121	1.883 ± 0.317	1.3	0.257
Itgb1	4.381 ± 0.365	5.353 ± 0.725	1.2	0.259
Sgce	0.146 ± 0.023	0.189 ± 0.028	1.3	0.262
Lamb2	0.911 ± 0.062	1.113 ± 0.161	1.2	0.266
Cxcl1	Undetermined	0.009 ± 0.005	∞	0.27
Vtn	0.088 ± 0.013	0.116 ± 0.020	1.3	0.274
Mmp9	0.017 ± 0.010	0.114 ± 0.085	6.7	0.283
Il1a	Undetermined	0.009 ± 0.006	∞	0.284
Tgfb1	0.665 ± 0.058	0.749 ± 0.051	1.1	0.299
Col4a2	5.831 ± 0.524	6.694 ± 0.662	1.1	0.331
Tnf	0.007 ± 0.001	0.008 ± 0.001	1.1	0.333
Cxcl13	0.006 ± 0.002	0.052 ± 0.045	8.7	0.337
Ccl17	0.037 ± 0.004	0.028 ± 0.008	0.8	0.343
Ccl3	0.027 ± 0.008	0.038 ± 0.008	1.4	0.346
Ccl5	0.086 ± 0.012	0.109 ± 0.020	1.3	0.347
Mmp11	0.017 ± 0.002	0.020 ± 0.003	1.2	0.356
Itgae	0.010 ± 0.001	0.008 ± 0.002	0.8	0.366
Cx3cl1	0.539 ± 0.047	0.463 ± 0.066	0.9	0.372
Adamts1	0.166 ± 0.038	0.131 ± 0.015	0.8	0.411
Col4a1	6.119 ± 0.675	6.969 ± 0.745	1.1	0.418
Lama2	0.278 ± 0.040	0.232 ± 0.037	0.8	0.421
Ccr5	0.352 ± 0.049	0.297 ± 0.047	0.8	0.44

Ctgf	15.013 ± 1.914	17.351 ± 2.404	1.2	0.464
Ncam1	0.226 ± 0.018	0.208 ± 0.017	0.9	0.496
Cd44	0.511 ± 0.040	0.550 ± 0.037	1.1	0.497
Itgax	0.084 ± 0.016	0.070 ± 0.012	0.8	0.502
Cdh3	0.002 ± 0.001	0.002 ± 0.001	1.0	0.518
Il10	0.005 ± 0.001	0.006 ± 0.002	1.2	0.524
Ccl11	Undetermined	0.002 ± 0.001	∞	0.544
Lamb3	0.005 ± 0.001	0.007 ± 0.002	1.4	0.551
Il10ra	0.165 ± 0.021	0.180 ± 0.017	1.1	0.586
Il11	0.006 ± 0.001	0.005 ± 0.001	0.8	0.588
Mmp12	0.004 ± 0.001	0.003 ± 0.001	0.8	0.632
Thbs1	8.274 ± 1.743	7.006 ± 2.115	0.8	0.653
Col1a1	31.163 ± 4.381	34.266 ± 5.285	1.1	0.661
Adamts2	2.438 ± 0.194	2.640 ± 0.410	1.1	0.665
Cxcl10	0.091 ± 0.023	0.105 ± 0.023	1.2	0.667
Casp1	0.151 ± 0.013	0.143 ± 0.014	0.9	0.675
Cxcr3	0.020 ± 0.003	0.018 ± 0.002	0.9	0.691
Tnc	0.909 ± 0.214	0.805 ± 0.169	0.9	0.712
Ctnnb1	1.623 ± 0.077	1.706 ± 0.208	1.1	0.714
Fn1	12.840 ± 1.790	14.030 ± 2.790	1.1	0.727
Ccl22	0.002 ± 0.001	0.002 ± 0.001	1.0	0.741
Xcr1	0.003 ± 0.001	0.003 ± 0.001	1.0	0.742
Cdh1	0.004 ± 0.001	0.004 ± 0.001	1.0	0.76
Thbs2	2.626 ± 0.328	2.871 ± 0.719	1.1	0.763
Ccr3	0.175 ± 0.029	0.162 ± 0.035	0.9	0.777
Col2a1	0.113 ± 0.026	0.100 ± 0.039	0.9	0.787
Ccr2	0.154 ± 0.026	0.143 ± 0.032	0.9	0.792
Ccr10	0.019 ± 0.002	0.021 ± 0.007	1.1	0.797
Ccr9	0.007 ± 0.001	0.007 ± 0.001	1.0	0.822
Cxcr5	0.004 ± 0.001	0.004 ± 0.001	1.0	0.833
Timp2	2.282 ± 0.157	2.220 ± 0.245	1.0	0.835
Col4a3	0.009 ± 0.003	0.009 ± 0.002	1.0	0.86
Ltb	0.016 ± 0.003	0.017 ± 0.005	1.1	0.863
Postn	9.391 ± 1.023	9.614 ± 1.487	1.0	0.904
Spp1	4.238 ± 0.581	4.133 ± 0.826	1.0	0.919
Ccr7	0.015 ± 0.003	0.014 ± 0.004	0.9	0.94
Lama1	0.002 ± 0.001	0.002 ± 0.001	1.0	0.943
Il6ra	0.205 ± 0.018	0.207 ± 0.028	1.0	0.944
Mmp14	1.089 ± 0.120	1.102 ± 0.141	1.0	0.944
Ccl4	0.071 ± 0.016	0.072± 0.014	1.0	0.953
Cdh2	1.073 ± 0.094	1.061 ± 0.162	1.0	0.953
Itgav	0.656 ± 0.024	0.652 ± 0.082	1.0	0.96
Ccl1	Undetermined	Undetermined	NA	NA
Ccl20	Undetermined	Undetermined	NA	NA
Ccl24	Undetermined	Undetermined	NA	NA
Ccr4	Undetermined	Undetermined	NA	NA
Ccr8	Undetermined	Undetermined	NA	NA
Cd40lg	Undetermined	Undetermined	NA	NA
Cntn1	Undetermined	Undetermined	NA	NA
Crp	Undetermined	Undetermined	NA	NA
Ctnna2	Undetermined	Undetermined	NA	NA

