Lp-PLA 2 Antagonizes Left Ventricular Healing After Myocardial Infarction by Impairing the Appearance of Reparative MacrophagesCLINICAL PERSPECTIVE

SUPPLEMENTAL MATERIAL

Supplemental Methods

Animal models and in vivo interventions. *Bone marrow chimeras:* 8 weeks old female C57BL/6J (wild-type[WT]) mice were lethally irradiated (950cGy) and reconstituted with WT (Controls) and *Lp-PLA*$_2^{-/-}$ bone marrow cells, respectively, for 10 weeks. *Model of myocardial infarction:* Myocardial infarction (MI) was induced by permanent ligation of the left anterior descending coronary artery (LAD). Mice were anesthetized with isoflurane (2%/2 liters O2), intubated and ventilated with an Inspira Advanced Safety Single Animal Pressure/Volume Controlled Ventilator (Harvard Apparatus). Left thoracotomy was performed in the forth intercostal space after shaving the chest wall. The left ventricle was visualized and the LAD was ligated with monofilament 8-0 suture (Ethicon, Somerville, NJ). The chest and skin were closed with a 7-0 nylon suture followed by removal of air from the thorax via a pleural catheter. The procedure was performed by the same surgeon blinded to genotypes. All protocols were conducted in accordance with the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were approved by the Animal Review Committee at Massachusetts General Hospital.

Cell isolation. Peripheral blood was collected by retroorbital bleeding in heparinized tubes, and erythrocytes were lysed in RBC Lysis buffer (Biolegend). Organs were harvested at the day of sacrifice. Femurs, tibias and hearts were excised after vascular perfusion with 10 ml sterile PBS. Flushed bone marrow was strained through a 40 µm-nylon mesh (BD Biosciences, San Jose, CA, USA). The healthy left ventricle or infarcted areas were excised including the border zone, minced and digested in 450 U/ml collagenase I, 125 U/ml collagenase XI, 60 U/ml DNase I and 60 U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) PBS for 1 h at 37°C while shaking. Cells were counted in a Neubauer chamber. Femur + tibias contains ~7% of all bone marrow cells. Bone marrow cell counts were extrapolated accordingly. *Cell sorting:* Heart tissues from *Lp-PLA*$_2^{-/-}$ and WT bone marrow chimeras were minced and digested as described above and stained for anti-Ly6C-Fitc, anti-Lin-PE (Lin (lineage) = CD3, CD90.2, CD19, NK1.1, Ly6G), anti-MHCII-Alexa Fluor 700, anti-F4/80-PECy7, anti-CD115-APC, anti-MHC II-Alexa Fluor 700, anti-CD11b-APCCy7 and anti-CD45.2-Pacific Blue. Macrophages were identified as shown in Figure 2. Cells were sorted on a FACS Aria II cell sorter (BD Biosciences) directly into RLT buffer for subsequent RNA isolation.

Serum analysis. Serum was collected from retro-orbital blood before and at 1, 3 and 7 days post MI. TNFα, IL-6, and IL-1ß serum levels were measured with Quantikine ELISA Kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Histology. Hearts were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), frozen in ice-cold 2-Methylbutane (Fisher Scientific, Fair Lawn, NJ) and sectioned into 6 µm slices yielding 30-40 sections per mouse. The following antibodies were used for immunohistology: anti-CD11b (clone M1/70, BD Biosciences), anti-Collagen I (ab21286, Abcam), anti-CD31 (clone MEC13.3, BD Biosciences) , anti-α-smooth muscle actin (ab5694; Abcam) for smooth muscle cells (SMA). Biotinylated secondary antibodies followed by VECTASTAIN ABC reagent (Vector Laboratories, Inc. Burlingame, CA) were applied and AEC substrate (Dako North America, Inc. Carpinteria, CA) was used for color development. Masson's Trichrome staining was performed in cross sections of hearts 7 days post MI. Image capture was performed using a Nanozoomer 2.0RS (Hamamatsu, Japan). Images were analyzed with ImageJ.

