SUPPLEMENT MATERIAL

ACE inhibition prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction

Leuschner: ACE inhibition impacts monocyte traffic post MI

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Detailed Methods

Animal models

Female C57BL/6J and apoE$^{-/-}$ mice were purchased from Jackson Labs. Cx3cr1$^{gfp/+}$ mice were obtained by breeding Cx3cr1$^{gfp/gfp}$ mice with C57BL/6J mice. Cx3cr1$^{gfp/+}$ mice have one Cx3cr1 allele replaced with cDNA encoding eGFP, and can be used to track monocytes [1]. ApoE$^{-/-}$ mice had an average age of 45 weeks and were on a high-cholesterol diet (Harlan Teklad, 0.2% total cholesterol), C57BL/6J and Cx3cr1$^{gfp/+}$ mice were 8-12 weeks old. The institutional animal welfare committee approved the research reported.

MI was induced by permanent coronary ligation as described before [2]. Briefly, mice were anesthetized with isoflurane (2% / 2L O2), and intubated and ventilated with an Inspira Advanced Safety Single Animal Pressure/Volume Controlled Ventilator (Harvard Apparatus, Holliston, MA). The chest wall was shaved and left thoracotomy was performed in the 4th left intercostal space. The left ventricle was visualized and the left coronary artery was permanently ligated with monofilament nylon 8-0 sutures (Ethicon, Somerville, NJ) at the site of its emergence from under the left atrium. The chest wall was closed with 7-0 nylon sutures and the skin was sealed with superglue. Splenectomy was done as described previously [3]. Mice were treated with a dose of 100 mg/kg [4, 5] Enalapril daily for the acute studies (sacrifice on day 1 after MI) or 20 mg/kg Enalapril daily [6, 7] for the chronic studies. Treatment was started two days before MI and continued for seven days after coronary ligation. In additional cohorts, we initiated Enalapril treatment one hour or twenty-four hours after coronary ligation. Hydralazine was given at a dose of 15 mg/kg [8] daily. Treatment was done by gastric gavage.

Histology

Histology of spleens was assessed for the following groups (n = 5-9 mice per group): wild type C57BL/6 mice and wild type mice 1 day after MI with or without Enalapril treatment. Histology of hearts was assessed for wild type mice 1 day after MI with or without Enalapril treatment. Furthermore, healing was assessed in apoE$^{-/-}$ mice with or without Enalapril treatment on day 4, 7 and 10 after MI. Spleens and hearts were excised, rinsed in PBS and embedded in OCT (Sakura Finetek). Fresh-frozen serial 6 µm thick sections were stained for monocytes and neutrophils (CD11b, BD Pharmingen, NIMP-R14, Santa Cruz Biotechnology), macrophages (MAC-3, BD Pharmingen), neovessels (CD31, BD Pharmingen) and collagen deposition (masson trichrome, Sigma Aldrich). The avidin-biotin peroxidase method was used for immunohistochemistry. The reaction was visualized with a 3-amino-9-athyl-carbazol substrate (AEC, DAKO California). For Immunofluorescence staining sections were incubated with anti-CD11b followed by biotinylated secondary antibody, and texas red-conjugated streptavidin (GE Healthcare). DAPI (Vector Laboratories) was used to identify cell nuclei. Microscopy was done on a Nikon 80i upright fluorescence scope and a Nikon 40 light microscope, both equipped with CCD cameras connected to a Macintosh workstation. Percentage positive area or cell numbers

were quantified using IPLab (version 3.9.3; Scanalytics, Inc., Fairfax, VA) analyzing 5 high power fields per section and per animal at magnification 200 or 400.

Flow Cytometry

Mice were sacrificed on days 1, 2, 3 and 5 after MI and 1 day after ischemia reperfusion injury for flow cytometry (n = 3 – 5 mice per group and time point). Spleens were removed, triturated in HBSS (Mediatech, Inc.) at 4° C with the end of a 3-ml syringe, and filtered through nylon mesh (BD Biosciences). The cell suspension was centrifuged at 300 g for 10 min at 4° C. Red blood cells were lysed with ACK lysis buffer, and the splenocytes were washed with HBSS and resuspended in HBSS supplemented with 0.2% (wt/vol) BSA and 1% (wt/vol) FCS. Heart tissue was harvested, minced with fine scissors, and placed into a cocktail of collagenase I, collagenase XI, DNase I, and hyaluronidase (Sigma-Aldrich) and shaken at 37° C for 1 h. Cells were then triturated through nylon mesh and centrifuged (15 min, 500 g , 4° C). Total spleen and cardiac cell numbers were determined with Trypan blue (Mediatech, Inc.). Peripheral blood was drawn via cardiac puncture with citrate solution (100 mM Na-citrate, 130 mM glucose, pH 6.5), as anti-coagulant and mononuclear cells were purified by density centrifugation. Total blood leukocyte numbers were determined using acetic acid lysis solution (3% HEMA 3 Solution II, 94% ddH 2 O, and 3% glacial acetic acid).

