Macrophage-Derived Matrix Vesicles:
An Alternative Novel Mechanism for Microcalcification in Atherosclerotic Plaques

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ABSTRACT

Rationale: We previously showed that early calcification of atherosclerotic plaques associates with macrophage accumulation. Chronic renal disease (CRD) and mineral imbalance accelerates calcification and the subsequent release of matrix vesicles (MVs) — precursors of microcalcification.

Objective: We tested the hypothesis that macrophage-derived MVs contribute directly to microcalcification.

Methods and Results: Macrophages associated with regions of calcified vesicular structures in human carotid plaques (n=136 patients). In vitro, macrophages released MVs with high calcification and aggregation potential. MVs expressed exosomal markers (CD9 and TSG101), and contained S100A9 and annexin V (Anx5). Silencing S100A9 in vitro and genetic deficiency in S100A9-/- mice reduced MV calcification, while stimulation with S100A9 increased calcification potential. Externalization of phosphatidylinerine (PS) after Ca/P stimulation and interaction of S100A9 and Anx5, indicated that a PS-Anx5-S100A9 membrane complex facilitates hydroxyapatite nucleation within the macrophage-derived MV membrane.

Conclusions: Our results support the novel concept that macrophages release calcifying MVs enriched in S100A9 and Anx5, which contribute to accelerated microcalcification in CRD.

Keywords: Extracellular vesicles, macrophages, calcification, chronic renal disease, atherosclerosis, inflammation, plaque rupture

Nonstandard Abbreviations:

Anx5  Annexin V
Ca  Calcium
CRD  Chronic renal disease
HA  Hydroxyapatite
hMac  Human macrophages
Mac-MV  Macrophage-derived MVs
MVs  Matrix vesicles
NIRF  Near-infrared fluorescence
P  Phosphate
PS  Phosphatidylinerine
SD  Standard deviation
siRNA  Small interfering RNA
TEM  Transmission electron microscopy
SMC  Smooth muscle cells

INTRODUCTION

Arterial microcalcification in the thin cap overlying the necrotic core in the intima of atherosclerotic plaques may cause compliance mismatch, increase local stress, and promote microfractures — leading to plaque rupture causing acute cardiovascular events\(^1\,^2\). Atherosclerotic plaques containing “spotty” calcifications consisting of calcified vesicles (precursors to microcalcification) have increased stress, thus making them more susceptible to rupture \(^3\).
Patients with chronic renal disease (CRD) have heightened susceptibility to atherosclerosis, a greater risk of intimal and medial calcification, and a high cardiovascular mortality. The added complication of hyperphosphatemia may trigger release of matrix vesicles (MVs) in both non-pathological and pathological conditions. Smooth muscle cell (SMC)-derived MVs contribute to medial calcification in CRD patients. Our molecular imaging studies co-localized early stages of atherosclerotic intimal calcification/microcalcification with macrophages. During this initial phase of calcification, coincident inflammation and microcalcification suggest a direct role for macrophages in calcification. In addition to the currently prevailing concept of osteogenic transition of SMCs, we hypothesized an alternative inflammation-related pathway of arterial calcification in which macrophages release microcalcification-generating MVs.

**METHODS**

Detailed Methods are available as an Online Supplement. Calcifying MVs were isolated from macrophages via ultracentrifugation, and characterized comprehensively using histological, biochemical, and imaging techniques. To address the mechanisms for the mineralization of macrophage-derived MVs, we examined the contribution of S100A9 in gain-of-function and loss-of-function studies.

**RESULTS**

**MV s released from macrophages contribute to intimal microcalcification.**

Immunohistochemical analysis of macrophages and SMCs (α-smooth muscle actin; α-SMA) in 136 human carotid endarterectomy samples and mouse arteries demonstrated that abundant macrophages associate with regions of MVs (Figure 1A), to a greater extent than α-SMA-positive or CD43-positive cells in areas of early spotty calcification, a characteristic associated with biomechanical instability (Figure 1B; Online Figures I-V). Ex vivo near-infrared fluorescence (NIRF) imaging of hydroxyapatite-binding nanoparticles visualized numerous calcifying MVs within calcified atherosclerotic plaques from humans (2784 ± 1073 MV/mm²; n=8; Figure 1C) and from apoE−/− mice (Figure 1D) with CRD, induced by 5/6 nephrectomy. We used transmission electron microscopy (TEM) to characterize MVs within plaques. Immunogold labeling visualized release of CD68-positive MVs from macrophages (Figure 1E). MVs abound in human and mouse plaques, and associate with cholesterol crystals (Figures 1F and 1G). Plaque areas rich in MVs showed microcalcification and dense hydroxyapatite crystals localized on the membranes of calcifying MVs (Figure 1H).

