Plasma Levels of Endothelial Microparticles Bearing Monomeric C-reactive Protein Are Increased in Peripheral Artery Disease

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Methods

Plasma Collection, MP and CRP Depletion of Plasma, and MP Isolation. Sodium-citrated blood was collected with written informed consent from asymptomatic volunteers or PAD patients following the principles outlined in the Declaration of Helsinki and specific approval from the Institutional Review Board of Baylor College of Medicine (H-20001 and H-20222; Houston, TX). Blood was collected into sodium citrate vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA). Tubes were centrifuged at 1570 x g at 4° C for 20 minutes, the top 90% of plasma was collected, and respun at 1570 x g at 4° C for 20 minutes. Plasma was aliquoted and stored at -80° C, depleted of MPs through high speed centrifugation or depleted of CRP with phosphocholine-coupled beads, or microparticles were further purified from the plasma by centrifugation at 20,000 x g at 4° C for 60 minutes. The supernatant was kept as MP-depleted plasma while the MPs were resuspended in HEPES saline buffer (20 mM HEPES, 140 mM NaCl, 0.1% human serum albumin (recombinant, Sigma-Aldrich, St. Louis, MO; 0.1 micron filtered). The MP-depleted plasma was further depleted of pCRP through overnight incubation with phosphocholine beads (Thermo Scientific, Grand Island, NY) in the presence of 2 mM calcium chloride and 1:100 heparin (Sigma-Aldrich) to prevent clotting. The CRP-depleted plasma gave an average measurement of <10 ± 10 ng/ml, which was below the detection level of our ELISA using antibodies specific for CRP isomers (10-40 ng/ml detection limit) and which is below the detection level of the Sekisui chemistry analyzer assay (50 ng/ml) and also below the detection level of the R&D Systems ELISA (0.78 ng/ml).

Volunteers were eligible for this study as the control population if they were asymptomatic with no prior documented cardiovascular disease. Plasma samples were also collected following the same protocol from patients with lower extremity arterial disease, also known as PAD. Subjects with PAD were recruited as a part of an ongoing study since 2006 to study biomarkers and genetics in subjects with PAD. Subjects were eligible for this study if they were between ages 18-90 and had some documented evidence of PAD including an ankle brachial index <0.9, by ultrasound, computed tomography, magnetic resonance imaging or angiogram. Subjects with active malignancies or conditions known to affect biomarkers of inflammation were excluded from the study. Aliquots from PAD patients were collected since 2006, while controls were collected since 2013.

Preparation of mCRP. Pentameric CRP (pCRP) (1707-CR-200/CF, R&D Systems, Minneapolis, MN) was incubated with 8 M urea in 10 mM EDTA for 2 hours at 37 °C. Samples were dialyzed into 20 mM phosphate buffer pH 7.4 using Slide-A-Lyzer mini dialysis devices with 3.5 MWCO (Thermo Scientific) for 2 hours at room temperature (RT) x 2, overnight at 4° C, and the 2 hour

incubations were repeated the next day 3x. Samples were passed through a 0.2 µm filter, protein concentration determined by BCA, and 0.1% human serum albumin was added for stability to protect the mCRP from the hydrophilic environment. The urea chelated mCRP was prepared fresh for each experiment and only stored briefly at 4° C.

ELISA. 96 well plates (Greiner Bio-One, Monroe, NC) were coated overnight at 4° C at 1:1000 dilution with sheep anti-CRP (AF1707, R&D Systems) for detecting mCRP or 10 µg/ml phosphocholine conjugated to Keyhole Limpet Hemocyanin (PC-KLH) (PC-1013, Biosearch Technologies, Petaluma, CA) for detecting pCRP. The KLH-PC capture is necessary for the anti-pentamer CRP antibody to maintain specificity for this isoform. Plates were washed 3 times with PBS/0.05% Tween-20. Plates were blocked in PBS-4% BSA for 1 hour at RT. 50 or 100 µl plasma or purified mCRP/pCRP in PBS with calcium was incubated overnight at 4° C, followed by washing. Mouse monoclonal anti-CRP antibodies, specific for monomer or pentamer, were added to the wells at a 1:100 dilution for 1 hour at RT. After washing, 1:10,000 dilution of HRP-conjugated goat $F(ab')_2$ anti-mouse IgG (Thermo Scientific) was added to the wells for 45 minutes at RT. After washing, TMB peroxidase substrate (Thermo Scientific) was added to the wells for 2-15 minutes at RT before stopping the reaction with 1 N H_2SO_4, and the plate was read at 450 nm on a SpectraMax 250 or VersaMax (Molecular Devices, Sunnyvale, CA). The isoform specificities of the CRP antibodies were verified using serial 1:5 dilutions of pCRP (R&D Systems) and urea-chelated mCRP starting at 1 µg/ml (Fig. S2 in the supplementary material).