Cxcl11	Undetermined	Undetermined	NA	NA
Cxcl15	Undetermined	Undetermined	NA	NA
Hapln1	Undetermined	Undetermined	NA	NA
Hc	Undetermined	Undetermined	NA	NA
Ifng	Undetermined	Undetermined	NA	NA
Il13	Undetermined	Undetermined	NA	NA
Il17	Undetermined	Undetermined	NA	NA
Il1f6	Undetermined	Undetermined	NA	NA
Il1f8	Undetermined	Undetermined	NA	NA
Il20	Undetermined	Undetermined	NA	NA
Il3	Undetermined	Undetermined	NA	NA
Il4	Undetermined	Undetermined	NA	NA
Il5ra	Undetermined	Undetermined	NA	NA
Lta	Undetermined	Undetermined	NA	NA
Mmp10	Undetermined	Undetermined	NA	NA
Mmp1a	Undetermined	Undetermined	NA	NA
Mmp7	Undetermined	Undetermined	NA	NA
Ncam2	Undetermined	Undetermined	NA	NA
Spock1	Undetermined	Undetermined	NA	NA
Syt1	Undetermined	Undetermined	NA	NA
Timp1	Undetermined	Undetermined	NA	NA

Values are mean ± SEM; ∞- infinity when MI D7 value was undetermined; NA- not applicable when values for both groups were undetermined; *Chemokine CC-motif ligand (Ccl)1, Ccl20, Ccl24, Chemokine CC-motif receptor (Ccr)4, Ccr8, Cd40 ligand, Contactin 1, C-reactive protein, Catenin (cadherin-associated protein) a2, Chemokine CX-motif ligand (Cxcl)11, Cxcl15, Hyaluronan and proteoglycan link protein 1, Hemolytic complement, Interferon gamma, Interleukin (Il)13, Il17b, Il1f6, Il1f8, Il20, Il3, Il4, Il5ra, Lymphotoxin-alpha (Lta), Matrix metalloproteinase (Mmp)10, Mmp1a, Mmp7, Neural cell adhesion molecule 2, Testican (Spock)1, Synaptotagmin 1, Tissue inhibitor of metalloproteinase 1; n=6/group; *p<0.05 vs WT MI+LPS*

Table S2 (Related to Methods). Primers used for M1 and M2 macrophage phenotyping (all from Applied Biosystems).

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

Table S3 (Related to Methods). Primers used for ECM genes (all from Applied Biosystems).

