Supplemental Material

Human Tissue Samples

Discarded human carotid endarectomy specimens (n=136 patients) were collected in accordance with a protocol approved by the Institutional Review Board of Brigham and Women's Hospital. Tissue samples were analyzed by immunohistochemistry and transmission electron microscopy. Human carotid endarectomy samples were collected and embedded in OCT compound. Cryosections of 6-μm thickness were stained for macrophages (CD68; Dako, Carpinteria, CA, USA) and smooth muscle cells (SMCs; α-smooth muscle actin [α-SMA; Dako, Carpinteria, CA, USA]).

Mice

Wild-type (WT) mice and apoE$^{-/-}$ mice in C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). S100A9$^{-/-}$ (MRP-14$^{-/-}$) mice in C57BL/6 background were provided by Dr. Nancy Hogg. S100A9$^{-/-}$ mice were crossbred with apolipoprotein E-deficient (ApoE$^{-/-}$) mice in a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME, USA) to generate compound mutant double-deficient mice (S100A9$^{-/-}$ApoE$^{-/-}$) [1]. Single mutant ApoE$^{-/-}$ mice were used as controls. All mice had a congenic C57BL/6 background and were maintained in animal facilities at Harvard Medical School. A subset of WT mice (n=8) were fed a high-fat diet (HFD). CRD was induced in apoE$^{-/-}$ mice by 5/6 nephrectomy, as previously described[2]. Animal care and procedures were approved by the Institutional Animal Care and Use Committees, and were performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health.

Immunohistochemistry/ Immunofluorescence

Immunohistochemistry was performed on fresh frozen sections of mouse aortic arches and human carotid endarterectomy specimens, as previously described[2]. Briefly, tissue samples were frozen in OCT compound (Sakura Finetech, Torrance, CA, USA), and 6-μm serial sections were cut. Antibodies included annexin V (1:100; Abcam, Cambridge, MA, USA), S100A9 (1: 100; R&D Systems, Minneapolis, MN, USA), CD68 (1:70; Dako, Carpinteria, CA, USA), and αSMA (1:100; Dako, Carpinteria, CA, USA). Immunohistochemistry used the avidin-biotin peroxidase method. The reaction was visualized with 3-amino-9-athyl-carbazol substrate (AEC; Sigma Chemical, St. Louis, MO, USA). Adjacent sections treated with PBS in place of a primary antibody were used as negative controls. Images were captured with a digital camera (Nikon DXM 1200F, Nikon Inc., Melville, NY, USA). Fluorescence visualized S100A9 and annexin V labeled with Alexa Fluor® 488/594. Images were captured and processed with the epifluorescence microscope (Eclipse 80i, Nikon Instruments Inc., Melville, NY, USA) with a cooled CCD camera (Cascade, Photometrics, Tucson, AZ, USA).

Detection and Quantification of Mineralization

Bisphosphonate-conjugated imaging agent that binds to hydroxyapatite (OsteoSense680, VisEn Medical Inc., Woburn, MA, USA), elaborating fluorescence evident through the near-infrared window (ex/em 650/680 nm), detected mineralization, as previously described[3,4]. Frozen sections of human carotid endarterectomy specimens were treated with OsteoSense680 for a minimum of 2 hours before imaging. ApoE$^{-/-}$ mice were intravenously injected with the NIRF imaging agent, and tissue was harvested after 24 hours. Frozen sections of the aortic arch were imaged *ex vivo*. Images were captured and processed with the epifluorescence microscope (Eclipse 80i, Nikon Instruments Inc., Melville, NY, USA) with a cooled CCD camera (Cascade, Photometrics, Tucson, AZ, USA). To quantify the calcifying vesicular structures, MVs, we utilized an imaging analysis technique developed in our laboratory (KY). Before analyzing a

binary image of Osteosense-labeled macrophage-rich plaques, the image was processed using an edge filter to delineate MVs in the images. We counted the number of calcifying vesicular structures in an average of three high-power fields (x400 magnification) in plaques from eight patients. A range threshold was obtained by calculating the cumulative frequency, and vesicular structures with pixel numbers ranging from 40 to 115 were counted as calcifying vesicles. Osteosense-labeled vesicular structures below 40 pixels were omitted as background, and structures above 115 pixels were considered micro/macrocalcification. In a similar manner, the percentage area of calcified MVs was quantified in an average of five high-power fields (x400 magnification) in plaques with either early-stage, mid-stage, or late-stage calcification from 10 patients per group.

Transmission Electron Microscopy (TEM) Analysis of MVs in Calcified Arteries

Tissue was immersion fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, in 0.1M cacodylate buffer pH 7.4 (modified Karnovsky's fixative) and post-fixed in 1% osmium tetroxide, dehydrated and embedded in Epon resin. Thin sections (80-nm thickness) were placed on carbon coated and glow discharged formvar coated copper slot grids; these were contrast stained with 2% uranyl acetate and lead citrate. Grids were imaged on a JEOL 1400 TEM equipped with a side mount Gatan Orius SC1000 digital camera.