hsCRP Assay. Quantitative measurements of CRP were performed using an AU480 Chemistry Analyzer (Beckman Coulter, Brea CA) with CRP Ultra Wide Range Reagent (082, Sekisui Diagnostics, Lexington, MA) after calibration with CRP Ultra Wide Range Calibrator (Sekisui Diagnostics) every 28 days as per the manufacturer's protocol. The analyzer's reportable range was 0.05 to 20 mg/L; samples exceeding 20 mg/L were diluted with 0.9% NaCl and the results were multiplied by the dilution factor to obtain the final concentration. Before measurement of samples, three concentrations of Liquid QC hsCRP Serum Protein controls (CLINIQA Corp, San Marcos, CA) were run at least twice to confirm that the instrument readings were within established ranges of the instrument and the CRP Ultra Wide Range Reagent.

CRP testing was conducted in 13 mm polystyrene test tubes. The plasma and purified MP sample volumes used for CRP measurement were a minimum of 60 µL up to a maximum of 1 mL. The calibrator and quality control volumes were 80-100 µL. For MP- and pCRP-depleted plasma, either 2 µg/ml pCRP (Sigma Aldrich) or urea-chelated mCRP was added for measurement. The MPs purified from plasma were incubated with pCRP (R&D Systems) or urea-chelated mCRP for 1 hour and then washed to remove unbound CRP isomers via centrifugation at 20,000 x g for 30 minutes. The presence of MPs (lactadherin$^+$, 0.5-1 µm particles) and CRP isoforms were verified using a Cell Lab Quanta SC flow cytometer (Beckman Coulter) using Quanta Analysis software (Fig. S3 in the supplementary material). hsCRP was measured for purified MPs and MPs enriched with CRP isoforms.

To measure CRP by a different method, the human C-Reactive Protein Quantikine ELISA kit (DCRP00) obtained from R&D Systems (Minneapolis, MN) was used according to the manufacturer's instructions. All samples were run in duplicate. Low and high controls from R&D Systems (QC70) with specified target ranges for CRP were also included in the assay. After color development, the plate was read with a Bio-Tek Synergy HT microplate reader at 450 nm with dual wavelength correction at 570 nm. A standard curve was generated using a four parameter

logistic curve fit. The data was linearized with a log/log plot. The included high and low controls were within the manufacturer's target range.

Western Blots. MPs were purified from 50 ml of blood as detailed above. Samples were mixed with native sample buffer with 0.1% SDS, resolved on 4-20% Tris-HCl gels (Bio-Rad, Hercules, CA), transferred to nitrocellulose membranes (Bio-Rad), probed with 1:1000 mouse anti-CRP antibody (C1688, clone SAP-8, Sigma Aldrich), and detected using 1:5000 anti-mouse IRDye 800 (LI-COR, Lincoln, NE) as per the manufacturer's protocols, visualized on an Odyssey CL (LI-COR).