Cell suspensions were incubated with a cocktail of mAbs against T cells (CD90-PE, 53-2.1), B cells (B220-PE, RA3-6B2), NK cells (CD49b-PE, DX5 and NK1.1-PE, PK136), granulocytes (Ly-6G-PE, 1A8), myeloid cells (CD11b-APC, M1/70), antigen-presenting cells (I-A^b (AF6-120.1)-biotin-Strep-PerCP) and monocyte subsets (Ly-6C-FITC, AL-21) (BD Biosciences). Monocytes were identified as $CD11b^{hi}$ $(CD90/B220/CD49b/NK1.1/Ly\text{-}6G)^{lo}$ $(F4/80/I\text{-}A^b/CD11c)^{lo}$ $Ly\text{-}6C^{hi/lo}$. Macrophages/dendritic cells were identified as $CD11b^{hi}$ $(CD90/B220/CD49b/NK1.1/Ly\text{-}6G)^{lo}$ $(F4/80/I\text{-}A^b/CD11c)^{hi}$ $Ly\text{-}6C^{lo}$. Neutrophils were identified as $CD11b^{hi}$ $(CD90/B220/CD49b/NK1.1/Ly\text{-}6G)^{hi}$ $(F4/80/I\text{-}A^b/CD11c)^{lo}$ $Ly\text{-}6C^{int}$. Reported cell numbers were calculated as the product of total living cells and percent cells within the monocyte/macrophage gate. Within this population, monocyte subsets were identified as $(F4/80/I\text{-}A^b/CD11c)^{lo}$ and either $Ly\text{-}6C^{hi}$ or $Ly\text{-}6C^{lo}$. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo v.8.5.2 (Tree Star, Inc.).

For analysis by PCR and Western blot, cells were sorted on a BD FACSAria (BD Biosciences). Splenic monocytes were enriched by lineage depletion using MACS LD columns (Miltenyi) and PE–conjugated antibodies against B220, CD49b, NK1.1, Ly-6G, CD90 and Ter-119 followed by anti-PE magnetic beads (Miltenyi). Lineage-depleted cells were further stained with specific antibodies as described above to allow for identification of monocytes.

ELISA for serum Ang-II levels

Blood was drawn from mice under anesthesia by cardiac puncture with a syringe pre-loaded with 80 µl of 100 mM EDTA anticoagulant. The blood was transferred to an eppendorf tube containing 50 µl protease inhibitor cocktail VI (Research Product Inc.), supplemented with 50mM p-hydroxymercuribenzoid acid (Sigma), centrifuged, and supernatant loaded onto Amprep Phenyl PH mini-columns (GE Biosciences) to isolate peptides from the sera. Methanol-eluted peptides were dried by vacuum centrifugation. Ang-II concentration was determined with an Ang-II ELISA (Cayman Chemical), and normalized to the volume of blood samples.

Intravital microscopy

During isoflurane anesthesia, the peritoneal cavity was opened with a transverse incision in the disinfected abdominal wall. The gastric-splenic ligament was dissected and the spleen carefully exteriorized. Robust blood flow was observed in the splenic artery during the duration of each experiment and splenic perfusion was confirmed by inspection through fluorescence microscopy upon tail vein injection of an intravascular imaging agent (AngioSense-680, Visen Medical). The exteriorized spleen was completely submerged in temperature-controlled saline solution.

Temperature was carefully monitored using an Omega HH12A thermometer with fine wire thermocouples (Omega Engineering Inc., Stamford, CT) and kept at 37ºC.