Immuno-EM on Calcified Arteries

Tissues were immersion fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, in 0.1M Cacodylate buffer pH 7.4 (modified Karnovsky's fixative). The tissues were dehydrated and embedded in acrylic resin. Thin sections (80-nm thickness) were placed on carbon coated and glow discharged formvar coated nickel slot grids. Blocked grids were incubated in primary antibody at RT for 1 hour, followed by an appropriate gold-conjugated secondary antibody for 1

hour at RT. After fixing with 1% glutaraldehyde in TBS, sections were contrast stained with uranyl acetate. Grids were imaged on a JEOL 1400 TEM equipped with a side mount Gatan Orius SC1000 digital camera. Primary antibodies included rabbit anti-human annexin V (1:200; Abcam, Cambridge, MA, USA), mouse anti-human S100A9 (1:200; R&D Systems, Minneapolis, MN, USA), and CD68 (1:100; Dako, Carpinteria, CA, USA). Secondary antibodies included goat anti-rabbit 10-nm colloidal and gold anti-mouse 10-nm colloidal gold (1:25; Abcam, Cambridge, MA, USA).

Culture and Stimulation of Murine Macrophages

Murine macrophage-like cells, RAW 264.7, were seeded at a density of 1.6 x 10^5 cells per cm^2. Prior to experiment, the cells were serum starved in DMEM containing 0.1% FCS. After 24 hours, either control media (DMEM containing 0.1% FCS) or calcifying media (DMEM containing 0.1% FCS, supplemented with stimuli as indicated). Stimuli included Ca/P (3 mmol/L calcium / 2 mmol/L phosphate), P (2 mmol/L phosphate), 20 ng/ml TNFα (PeproTech, Rocky Hill, NJ, USA), Ca/P + TNFα (3 mmol/L calcium / 2 mmol/L phosphate and 20 ng/ml TNFα), S100A9 (50 ng/ml recombinant mouse S100A9 [Novus Biologicals, Littleton, CO, USA]). $CaCl_2$ and NaH_2PO_4 (Sigma-Aldrich Corp., St. Louis, MO, USA) were added to supplement calcium and phosphate in the media.

Time-Lapse Imaging of Macrophages

Macrophages, seeded onto glass-bottomed dishes, were serum starved as previously described. After 24 hours, the cells were loaded with 5 μmol/L Fluo-3 (Life Technologies, Grand Island, NY, USA). Calcium influx was visualized by imaging cells before and after stimulation with Ca/P in Tyrode solution. In the same vein, vesicle release was visualized after Ca/P stimulation by time-lapse microscopy. In both protocols, the following were used: a 60x oil

immersion objective, the Eclipse 80i microscope (488 nm, Nikon, Melville, VY), and the NIS element software (Nikon, Melville, VY). For the visualization of MV release, images were taken every 10 seconds over 24 hours.

Visualization of Phosphatidylserine (PS) Externalization

Murine macrophage-like cells, RAW 264.7, were seeded onto glass-bottomed dishes and serum starved as previously described. After 24 hours, the medium was exchanged for control medium, and 20 µl/ml pSIVA-IANBD (Abcam, Cambridge, MA, USA) was added directly to the cell cultures before stimulation. pSIVA^{TM} is an annexin XII-based polarity sensitive probe with a high affinity for PS that binds reversibly to the cell membrane, enabling the detection of irreversible as well as transient PS exposure. pSIVA^{TM} is conjugated to IANBD, which fluoresces only when pSIVA is bound to the cell membrane, thus allowing the detection of PS exposure. The cells were visualized before and after Ca/P stimulation using a 60x oil immersion objective, the Eclipse 80i microscope (488 nm, Nikon, Melville, VY), and the NIS element software (Nikon, Melville, VY).

Isolation of Peritoneal Macrophages from Mice

Peritoneal macrophages were elicited by injecting 1.5 ml 4% aged thioglycolate into the peritoneal cavity. After 4 days, the peritoneal macrophages were harvested by injecting RPMI 1640 medium into the peritoneal cavity, gently massaging the abdominal area, and removing the liquid containing the resident macrophages. After washing with PBS, the cells were seeded at a density of 1-2 x 10^6 cells per well in serum-free medium. After 2 hours, the cells were washed with PBS to remove non-adherent cells, and the adherent cells were incubated with RPMI 1640 containing 10% fetal bovine serum for 48 hours. Due to the high phosphate concentration already present in RPMI-1640, the cells were switched to α-MEM prior to

stimulation. Peritoneal macrophages were serum starved in α-MEM containing 0.1% fetal bovine serum for 24 hours before addition of either control media (α-MEM containing 0.1% fetal bovine serum) or calcification media (α-MEM containing 0.1% fetal bovine serum, supplemented with 3 mmol/L calcium chloride / 2 mmol/L sodium phosphate) for an additional 24 hours prior to analysis.