Primers	
aSMA (Mm00725412_s1)	Ctgf (Mm01192932_g1)
Col1a1 (Mm00801666_g1)	Fn (Mm01256744_m1)

Col3a1 (Mm01254476_m1) Tgfβ (Mm01178820_m1)

Figure Legends

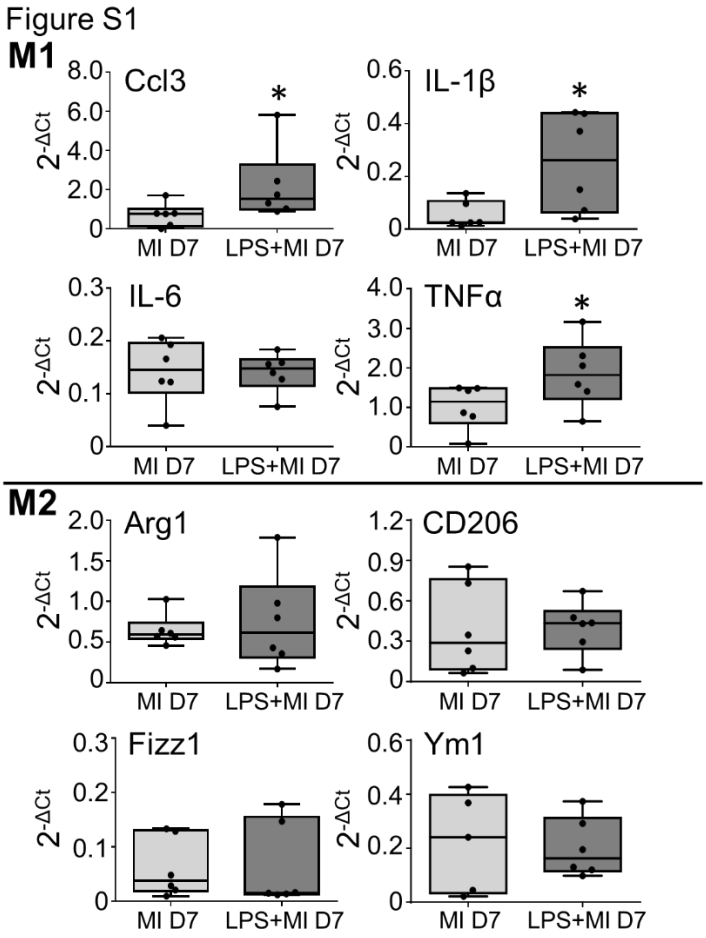

Figure S1. Lipopolysaccharide (LPS) pre-exposure prolonged pro-inflammatory macrophage polarization at day 7 post-myocardial infarction (MI). Pro-inflammatory M1 marker expression (Ccl2, IL1β, and Tnfα) in isolated macrophages were increased in LPS+MI compared to MI controls. No effect was observed in anti-inflammatory M2 marker expression. n=6/group (3M, 3F); MI=4.0±0.1 months; LPS+MI=4.4±0.1 months); *p<0.05 vs. WT MI; n=6/group.