Flow Cytometry. Cell suspensions were stained in PBS supplemented with sterile 2% FBS and 0.5% BSA. The following monoclonal antibodies were used for flow cytometric analysis: anti-Ly6C (clone AL-21, BD Biosciences), anti-CD45.2 (clone 104, BD Biosciences), anti-CD3e (clone 145-2C11, ebioscience), anti-CD90.2 (clone 53-2.1, BD Biosciences), anti-CD19 (clone 6D5, Biolegend), anti-MHCII (clone AF6-120.1, BD Biosciences), anti-F4/80 (clone BM8, Biolegend), anti-NK1.1 (clone PK136, BD Biosciences), anti-Ly6G (clone 1A8, BD Biosciences), anti-CD11b (clone

M1/70, BD Biosciences), anti-CD11c (clone HL3, BD Biosciences), anti-CD115 (clone AFS98, ebioscience). Data were acquired on a LSRII and analyzed with FlowJo (Tree Star, Ashland, OR, USA). Specifically, monocytes were identified as $CD45^+$, Lin^- (Lin = CD3, CD90.2, CD19, NK1.1, Ly6G), $CD11b^+$, $F4/80^{low}$, $MHCII^{low}$, $CD11c^{low}$, $CD115^+$ cells, subdivided into $Ly6C^{high}$ and $Ly6C^{low}$ subsets. Neutrophils were identified as $CD45^+$, Lin^+, $CD11b^+$, $MHCII^{low}$, $CD11c^{low}$, SSC^{high}, $Ly6C^{int}$ cells. Cardiac macrophages were identified as $CD45^+$, Lin^- (Lin = CD3, CD90.2, CD19, NK1.1, Ly6G), $CD11b^+$, $F4/80^{high}$, $Ly6C^{low}$, $MHCII^+$ cells.

Real-time PCR. 2-4 x 10^4 sorted macrophages from heart were lysed in RLT buffer. RNA was isolated using the RNeasy Micro Kit (Qiagen, Venlo, Netherlands) followed by cDNA transcription with the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. Quantitative real-time TaqMan PCR was performed using the following TaqMan gene expression assays (Applied Biosystems): MMP3 (Mm00440295_m1), MMP9 (Mm00442991_m1), TLR4 (Mm00445273_m1), TLR9 (Mm00446193_m1), Fas (Mm01204974_m1) Bax (Mm00432051_m1), Bcl-2 (Mm00477631_m1), Arg1 ((Mm00475988_m1), IL-10 (Mm00439614_m1), Msr1 (Mm00446214_m1), CD36 (Mm01135198_m1), Fizz (Mm00445109_m1), VEGF (Mm01281449_m1) and housekeeping gene β-actin (4352341E). PCR was run on a 7500 PCR thermal cycler (Applied Biosystems). Data were quantified with the $2^{-\Delta\Delta Ct}$ method.