**Pro-inflammatory macrophages release microcalcification-generating MVs.**

Time-lapse imaging of macrophages loaded with Fluo-3, a calcium indicator, showed vesicle release from membranous protrusions (Figure 2A; videos provided in Online Supplement) and calcium influx after Ca/P-stimulation (Figures 2B and 2C). Ca/P-stimulated macrophages released MVs (Mac-MVs) capable of mineralization, with increased calcium content (Figures 2D and 2E), alkaline phosphatase (ALP) activity (Online Figure VI), and suggested aggregation potential (Figures 2F and 2G). TEM of MVs released by control macrophages in vitro identified membrane-bound vesicles 30–300 nm, showing hydroxyapatite nucleation on the membrane and inside the vesicles after Ca/P stimulation (Figure 2H). Mac-MVs possessed exosomal markers (Figure 2I) and were smaller than those described for apoptotic bodies. Additionally, Ca/P stimulation did not affect the viability or apoptotic levels of
mouse macrophages (Online Figure VII). Mouse macrophages stimulated with Ca/P possessed higher levels of mRNA encoding inducible nitric oxide synthase (iNOS) and interleukin-6 (IL-6) (Figure 2J), markers in mice of a pro-inflammatory “M1” macrophage polarization, while “M2” markers tended to decrease.

PS-S100A9-annexin V complex facilitates MV mineralization.

Annexins contribute to SMCs and osteoblast-derived MV-mediated calcification. Calcified plaques of CRD apoE-/- mice showed expression of both S100A9 and Anx5 (Figure 3A; Online Figure VIII). In addition, S100A9 and Anx5 co-localized with MVs in calcified regions of human atheroma (immunogold TEM, Figures 3B and 3C). Ca/P stimulation enriched S100A9 and Anx5 expression in mouse Mac-MVs (Figure 3D). We hypothesized that S100A9 and Anx5 form a complex in Mac-MVs, similar to the complex formation of S100A9 and annexin VI. The co-localization and co-immunoprecipitation analysis of S100A9 and Anx5 in cultured mouse macrophages supported this conjecture (Figures 3E and 3F). Ca/P stimulation of mouse macrophages augmented the association between S100A9 and Anx5 (Figure 3F). Anx5 and phosphatidylserine (PS) are critical for growth plate-MV mineral nucleation, but whether nucleation occurs on the inside or outside of the vesicle is unclear. Ca/P-stimulated macrophages externalized PS, suggesting that nucleation may take place on the outer Mac-MV membrane (Figure 3G).

S100A9 as a mediator of mineralization.

The addition of S100A9 in vitro enhanced the calcific potential of Mac-MVs (Figure 4A). S100A9 siRNA treatment in human macrophages (see Online Figure IX for silencing efficacy) limited the calcific potential of Mac-MVs (Figure 4B). To furnish further evidence in vivo for the function of S100A9 as a mediator of the calcific potential of Mac-MVs, we performed experiments using S100A9-/- apoE-/- mice. The level of MVs quantified in mouse plasma of fat-fed apoE-/- mice trended higher than in fat-fed wild-type (WT) mice, but did not achieve statistical significance with the number of animals studied. Inactivation of the S100A9 gene in apoE-/- mice tended to decrease MVs in plasma to the levels in WT mice (Figure 4C). In addition, Ca/P-stimulated S100A9-/- mouse peritoneal macrophages released Mac-MVs with lower calcific potential than those isolated from WT mice (Figure 4D).