Isolation and Culture of Human Macrophages

Human peripheral blood mononuclear cells were isolated by density gradient centrifugation, as described previously[5], and were cultured in IMDM containing 5% human serum for 7 days. After 7 days, the cells morphologically appeared to have differentiated into macrophages, and the cells were then cultured in DMEM. After 10 days, some differentiated macrophages were either transfected with siRNA or serum starved in DMEM containing 1% human serum for 24 hours, before the addition of control media (DMEM containing 1% human serum) or calcification media (DMEM containing 1% human serum, supplemented with 3 mmol/L calcium chloride / 2 mmol/L sodium phosphate) for an additional 24 hours prior to analysis. The data are presented as fold-change of calcium content comparing Ca/P to control, in the presence and absence of siRNA against S100A9. Data were analyzed using the Mann-Whitney test.

siRNA Transfection of Human Macrophages

Short interfering RNA (siRNA) oligonucleotides predesigned by Dharmacon Thermo Scientific included ON-TARGETplus Non-targeting Pool, Human S100A9 ON-TARGETplus SMARTpool, and Human ANXA5 ON-TARGETplus SMARTpool. Transfection of human macrophages was performed using Dharmafect 4 transfection reagent (Thermo Scientific, Lafayette, CO, USA), according to the manufacturer's protocol. Briefly, 100nM siRNA was diluted in OPTIMEM, mixed with Dharmafect 4, and incubated for 20 minutes at room temperature. This mixture was added

drop-wise on to human macrophages, which were then incubated in DMEM supplemented with 5% human serum. After 48 hours, the cells were serum starved in DMEM containing 1% human serum and antibiotics for 24 hours. The cells were then incubated in control media (DMEM containing 1% human serum and antibiotics) or in calcifying media (DMEM containing 1% human serum and antibiotics and supplemented with 3 mmol/L calcium chloride / 2 mmol/L sodium phosphate) for 24 hours. MVs were analyzed using the calcium or ALP assay (BioVision, Inc., Milpitas, CA, USA), or lysed for analysis by Western blot, and the cells were lysed using Trizol and analyzed by RT-PCR to determine efficiency of the knockdown.

Analysis of Cell Viability and Apoptosis

Cell viability, assessed by measuring live-cell protease activity, and apoptosis, assessed via detection of Caspase 3/7 activation, was analyzed using ApoLive-Glo Multiplex assay (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Reverse Transcription and Quantitative Polymerase Chain Reaction

Total RNA was isolated from cell isolates and reverse transcribed using an Applied Biosystems 2720 thermal cycler (Applied Biosystems, Foster City, CA). TaqMan quantitative polymerase chain reaction (PCR) detection of human S100A9 and annexin V, and of mouse inducible nitric oxide synthase (iNOS), mannose receptor 1 (MRC-1), IL6, and IL1β, was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Quantitative PCR values were normalized to β-actin. Relative fold changes in value were calculated by the comparative threshold cycles (C_t) method, $2^{-\Delta\Delta CT}$.

Isolation of Macrophage-derived MVs

Cell media were collected after approximately 24 hours and subjected to centrifugation at 1000 g for 5 minutes to remove cell debris, followed by 16500 g for 5 minutes to remove apoptotic bodies and any larger vesicles. MV fraction was harvested from the media by ultracentrifugation at 100000 g for 40 minutes at 4ºC (Optima Max Ultracentrifuge, Beckman Coulter, Inc., Indianapolis, IN, USA).

Calcium Content Analysis

MVs were analyzed using the calcium colorimetric assay (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's specifications.

Alkaline Phosphatase (ALP) Activity

MVs were analyzed using the ALP assay (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's specifications.

Protein Concentration

Protein concentration was determined by the Pierce BCA assay (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's specifications.

Negative Staining of Isolated MVs from RAW 264.7 Cells

Nickel grids with 200 mesh, formvar, carbon coating, and freshly glow discharged were used for the negative staining of MVs. Each grid was placed on a 20-µl droplet of sample (approximately 1 mg/ml in PBS) for 5 minutes. After washing with water, grids were negatively stained with phosphotungstic acid. Grids were imaged on a JEOL 1400 TEM equipped with a side mount Gatan Orius SC1000 digital camera.

Quantification of MVs in Mouse Plasma

Flow cytometry was used to detect MVs in mouse plasma from WT, apoE$^{-/-}$, and apoE$^{-/-}$ S100A9$^{-/-}$ mice[6], fed a HFD. Mouse blood was separated into plasma by 1500 g centrifugation for 15 minutes. A platelet-poor fraction was obtained by spinning the plasma for an additional 2 minutes at 13000 g. The MV flow cytometry protocol combined 20 µL of mouse platelet-poor plasma, 42.5 µL of filtered (0.22 µm) annexin V binding buffer (1X Tris Buffered Saline with 2.5 mM $CaCl_2$), and 2.5µL of Annexin V-FITC (10 µg/mL, Bender MedSystems [eBioscience], San Diego, CA, USA) to enable quantification of phosphatidylserine-positive MVs. Prior to flow cytometry, annexin V-labeled MVs were combined with 385 µL of annexin V binding buffer and 50 µL of fluorescent counting beads, which enabled determination of flow rate and MV concentration (Flow-Count Fluorospheres, Beckman Coulter, Brea, CA, USA). Sample analysis was performed on a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and we analyzed the flow cytometry data with FCS Express software 3.0 (DeNovo Software, Los Angeles, CA, USA). MVs were identified by side scatter size compared to sizing beads (Megamix, Biocytex 7801, France), and by annexin V binding as described previously. We defined the MV gate as annexin V-positive events sized < 1 µm. Annexin V binding to phosphatidylserine-containing plasma membranes is calcium dependent; thus, samples treated with the calcium-chelating agent ethylenediaminetetraacetic acid (EDTA, 20 mM) served as a negative control for annexin V gating. The threshold of annexin V-positive MV events was set above the 99.99th percentile of the EDTA-treated negative control sample. The FACS analysis of the mouse plasma samples was performed in a blinded fashion. The groups were identified only when performing the final data compilation.