Lp-PLA_2/PAF Acetyl-Hydrolase Activity Assay. The PAF hydrolase activity assay was performed as previously described with the following modifications[1]. Lp-PLA2 activity was measured using [^{3}H]PAF (Platelet Activating Factor, 1-O-Hexadecyl-[Acetyl-^{3}H(N)]-, Hexadecyl PAF) as a substrate. Where required, reagent and assay volume was supplemented with buffer comprised of 50 mM HEPES, 150 mM NaCl, pH 7.5. The 1 mM [^{3}H]PAF stock solution was initially prepared by combining 78 nmoles PAF with 6 µCi [^{3}H]PAF. The substrate solvents were evaporated under a gentle stream of nitrogen before adjusting the final volume to 780 µl with buffer. All reactions were evaluated in Immunotubes (Nunc, 62409-170) in a final reaction volume of 100 µl comprised of two additions of 90 ul buffer and 10 ul of plasma derived from WT controls or *bmLp-$PLA_2^{-/-}$* mice as the source of enzyme. All samples were pre-incubated at 37˚C for 10 minutes; reactions were initiated with the addition of 5 µl 1 mM [^{3}H]PAF (50 µM final reaction concentration containing 0.04 µCi [^{3}H]PAF). Following a 2 min incubation with substrate, the reactions were terminated by the addition of 600 µl CHCl3:CH3OH (2:1). An additional 100 µl buffer was added to quenched reactions to adjust the aqueous phase to 200 ul. The aqueous layer was separated by centrifugation (2000 rpm, 5 minutes) whereby 200 µl was removed and mixed with an additional 250 µl CHCl3. Following a second centrifugation, 100 µl of the aqueous layer was removed and the amount of [^{3}H]acetate extracted was quantitated by liquid scintillation counting in 5 ml of Bio-Safe II. Where indicated, the inhibition of LpPLA2 associated activity was evaluated in the presence of 10 µM SB-435495 suspended in DMSO (1% (v:v) final reaction concentration) whereby samples were pre-incubuated with compound at 37˚C for 10 minutes before the addition of [^{3}H]PAF, and were processed as described. We confirmed the contribution of Lp-PLA_2 by adding a saturating concentration of a selective inhibitor. When plasma samples were assayed in the presence of a saturating concentration of SB-435495[2] we observed the remaining activity to be uniform across all treatment samples, averaging 2.4 0.2 nmol min-1 ml-1.

MRI. We performed in vivo MRI on days 1 and 21 after MI in mice (n = 8–10 per group). A 7 Tesla horizontal bore Pharmascan (Bruker) and a custom-made mouse cardiac coil in birdcage design (Rapid Biomedical) were used to obtain cine images of the left ventricular short axis. We used ECG and respiratory gating using a gradient echo sequence (echo time 2.7 ms, 16 frames per RR interval; flip angle 30 degrees [or 60 degrees for delayed enhancement imaging]; in-plane resolution 200 Å~ 200 µm; slice thickness 1 mm). The infarcted area was identified in end-diastolic frames as: (a) hypokinetic in cine-loops and (b) hyperenhancing after injection of gadolinium-diethylenetriaminepentaacetic acid (Berlex) at a dosage of 0.3 mmol/kg. Cardiac volumes and infarct size were quantitated from 6–8 short axis imaging slices covering the left ventricle, as described previously[3]. Ejection fraction was calculated as End diastolic volume - End systolic volume) / End Diastolic volume and expressed as percentage.

Supplemental References

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Table 1: Functional Characteristics of the Heart after MI

		EDV (µL)	ESV (µL)	SV (µL)	EF (%)	LVM (mg)	HR (bpm)	CO (mL/min)
Day 1	*bmLp-PLA$_2^{-/-}$*	69	43	26	38	82.6	391	10.17
		65	41	24	37	84.5	403	9.67
		62	34	28	45	87.3	371	10.38
		53	27	26	49	79.7	407	10.58
		51	26	25	49	75.5	430	10.75
		70	42	28	41	94	427	12.14
	WT	56	35	21	38	78.9	431	9.05
		50	24	26	52	71.4	412	10.71
		56	35	21	38	73.9	395	8.29
		70	47	23	3	89.4	379	8.72
		71	50	21	30	99.9	408	8.56
		69	49	20	29	88.1	434	8.67
		62	36	26	42	83.2	436	11.34
Day 21	*bmLp-PLA$_2^{-/-}$*	66	39	27	41	94	383	10.34
		71	47	24	34	91.3	376	9.02
		56	34	22	39	94.3	395	8.68
		45	21	24	53	103.1	411	9.87
		41	19	22	54	96.8	436	9.59
		89	66	23	38	124	413	9.49
	WT	66	41	25	38	90.4	393	9.82
		50	28	22	44	82.1	415	9.13
		70	47	23	33	97.5	409	9.41
		104	77	27	26	115.7	413	11.16
		101	80	21	21	101.7	444	9.32
		82	57	25	30	120.9	441	11.02
		68	48	20	29	115.8	433	8.65