Human cardiac microvascular endothelial cells (HCMEC) (Lonza, Walkersville, MD), passages 6-9, were maintained in complete EBM-2 media (Lonza). To induce MP production, the cells were stimulated overnight in the presence of 10 ng/ml TNF-α (R&D Systems). Cells were removed by centrifugation at 300 x g for 5 minutes, MPs were collected through centrifugation at 20,000 x g for 60 minutes at 4° C, and the MP pellet was resuspended in 60 µl HEPES buffered saline with calcium (20 mM HEPES, 140 mM NaCl, 10 mM $CaCl_2$, 0.5% HSA). pCRP was incubated with the HCMEC-derived MPs for up to 3 hours at 37 °C, and the CRP-containing supernatants were collected at 3 time points through centrifugation at 20,000 x g for 20 minutes at 4° C. The supernatants were run in a native gel and visualized as above.

Flow Cytometry. Flow cytometry has been reported to measure differences in MPs for cardiovascular diseases [1-3], but there are significant limitations with conventional flow cytometers measuring such small particles [4,5]. Detection of MPs through imaging flow cytometry has the advantage of measuring MPs below 0.4-0.5 µm, the limit in a conventional flow cytometer. The precise fluid dynamics of the image cytometer records each MP individually, while conventional flow cytometers have their fluidics designed to pass much larger cells which can lead to multiple MPs being read as a single event. The imaging cytometer takes a picture of every event in addition to representing the events in histograms, allowing judgements to be made concerning the gating strategy for multiple markers, which helps reduce false positives.

For cytometry, plasma was thawed at room temperature and spun at 1500 x g for 5 minutes to remove debris. 100 ng of antibodies for specific cell markers conjugated to APC or VioletFluor 421 were added to 20 µl of plasma. Samples were briefly vortexed and incubated for 30 minutes at RT in the dark. The plasma was mixed with 20 µl HEPES saline buffer + 0.05% HSA containing 3 ng lactadherin-PE, 75 ng anti-monomer (8C10) or 10 ng anti-pentamer (1D6) antibody conjugated to Dylight 488. After a 30 minute incubation, samples were analyzed on the ImagestreamX MkII (Amnis, Seattle, WA) using a X60 magnification with low flow rate/high sensitivity and having "Hide beads" in the INSPIRE acquisition software unchecked [4]. Events were gated against side scatter to discriminate against the calibration speed beads. Channels 1 and 9 were collected as brightfield images, channel 12 as side scatter, and fluorescence channels at the following laser power: channel 2 (488 nm) at 150 mW, channel 3 (561 nm) at 50 mW, channel 7 (405 nm) at 120 mW, and channel 11 (658 nm) at 150 mW. Microparticles were measured as low side scatter, lactadherin$^+$ particles in the size range of 0.1-1 µm as gated using a Flow Cytometry Sub-micron Particle Size Reference Kit (F13839, Thermo Scientific). The gating strategy is visualized in Fig. S1 in the supplementary material. The isotype negative controls had no or negligible signal. The cytometer was able to give absolute cell counts by using well-defined volumes and imaging every object in the sample without the need for calibration beads.

The cell markers used in flow cytometry were CD3 (20-0037, clone OKT3, Tonbo Biosciences, San Diego, CA), CD14 (75-0149, clone 61D3, Tonbo Biosciences), CD19 (555415,

clone HIB19, BD Biosciences), CD31 (303124, clone WM59, Biolegend), CD41a (303710, clone HIP8, Biolegend), CD45 (20-0459, clone HI30, Tonbo Biosciences), CD66b (17-0666-42, clone G10F5, Ebioscience), CD144 (A18384, clone 16B1, Thermo Scientific), and CD235ab (306608, clone HIR2, Biolegend).

Before the antibodies were added to plasma, the antibodies were initially spun for 5 minutes at 10,000 x g to remove aggregates. However, this resulted in an overestimation of lactadherin^{+} particles so the plasma MPs were measured only after the antibodies were spun at 18,000 x g for 20 minutes to pellet any aggregates or potential MP contaminates for any antibodies prepared from cell culture/ascites fluid.

False positives for antibody positive events were eliminated using visual inspection of the MP images and setting the appropriate gates using the antibody signal intensity for the x axis and the raw max pixel values for the y axis in the IDEAS software (Amnis). False positives were identified as particles having a signal intensity > 0.7-1 x 10^3 but not having any staining in the visual inspection of the images. These false positives tended to have a low raw max pixel value that allowed for gating to remove the false positives (Fig. S1d).