Aggregation potential of MVs

MV size was measured using a time-resolved particle analyzer (EX-300, Kowa Company, Ltd., Nagoya, Japan). Some remodeling was performed to improve the sensitivity against small particles, including equipping the device with preamps and an analog/digital converter. According to a fitting curve (size versus scattering light intensity) obtained from measurements of size-standard nanoparticles (Nanosphere with 20, 60, 150, and 500 nm in diameter; Thermo Scientific, Fremont, CA, USA) and refractive indices (RI) of Nanosphere (RI=1.59) and MVs (using the previously reported[7] RI of macrophages; RI=1.38 ± 0.02), we calculated actual sizes of MVs. The aggregation potential of MVs was evaluated by measuring their size growth under continuous stirring of the media (control or Ca/P stimulation) for 10 minutes.

Western Blotting

Cell or MV samples were lysed in RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The lysates were centrifuged at 13000 g for 10 minutes. Supernatants were collected, and the protein concentration was determined using the BCA assay (Thermo Scientific, Rockford, IL, USA). For Western Blotting, 10-20 μg of protein lysate were separated on 15% SDS-PAGE and transferred to a PVDF membrane using the iBlot dry transfer system (Life Sciences, Grand Island, NY, USA). The membrane was blocked for non-specific binding in blocking buffer (TBS-T [Tris-buffered saline with 0.05% Tween-20] containing 3% dry milk), and incubated with primary antibody. Antibodies for detection included annexin V (1:250) S100A9 (1:1000), CD9 (1:500; Abcam, Cambridge, MA, USA), and TSG101 (1:500; GeneTex Inc., Irvine, CA, USA). Following incubation with primary antibodies, and washing wells with TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000, Amersham Biosciences, Piscataway, NJ, USA) and visualized using the ECL system (PerkinElmer, Billerica, MA, USA). β-actin (1:5000; Novus Biologicals, Littleton, CO, USA) was used as a loading control to demonstrate equal sample loading. Membranes were imaged using

the ImageQuant™ LAS 4000 (GE Healthcare, Piscataway, NJ, USA) and image band intensity was measured using ImageJ 1.45 software.

Co-immunoprecipitation of S100A9-Anx5

Antibodies – Anx5 antibody (Abcam, Cambridge, MA, USA) and IgG antibody (Santa Cruz, Santa Cruz, CA, USA) – were non-covalently bound to beads by incubating Dynabeads® Protein G (Invitrogen, Grand Island, NY, USA) with the appropriate antibody under gentle rotation overnight at 4°C. Beads were then washed 3 times with 1 x PBS prior to incubating with protein under gentle rotation at 4°C for 4 hours. Elution in SDS was performed by heating the beads for 5 minutes at 95°C in SDS-reducing buffer (Boston BioProducts Inc., Boston, MA, USA). Eluates were then subjected to Western blot analysis to demonstrate the interaction between Anx5 and S100A9.

Statistical Analysis

Data were analyzed by *t*-test or one-way ANOVA with the Bonferroni post hoc test using PRISM software (GraphPad, San Diego, CA). Data with multiple interactions were analyzed by two-way ANOVA with the Bonferroni post hoc test. Data show mean±SD. $P<0.05$ was considered statistically significant. The *n* values represent the number of patients or donors studied, or the number of times the experiment was performed (the latter refers to experiments using RAW267.4, the mouse macrophage-like cell line).