Images were collected with a prototypical intravital laser scanning microscope (IV100, Olympus Corporation, Tokyo, Japan) [3, 9] using an Olympus 20x UplanFL (NA. 0.5) objective and the Olympus FluoView FV300 version 4.3 program. Samples were excited at 488 nm with an air-cooled argon laser (Melles Griot, Carlsbad, CA) for visualization of GFP+ cells, and at 748 nm with a red diode laser (Model FV10-LD748, Olympus Corporation, Tokyo, Japan) for visualization of the blood pool agent AngioSense-680. Light was collected using custom-built dichroic mirrors SDM-570 and SDM-750, and emission filters BA 505-550 and BA 770 nm IF (Olympus Corporation, Tokyo, Japan). Both channels were collected simultaneously. A prototypical tissue stabilizer (Olympus Corporation, Tokyo, Japan) was used to reduce motion and stabilize the focal plane. The stabilizer was attached to the objective and its z-position was adjusted using a micrometer screw to apply soft pressure on the tissue. Time-lapse recordings were made by collecting images of 256x256 resolution at 15 s intervals over 1 hour in a single plane (2D) of focus. Mice were analyzed in the steady-state or 24 h after MI either treated with Enalapril or untreated.

All GFP+ cells were identified manually in each recording. To determine the displacement over time of individual cells, the centroid position (x-y dimension) of these cells was recorded at the first and last time-point when they could be identified during a recording; then the distance between these two points was calculated, and divided by the elapsed time. Single cell tracks for GFP+ cells were generated based on the position of cell centroids from a series of images recorded at 15 s intervals, and ImageJ and the Manual Tracking plugin (http://rsbweb.nih.gov/ij/plugins/track/track.html) were used for display and quantification. Motion-artifacts in recordings were corrected using the auto-alignment plugin (stackreg) of ImageJ (http://rsb.info.nih.gov/ij/).

In vitro migration

Migration experiments using Ang-II as a chemoattractant were performed in BD BioCoat invasion chambers (BD Bioscience). Sorted monocytes from spleen were suspended in RPMI 1640 media (Cellgro, Mediatech, Inc, VA) supplemented with 0.2% FCS (Valley Biomedical, Inc.); $2x10^5$ cells were placed on the matrigel-coated 8 micron pore size PET membrane and incubated in a humidified incubator at 37°C, 5% CO_2 for 1h, allowing the cells to attach to the matrigel. For the receptor blocking condition, cells were pretreated with the AT_1R antagonist losartan (100 μM) (Merck) for 30 min at 37 C, 5% CO2. Migration was induced by addition of Ang-II (1 μM) (Bachem, Torrance, CA) to the lower compartment. After 2h, non-migrating cells were removed with a cotton tip and the membranes were fixed and stained with Protocol HEMA 3 staining set (Fisher Scientific Company L.L.C.) to identify cells that had migrated to the lower surface of the membrane. The number of migrated cells was determined per x 200 high-power field. Cells that had migrated to the lower chamber were counted using Trypan Blue solution (Cellgro, Mediatech, Inc, VA). Experiments were performed in triplicate.

Western for Ang-II Type 1 (AT_1R) receptor on splenic monocytes

Monocytes were isolated by FACS as described above. Sample pellets of ~200,000 monocytes were resuspended in Laemmli buffer (BioRad), and sonicated to lyse cells and shear genomic DNA. Samples were developed by electrophoresis on a 4-15% polyacrilamide gel (BioRad). The proteins were transferred to a polyvinylidene difluoride membrane (Fischer) by semidry transfer. Membranes were blocked with carnation milk and PBS supplemented with 0.05% Tween 20 overnight. Membranes were washed, stained initially with anti-AT_1R receptor antibody (Abcam), stripped with Restore buffer (Pierce), and stained with anti-glyceraldehyde-3-phosphate (GAPDH) (Rockland Immunochemicals for Research). Blots were developed with Western Lightning Chemiluminescence reagent (PerkinElmer Instruments) and molecular weights were compared to bands for Precision Plus Protein Western C standards (BioRad).

Quantitative PCR
Total mRNA from heart tissue and isolated cells was isolated by Qiagen RNeasy Mini kit and RNeasy Micro kit, respectively. Oligo(dT)-based cDNA was generated by use of the SuperScript® III First-Strand Synthesis kit (Invitrogen), which made cDNA only from the mRNA portion of the total RNA pool. Multiplex quantitative PCR was performed on triplicate samples using Applied Biosystems TaqMan® Assays. Infarct tissue was examined for expression of markers of inflammatory monocytes (Ly-6C), differentiated macrophages (CD68; Mac3), TNF-α, TGF-β, myeloperoxidase, and appropriate controls (GAPDH). Gene expression was determined as x-fold difference after normalizing to GAPDH loading control [10].