Lipid Profile. Lipid profile analysis was performed using the Beckman Coulter AU480 automated chemistry analyzer. Lipid panel testing was conducted using the total cholesterol (OSR6116), high-density lipoprotein cholesterol (OSR6195), low-density lipoprotein cholesterol (OSR6196), and triglyceride (OSR60118) reagents (Beckman Coulter). The cholesterol and triglyceride tests were calibrated with the Lyophilized Chemistry Calibrator Level 2 (DR0070-2). The HDL and LDL tests were calibrated with the HDL-cholesterol (ODC0023) and LDL-cholesterol (ODC0024) calibrator, respectively (Beckman Coulter). All calibrators, controls and reagents were stored and used according to the manufacturer's instructions.

Transendothelial Migration (TEM). Blood was collected from healthy volunteers, and mononuclear leukocytes (MNLs) were then fractioned from the blood by Ficoll-Paque (Sigma Aldrich) density centrifugation. Human cardiac microvascular endothelial cells (HCMEC) (Lonza, Walkersville, MD), passages 3-8, were seeded onto inserts with 8 µm pores (Corning, Tewksbury, MA) in RPMI1640 with 10% FBS. MNLs were applied to the top insert and allowed to migrate through the HCMECs in response to the chemoattractant MCP-1 (R&D Systems) in the well below at 128 ng/ml. In addition to MNLs, stimulating agents pCRP (R&D Systems) or mCRP (urea-chelated, detailed above) at 5 µg/ml were added above the insert. MNLs were allowed to migrate 4 days and the number of adherent spindle-shaped cells was counted in the bottom of the wells. Cells that transmigrated in the TEM assay onto poly D-lysine coated coverslips (NeuVitro, Vancouver, WA) were stained and imaged as described previously [6] using anti-CD86 (clone IT2.2, Biolegend), CD206 (clone EPR6828(B), Abcam), or appropriate isotype controls. Controls were measured in at least triplicate and the number of cells counted was normalized to untreated controls (no stimulating agent). For endothelial MP studies, MNLs were not added to every insert so that in inserts lacking MNLs, only endothelial MPs would be generated. Additionally, the supernatants containing the MPs were collected after 18 hours instead of 4 days.

Protein Arrays. After 96 hours of TEM, the migrated cells were expanded in medium plus 5% FBS (Hyclone) supplemented with PMA (Sigma) and calcium ionomycin (Sigma) for 3 days, and on the final day before extraction Brefeldin A (Sigma) was added for 6 hours. The cells were lysed and protein was extracted using lysis buffer supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific). Protein was loaded and analyzed on a standard human cytokine array

membrane (ARY005, R&D Systems) as per the manufacturer's protocol. Images on film were scanned, and densitometry was measured by ImageJ software. Data are expressed as integrated mean pixel density normalized to the positive plate controls and with the background subtracted.

Statistics. Results are presented as mean ± SE. Since data were not always normally distributed, the nonparametric Mann-Whitney U-test was used for comparison of two data sets, whereas the Kruskal-Wallis test with Dunn's multiple comparison posttest was used for more than two. A p value <0.05 was considered statistically significant.