References

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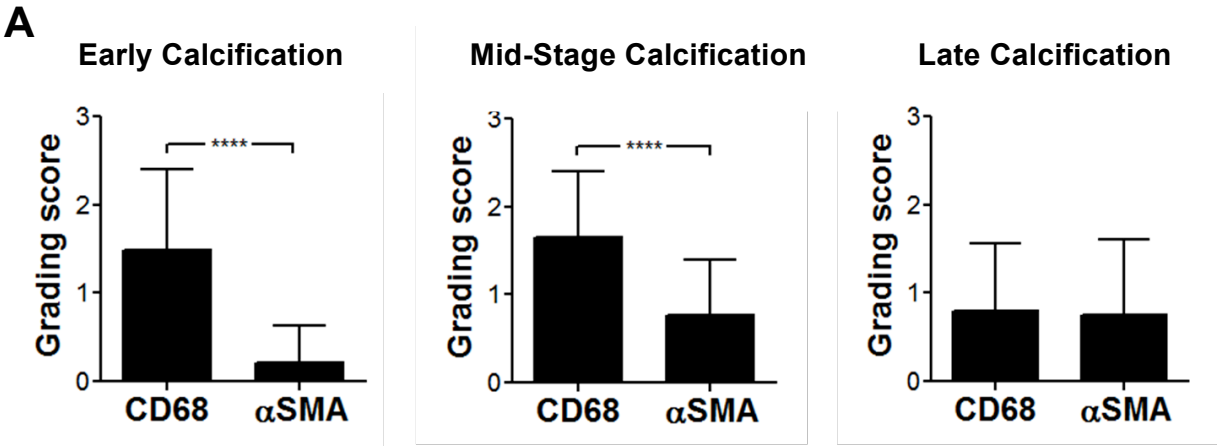
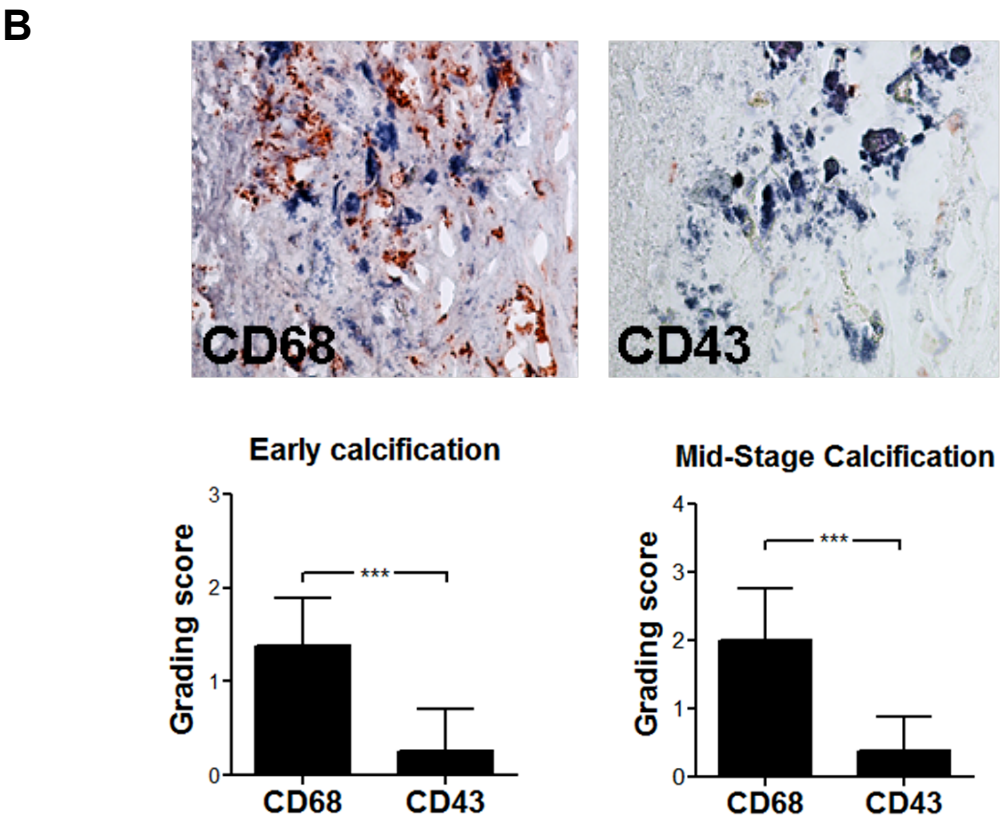
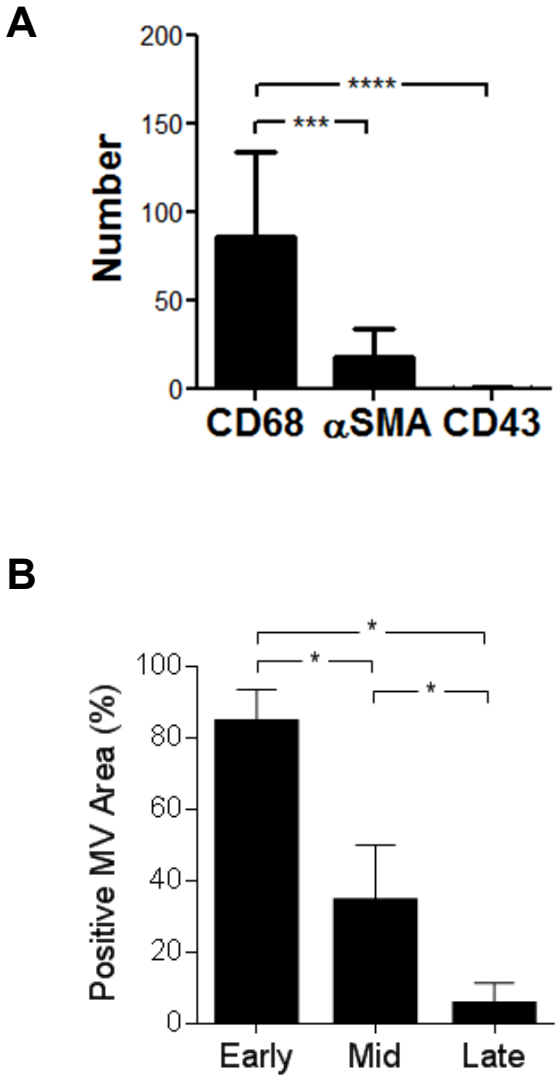
Supplemental Figures