FMT-CT
On day 1 after MI we performed FMT-CT imaging [11-13] to interrogate the magnitude of inflammation 24 hours after injection of 5 nmol of a pan-cathepsin protease sensor (Prosense-680, PerkinElmer) in apoE$^{-/-}$ mice with and without Enalapril treatment and apoE$^{-/-}$ mice that were splenectomized at the time of coronary ligation (n = 8-12 per group). A 3D dataset was reconstructed in which fluorescence per voxel was expressed in nM. To robustly identify the region of interest in the heart, anatomic imaging with CT immediately followed free-space FMT at 680/700nm excitation/emission (FMT-2500, Visen Medical). The imaging cartridge containing the anesthetized mouse was placed into the custom machined Plexiglas holder that supplied isoflurane, warm air and optimal positioning in the CT (Inveon PET-CT, Siemens). The CT x-ray source operated at 80 kVp and 500 μA with an exposure time of 370 - 400 ms. The effective 3D resolution was 80 μm isotropic. Isovue-370 was infused intravenously at 55 μL/min through a tail vein catheter. The CT reconstruction protocol performed bilinear interpolation, used a Shepp-Logan filter, and scaled pixels to Hounsfield units. Infarct size was evaluated by iodine-enhanced CT as described before [14]. At midventricular level, the enhancing scar and the non-enhancing remote myocardium were tracked manually using OsiriX software (The OsiriX foundation, Geneva, Switzerland). Infarct size was then calculated as percent enhanced area.

Image fusion
The fusion method realized three-dimensional mapping of fluorescence within the anatomical reference CT. The approach was based on a multimodality-compatible animal holding device that provides fiducial landmarks on its frame. The imaging cartridge lightly compressed the anesthetized mouse between optically translucent windows and thereby prevented motion during transfer between modalities. The three-dimensional distribution of the fiducials enabled co-registration of datasets in an automated fashion. The point based coregistration tool kit in OsiriX shareware (64 bit, version 3.5.1) fused images after identification of fiducials in respective modalities. Fiducials were tagged with point markers to define their XYZ coordinates. Using these coordinates, data were resampled, rotated and translated to match the image matrices, and finally fused. Fusion was done on a Macintosh computer with a quad-core processor, 16GB RAM and an NVIDIA GeForce graphic card.

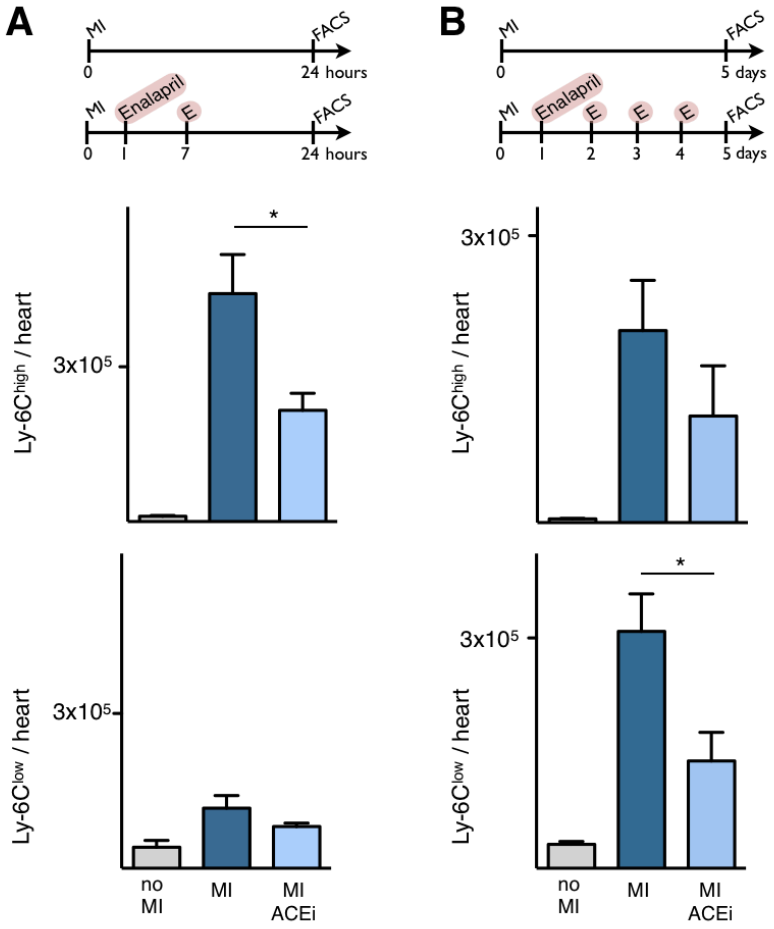
MRI
We performed in vivo MRI on day 21 after MI in apoE$^{-/-}$ mice with and without Enalapril treatment and in apoE$^{-/-}$ mice that were splenectomized at the time of coronary ligation (n = 8-12 per group). A 7 Tesla horizontal bore Pharmascan (Bruker) and a custom-made mouse cardiac coil in birdcage design (Rapid Biomedical, Wuerzburg, Germany) was used to obtain cine images of the left ventricular short axis. We employed ECG and respiratory gating using a gradient echo sequence (echo time 2.7 ms, 16 frames per RR interval; flip angle 30 degrees; in-plane resolution 200x200 μm; slice thickness 1 mm). Cardiac volumes were quantitated from 6-8 short axis imaging slices covering the left ventricle as described previously [2].