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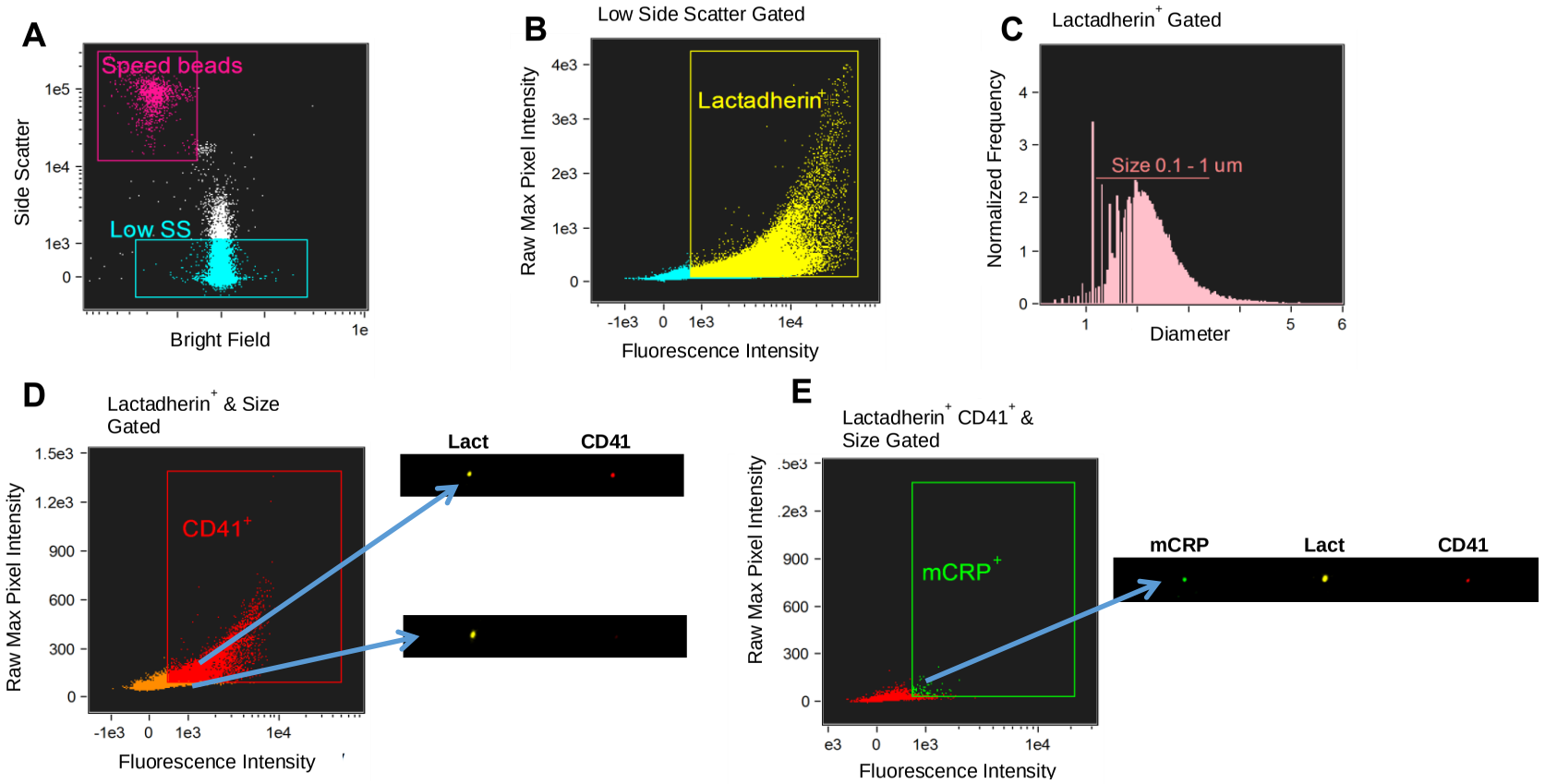
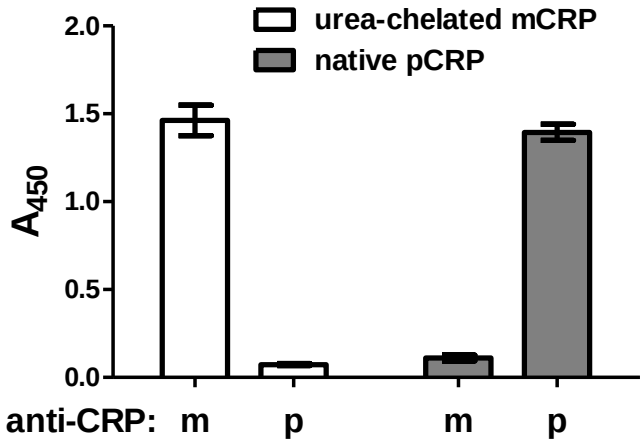
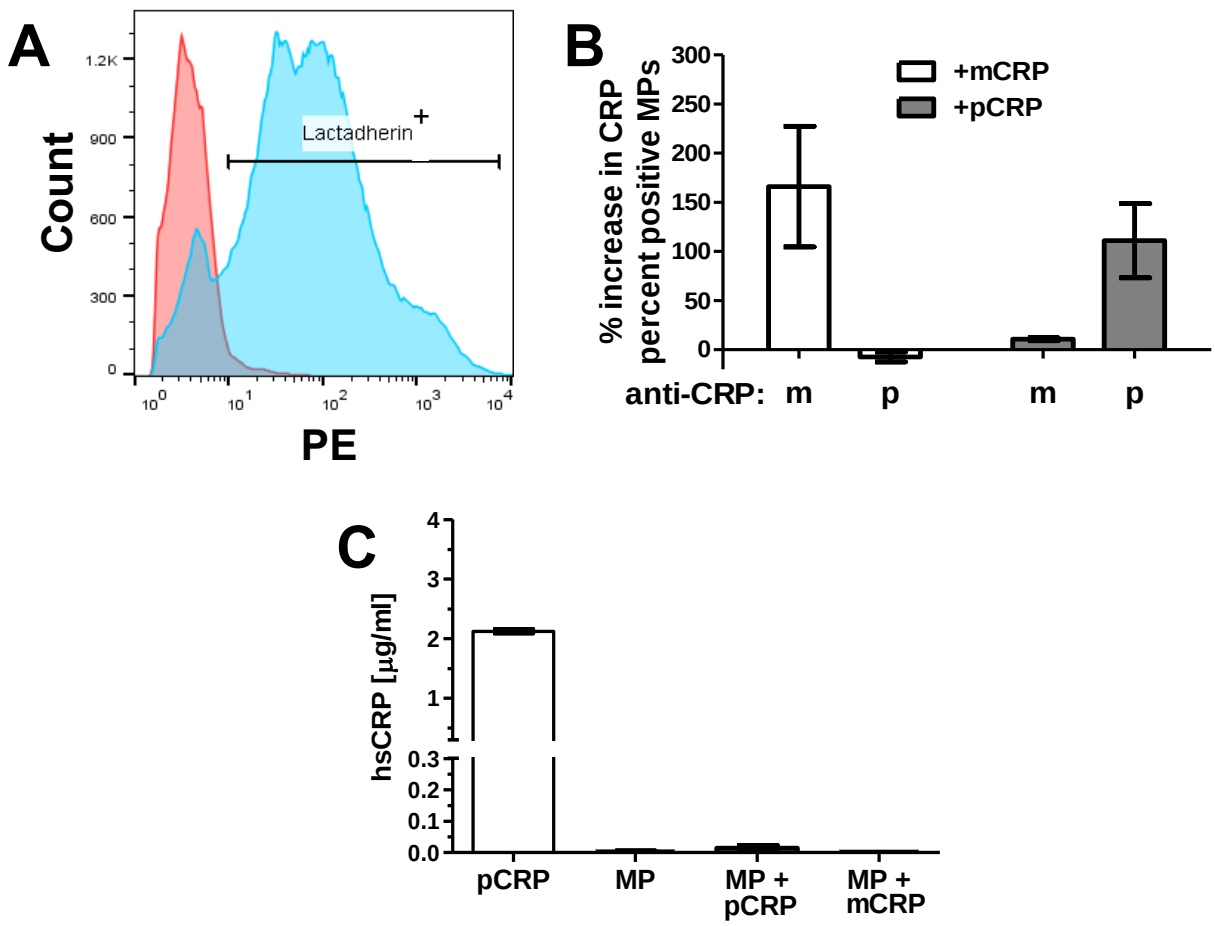