DISCUSSION

We report a novel pathway of microcalcification that involves macrophage-derived MVs. Concentrations of extracellular Ca and P, similar to those found in serum of CRD patients on dialysis, induced the release of Mac-MVs with higher calcification potential. These findings support the operation of a new additional mechanism of arterial calcification that likely accelerates the excessive intimal and medial calcification associated with CRD. Due to the relative abundance of macrophages over SMCs in rupture-prone plaques, we hypothesize that macrophage-derived MVs participate in the initiation of microcalcification.

Mac-MVs were similar to previously reported chondrocyte-derived MVs (e.g., PS and Anx5 expression) and exosomes (CD9 and TSG101). Mineralization of growth plate chondrocytes by MVs requires PS and Anx5; indeed, other annexins may compensate for the loss of Anx5. This study found increased S100A9 and Anx5 expression in calcifying mac-MVs, highlighting their potential importance in MV calcification. The results provide evidence that S100A9 interacts with Anx5, forming a PS-Anx5-S100A9 membrane complex, which acts as a nucleation site for hydroxyapatite (Figure 4E).
The use of compound mutant mice, in addition to gain-of-function and loss-of-function in vitro experiments, permitted dissection of the role of S100A9 in the calcification of macrophage-derived MVs in vivo. Previous studies identified S100A9-positive macrophages in early atherosclerotic lesions and suggested the role of this calcium-binding protein in vascular inflammation. The present evidence implicates S100A9 in the mineralization of macrophage-derived MVs.

This study provides new insight into the pathogenesis of vascular calcification, particularly microcalcification — a contributor to the acute thrombotic complications of atherosclerosis. Despite its clinical challenge, we currently lack treatments and diagnostic tools to prevent or reverse cardiovascular calcification. Molecular imaging provided a novel technique for visualizing calcifying vesicular structures that could be translated to the clinic. The evidence provided should help to develop therapies for this unmet medical need, including valvular as well as vascular calcification. Specifically, modulation of expression or function of S100A9 and/or Anx5 may suppress the nucleation of hydroxyapatite by macrophage-derived MVs and the generation of microcalcification in “spotty” plaques prone to rupture — thus preventing the onset of devastating clinical complications.

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DISCLOSURES
None.

REFERENCES


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FIGURE LEGENDS

Figure 1. Macrophage-derived MVs contribute to atherosclerotic microcalcification. A, Calcified vesicular structures (hematoxylin; blue) adjacent to macrophages (CD68; red) in human plaques (n=127). B, Few to no αSMA-positive SMCs were noted in the same area. C, Ex vivo hydroxyapatite-binding NIRF imaging agent detected vesicular microcalcification (red) within human and, D, apoE⁻/⁻ mouse plaques. E-F, Ultrastructure of human calcified plaques: E, Macrophage releases CD68-positive MVs (immunogold). F, Vesicles of different sizes and appearance. G-H, Ultrastructure of calcified CRD apoE⁻/⁻ mouse plaque: G, MVs near cholesterol crystals (ChC; AB apoptotic bodies). H, MV (30-300 nm) hydroxyapatite crystallization.

Figure 2. Macrophages release microcalcification-generating MVs. A, Time-lapse imaging of mouse MV release from RAW264.7 (t = 0-20 seconds; video available in Online Supplement), B-C, The intensity of Fluo-3, a calcium indicator, within human macrophages (hMac) increased with Ca/P-stimulation, demonstrating calcium influx upon Ca/P stimulation. The arrow represents the x-axis on the graph. D-E, Various stimuli induce release of MVs with increased calcium content from mouse and human macrophages (n=3-7, *p<0.05, **p<0.001, ***p<0.0001; mean±SD). F-G, Mac-MVs from Ca/P-stimulated mouse macrophages were larger in size and potentially aggregated over time (n=3). H, Ultrastructure of mouse Mac-MVs released in vitro, showing membrane-bound MVs from control cells (top) and calcifying MVs (bottom). I, MVs expressed exosomal markers (TSG101 and CD9). J, Ca/P-stimulated mouse macrophages expressed pro-inflammatory markers (M1 phenotype) (n=3, *p<0.01 vs. control). Ca/P = 3 mmol/L calcium/2 mmol/L phosphate.