Ischemia reperfusion injury
Ischemia reperfusion injury (IRI) was studied in wild type mice, apoE$^{-/-}$ with and without Enalapril treatment, and in apoE$^{-/-}$ that were splenectomized immediately prior to ischemia. Five minutes after ligation of the coronary artery, 360,000 fluorescent microspheres (10 µm size, excitation/ emission wavelength 580/605 nm; Fluospheres, Invitrogen) were slowly injected in a volume of 100 µl into the left ventricle, and distributed to myocardial areas with intact perfusion. Thirty-five minutes after ligation, the suture was released. Twenty-four hours after IRI, 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich) staining was conducted for evaluation of myocardial infarction and fluorescence reflectance imaging (FRI) to quantify the ischemic area at risk. The heart was harvested, fixed and cut into 1 mm thick myocardial rings. The myocardial rings were scanned using a flat bed scanner (Hewlett Packard). Thereafter, FRI was performed using an epifluorescence microscope (OV110, Olympus). Images were analyzed for TTC-negative infarction area and the ischemic area at risk defined as fluorescence-negative area using Osirix software. Percent infarction over area at risk was calculated as TTC-negative area / FRI-negative area * 100%.

Blood pressure measurements
Blood pressure was monitored in various treatment groups (n = 5-8 per group) with the non-invasive tail cuff CODA System (Kent Scientific) as described previously [15]. In mice after coronary ligation, the procedure was done on day 10 after MI.

Statistics
Results are expressed as mean ± SEM. Statistical comparisons between two groups were evaluated by Student's t-test and corrected by ANOVA for multiple comparisons. A value of $p < 0.05$ was considered to indicate statistical significance.

Online Figure I



Online Figure I: Start of Enalapril one and twenty-four hours after MI
Top: Flow chart illustrating the set up of the study.
A. Mice were started on Enalapril (E) one hour after coronary ligation, and monocyte numbers were analyzed by flow cytometry 24 hours later.
B. Treatment started 24 hours after coronary ligation and was continued for five days.
Mean ± SEM; * $p < 0.05$.

Online Table I

Table 1 - Systolic and diastolic blood pressure

	wild type		apoE$^{-/-}$	
	systolic	diastolic	systolic	diastolic
control	120±2 *†‡	91±2 *†‡	121±2 *‡	87±2 *‡
Enalapril	108±3 †	81±3 †	104±2	67±2
Hydralazine	99±2	65±3	N/A	N/A
MI	113±8 ‡	91±7	118±8	90±7 ‡
MI, Enalapril	93±5	76±4	95±1	65±2

* $p < 0.05$ versus Enalapril, † $p < 0.05$ vs. Hydralazine, ‡ $p < 0.05$ vs. MI, Enalapril. Group sizes were n = 5-8. Data are reported as mean ± standard error of mean.

Supplemental References

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Legends for the Video file:

Intravital microscopy of monocytic migratory activity in the subcapsular spleen red pulp 1 day after MI

a. The movie shows GFP+ cells (green) in the subcapsular red pulp of a live Cx3cr1$^{gfp/+}$ mouse on day 1 after MI. Tracks indicate the path of the centroid of each GFP+ cell at 15 second intervals. Larger steady green cells are dendritic cells / macrophages.
b. GFP+ monocytes in the subcapsular red pulp of the spleen from a Cx3cr1$^{gfp/+}$ mouse on day 1 after MI treated with Enalapril. Time is shown in minutes and seconds. Scale bar = 100 μm.
c. and d. The animation shows isolated tracks of monocytes computed from panels a and b.