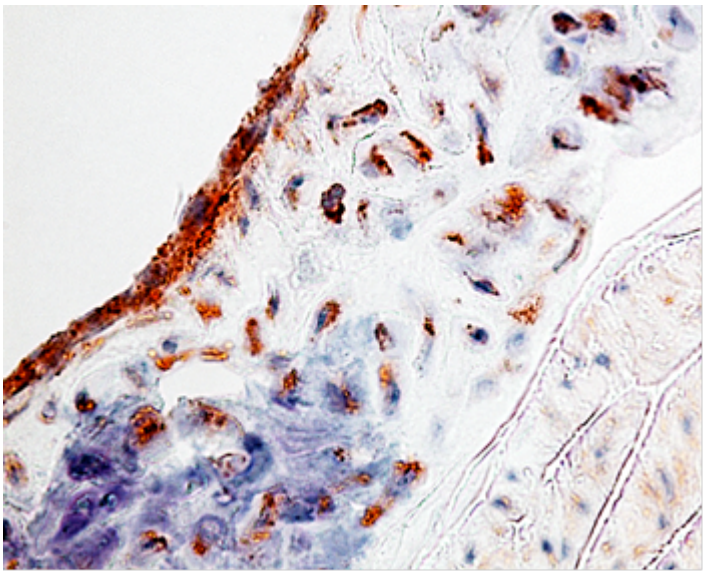




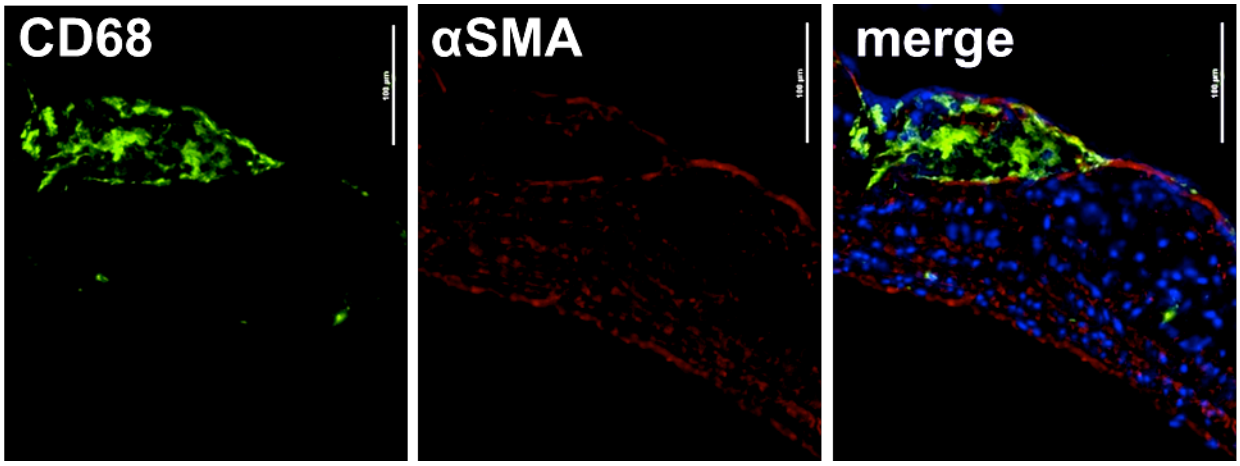
Online Figure I. A, Macrophages associate with MV-rich areas in human calcified atherosclerotic plaques (****p <0.0001). Regions of early calcification contained calcifying vesicular structures (less than 10 μm in size) only, and no larger calcifications (87 sections from 75 patients); mid-stage calcification areas contained a combination of calcifying vesicular structures and larger calcification/microcalcification (101 sections from 89 patients), and regions of late calcification/macrocalcification were large areas with no evidence of vesicles (20 sections from 19 patients). Co-localization of CD68-positive macrophages and vesicular structures was identified in regions of both early calcification and mid-stage calcification (161 sections from 127 patients). The number of sections analyzed overall was 176 from 136 patients. **B,** Negligible neutrophil content in regions of either early (n=8; ***p<0.001) or mid-stage calcification in human plaques (n=8; ***p<0.0001).