Figure 3. PS-Anx5-S100A9 complex facilitates MV mineralization. A, Expression of S100A9 and Anx5 in calcified CRD apoE⁻/⁻ mouse plaques (arrows). B, S100A9 and C, Anx5 co-localized with MVs in calcified human plaques (immunogold) (arrows point to MVs). D, Enriched S100A9 and Anx5 expression in calcifying mouse Mac-MVs (n=3). E, S100A9 (green) and annexin V (red) co-localized (yellow) in mouse macrophages; interaction confirmed by co-immunoprecipitation (F, n=3). G, PS externalization (green) noted with Ca/P stimulation.

Figure 4. S100A9 participates in MV calcification. A, Addition of S100A9 enhanced the calcific potential of Mac-MVs released by mouse macrophages (n=11). [*p<0.05, **p<0.01, †p=0.057, mean±SD]. B, S100A9 siRNA transfection of human macrophages tended to decrease the calcific potential of MVs (n=4). C, MVs increased in apoE⁻/⁻ mouse plasma (n=5) compared to WT mice (n=8). ApoE⁻/⁻S100A9⁻/⁻ mice (n=5) had a lower MV concentration compared to apoE⁻/⁻ mice (p=ns). D, Peritoneal macrophages from S100A9⁻/⁻ mice tended to release MVs with a lower calcific potential, compared to WT mice (n=9). E, Proposed mechanism: Stimulated macrophages release MVs with a PS-Anx5-S100A9 complex that facilitates the nucleation of hydroxyapatite and generation of microcalcification.
Novelty and Significance

What Is Known?

- Chronic renal disease (CRD) accelerates calcification in atherosclerotic plaques.
- Microcalcification-rich plaques cause fatal ruptures.

What New Information Does This Article Contribute?

- Macrophages directly contribute to the formation of microcalcification.
- Pro-inflammatory macrophages release MVs capable of mineralization via formation of the phosphatidylserine-annexin 5-S100A9 membrane complex.
- Disruption of MV formation may provide new therapeutic targets to combat calcific cardiovascular disease.

CRD patients are prone to medial and intimal calcification. The abundant macrophages in early microcalcification-rich atherosclerotic plaques suggest their importance in intimal calcification. Here, we demonstrate the direct contribution of macrophages to calcification via release of microcalcification-generating MVs, and provide new insight into their exosomal nature. These results reveal an alternative inflammation-related pathway of intimal calcification in atherosclerotic plaques, which is in contrast to the currently prevailing concept of osteogenic transition of smooth muscle cells. This understanding may open new therapeutic avenues to combat calcific arterial disease.
Figure 4

A

Calcium Content (µg/µg)

B

Calcium Content, fold increase

C

Number of MV (µL)

D

Calcium Content (µg/µg)

E

CRD Milieu

Macrophage

AnxA5
PS
HA
S100A9

WT
ApoE−/−
ApoE−/−
S100A9−/−

WT
S100A9−/−

(HFD)

Ctl rS100A9

scr siS100A9

Ctl

Ca/P

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Supplemental Material

Human Tissue Samples

Discarded human carotid endarterectomy 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 endarterectomy 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 described2. 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. 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-ethyl-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. 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]). CaCl₂ and NaH₂PO₄ (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, NY), and the NIS element software (Nikon, Melville, NY). 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\textsuperscript{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\textsuperscript{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, NY), and the NIS element software (Nikon, Melville, NY).

**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\textsuperscript{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 (Ct) 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 4°C (Optima Max Ultracentrifuge, Beckman Coulter, Inc., Indianapolis, IN, USA).

**Calcium Content Analysis**

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

**Alkaline Phosphatase (ALP) Activity**

MVVs 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⁻/⁻ mice⁶, 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 CaCl₂), 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 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


Supplemental Figures

A

**Early Calcification**

![Early Calcification Graph](image)

**Mid-Stage Calcification**

![Mid-Stage Calcification Graph](image)

**Late Calcification**

![Late Calcification Graph](image)

B

![CD68 and CD43 images](image)

**Early calcification**

![Early Calcification Graph](image)

**Mid-Stage Calcification**

![Mid-Stage Calcification Graph](image)
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<sup>−/−</sup> 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.