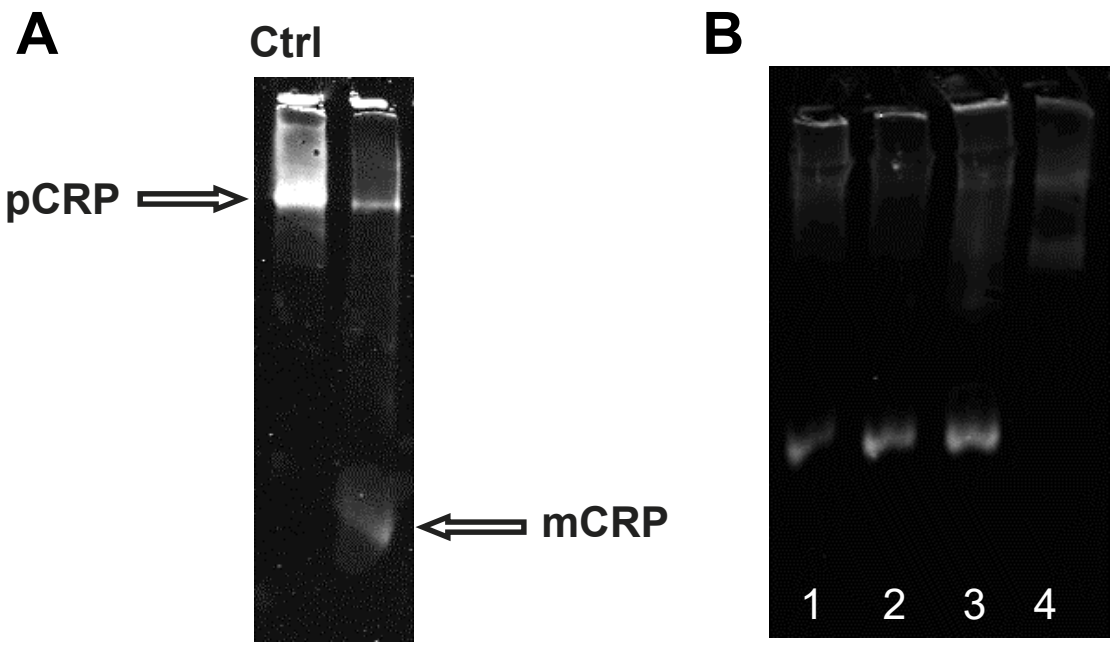
Supplementary Fig. S1 Gating strategy and elimination of false positives. For image-based cytometry, the data were initially gated for **(a)** low side scatter according to prior published data. MPs were **(b)** lactadherin$^+$ particles in the **(c)** 0.1-1 µm range with the range limits set by comparison to a sub-micron bead set (Thermo Scientific). The MPs were further identified with antibodies against **(d)** cell markers and **(e)** CRP. To eliminate false positives, the gates were set on scatterplots graphing fluorescence intensity against raw max pixel intensity, which **(d)** allowed for visual inspection of individual MPs to best set the gating limits