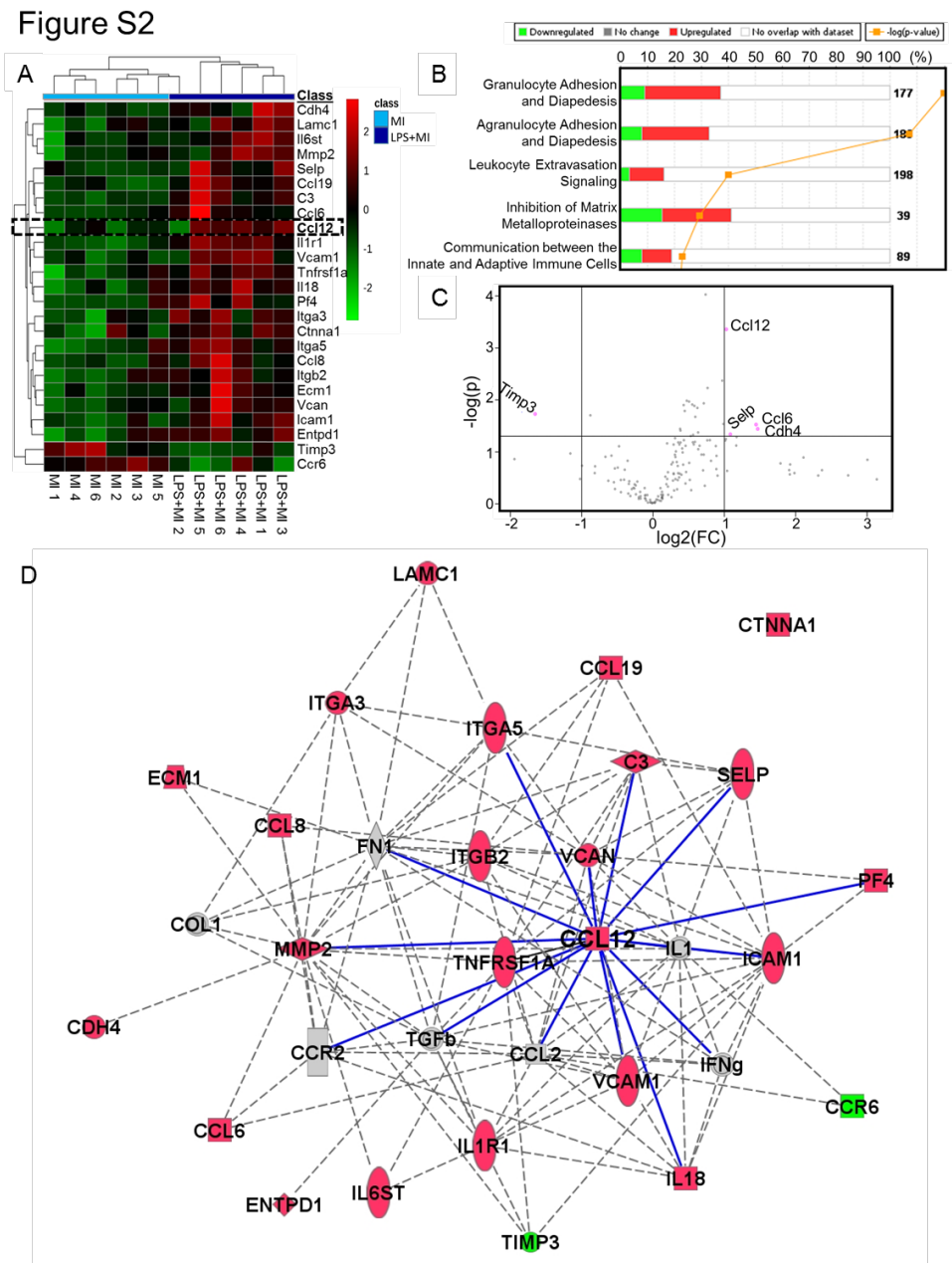

Figure S2. Lipopolysaccharide (LPS) induced chronic inflammation increased cardiac expression of macrophage related inflammatory factors. (A) Infarct tissue taken at day 7 post-MI was analyzed for 165 inflammatory and fibrotic genes. Hierarchical clustering of myocardial infarction (MI) controls and LPS+MI mice was performed analyzing genes that were found differentially expressed between the two groups. A red-green color scale depicts normalized mRNA expression levels in $2^{-\Delta Ct}$ values (Red: high, Green: low; $p<0.05$). Out of 165

inflammatory and fibrotic genes, 25 genes were significantly different between groups and 23 were increased in LPS+MI mice. (B) Integrated pathway analysis indicated these features were associated with leukocyte migration and inflammation. (C) Ccl12 was the top candidate, as it had the smallest p-value and was more than 2 times higher in LPS exposed mice. (D) Of the 25 genes that were found to be significantly different between LPS+MI and MI mice, Ccl12 was a central gene within the network. Up-regulated genes are shown in red, down-regulated genes are shown in green, and genes that were not significantly different between groups are shown in grey. Ccl12 relationships are indicated by the blue lines. (n=6/group (3M, 3F); MI=5.2±0.1 months; LPS+MI=6.0±0.1 months).