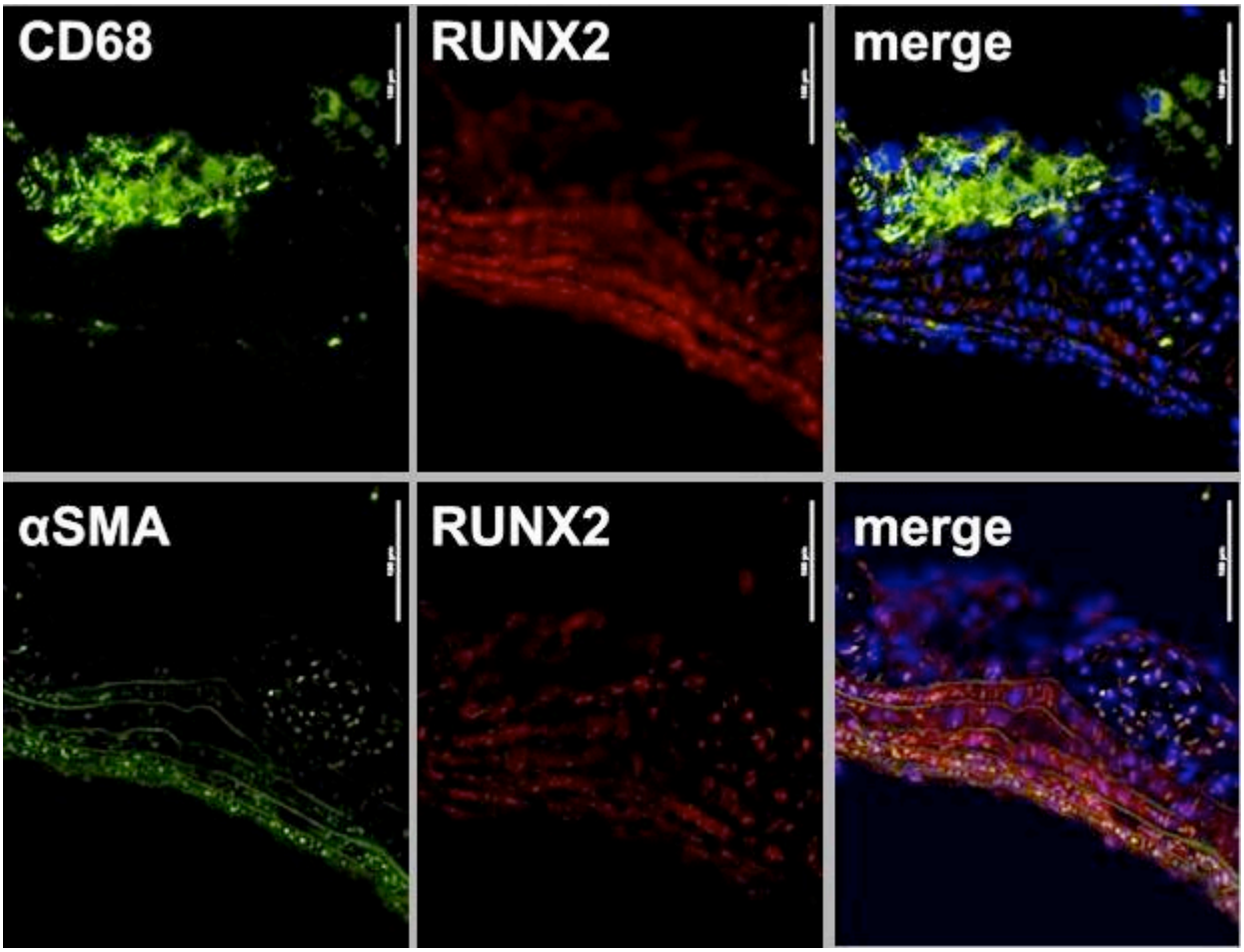
Online Figure II. A, MV-rich areas in human calcified atherosclerotic plaques contain abundant macrophages (CD68-positive cells) (n=8, ***p<0.001, ****p <0.0001), demonstrating the frequency of macrophages releasing MVs in the plaque compared to smooth muscle α-SMA positive cells and neutrophils (CD43-positive cells). **B,** MV burden is greatest in early calcified plaques and decreases with the formation of larger calcification areas in the later stages of plaque progression (p<0.05).



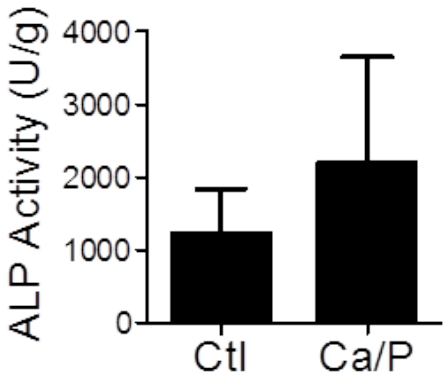
Online Figure III. Mac3-positive cells (red reaction product) localize in the region of calcification (hematoxylin; blue) in atherosclerotic plaques of apoE$^{-/-}$ mice with 5/6-nephrectomy.



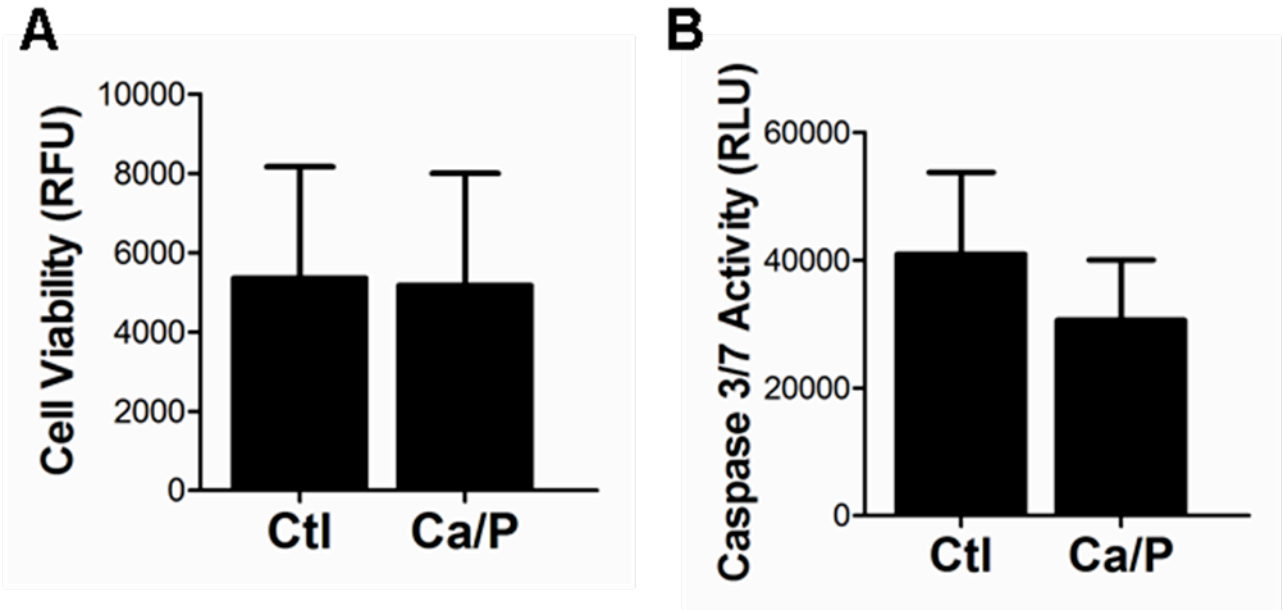
Online Figure IV. Little to no co-localization of CD68-positive and α-SMA-positive areas in calcified plaques from CRD apoE$^{-/-}$ mice.