Supplementary Fig. S2 Specificity of CRP antibodies towards monomer and pentamer isoforms of CRP determined by ELISA. The anti-CRP: m and p stand for anti-mCRP antibody and anti-pCRP antibody, respectively. (n=3)



Supplementary Fig. S3 Purification of MPs and enrichment with pCRP or mCRP. MPs were isolated from plasma, loaded with pCRP or mCRP, and washed. **a** Compared to buffer only controls (red), the purified MPs (blue) were identified as lactadherin^{+} particles in the 0.5 – 1 µm size range (PE = phycoerythrin fluorescence intensity). **b** When purified plasma MPs were loaded with additional mCRP or pCRP, the number of CRP^{+} MPs measured by cytometry increased for the isomer in question but not the alternate isomer. The anti-CRP: m and p stand for anti-mCRP antibody and anti-pCRP antibody, respectively. (n=3) **c** When the mCRP or pCRP loaded MPs were placed into the hsCRP assay, they did not give a positive measurement



Supplementary Fig. S4 Detection of CRP isoforms bound to MPs and conversion of pCRP to mCRP by HCMECs. **a** MPs isolated from the blood of a normal donor can contain both pentameric and monomeric CRP as determined by native Western blot. **b** Human cardiac microvascular endothelial cells (HCMEC) converted pCRP to mCRP as measured by native Western blot. Lane 1, 20 minute incubation; lane 2, 1 hour incubation; lane 3, 3 hour incubation; lane 4, CRP in plasma