Figure S3

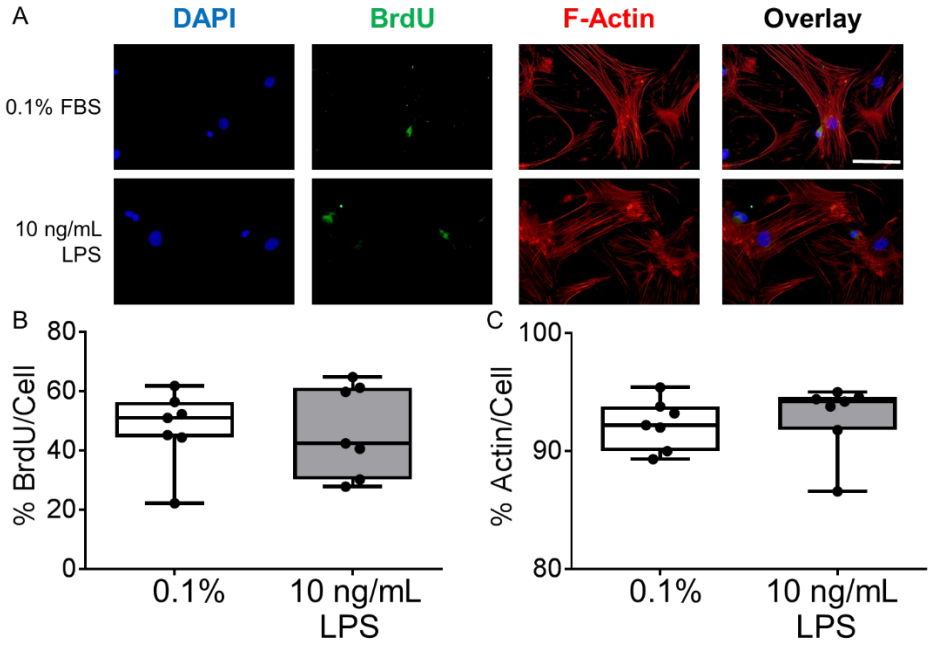

Figure S3. *Porphyromonas gingivalis* lipopolysaccharide (LPS) stimulation of control naïve cardiac fibroblasts induced cytoskeletal rearrangement. (A) Imaging of naïve cardiac fibroblasts showed LPS had (B) no effect on proliferation (BrdU-positive cells) and (C) increased F-actin filaments. This indicates the *in vivo* fibroblast phenotype was not due to direct LPS effects on fibroblasts. Scale bar is 100 μm. n=6/group (6M); 4.0±0.1 months for all groups. All in vitro stimulation experiments were paired. *p<0.05 vs unstimulated.

Figure S4

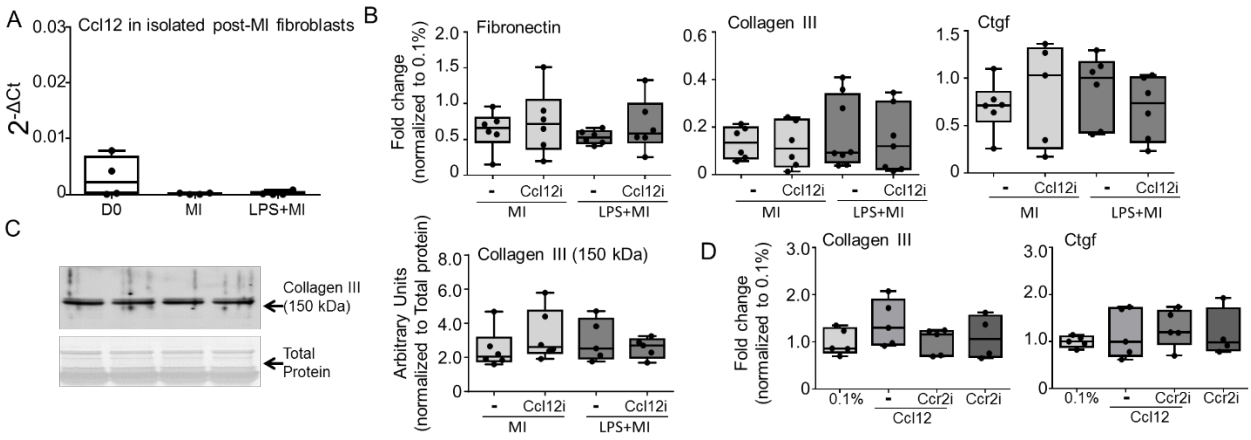

Figure S4. (A) The effect of Ccl12 on fibroblast function was not an autocrine response as fibroblast isolated from unoperated controls (D0), myocardial infarction (MI) mice, or lipopolysaccharide (LPS)+MI mice expressed very low amounts of Ccl12. (n=6/group (3M, 3F); MI=5.2±0.1 months; LPS+MI=6.0±0.1 months). (B) Stimulation of naïve fibroblast with macrophage conditioned media did not affect fibroblast gene expression of fibronectin, collagen III, or connective tissue growth factor (Ctgf). Ccl12 blocking antibody (Ccl12i) had no effect on these genes. n=6/group (4M); 4.0±0.1 months for all groups. (C) Collagen III protein secretion was not effected by macrophage conditioned media. (D) *In vitro* Ccl12 stimulation did not affect collagen III, or Ctgf. n=5/group (5M); 4.5±0.1 months for all groups. All in vitro stimulation experiments were paired. *p<0.05 vs 0.1%.