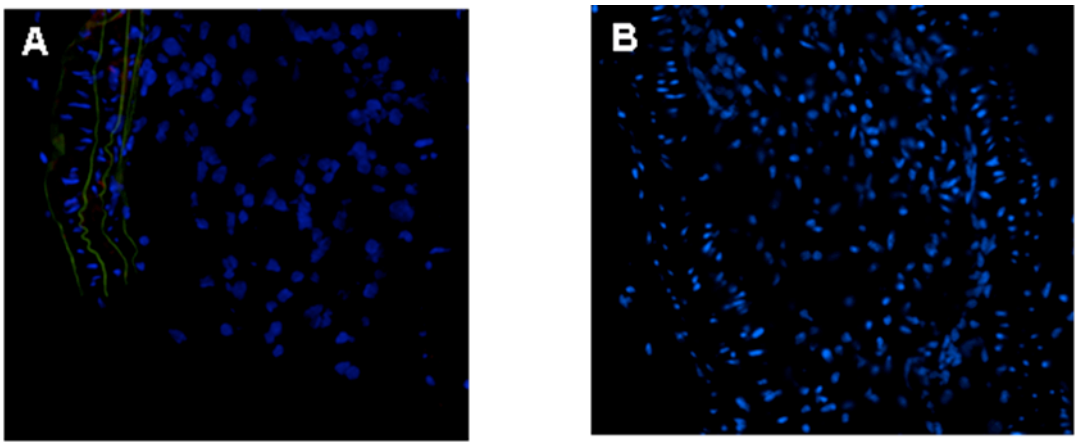
Online Figure V. CD68-positive regions did not co-localize with areas of osteogenic activity, determined via the use of Runx2. α-SMA-positive cells, however, co-localized with Runx2.



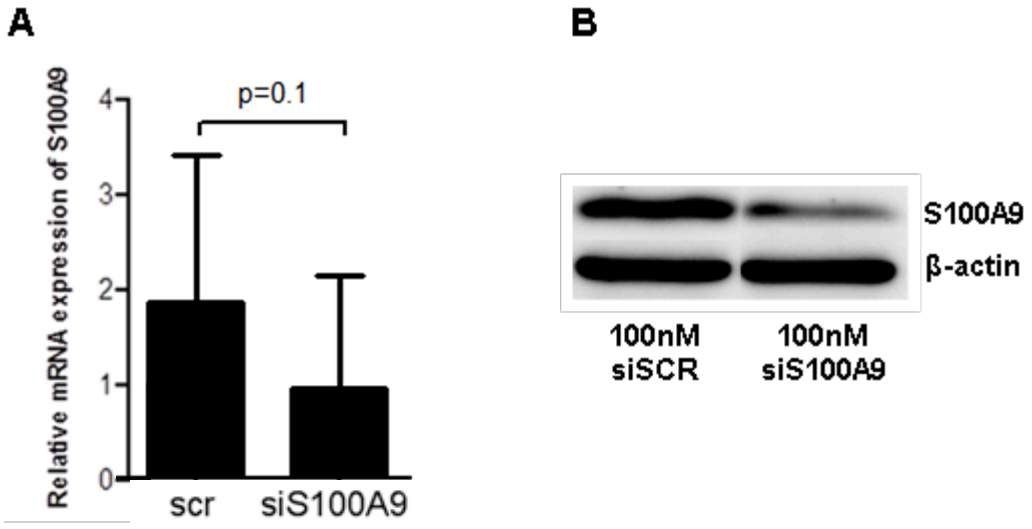
Online Figure VI. MVs released from murine macrophage-like cells under calcifying conditions have higher ALP activity than MVs released under non-calcifying conditions (n=5).



Online Figure VII. A, Calcifying conditions used for *in vitro* experiments did not affect viability of the murine macrophage-like cells (n=4). **B,** Analysis of caspase 3/7 activity in the same cells demonstrated that the calcifying conditions used for *in vitro* experiments did not induce apoptosis (n=4).



Online Figure VIII. A, Negative control for S100A9 immunofluorescence (Figure 3A). **B,** Negative control for annexin V immunofluorescence (Figure 3B).



Online Figure IX. A, mRNA expression of S100A9 after transfection of human macrophages with siRNA (n=4; p=0.1). The data are presented as fold-change of S100A9 mRNA expression comparing Ca/P to control, in the presence and absence of siRNA against S100A9. **B,** Protein expression of S100A9 by Western blotting was knocked down (~70%) by siRNA in human macrophages in vitro.