

A Macrophage Rac1-IL-1B Signaling Axis Directs Atherosclerotic Calcification

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Thesis

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A Macrophage Rac1-IL-1 β Signaling Axis Directs Atherosclerotic Calcification

Abstract

Atherosclerotic plaque calcification is associated increased risk of cardiovascular events. Inflammation plays a key role in the development of atherosclerosis and is thought to potentially participate in vascular calcification. 3-hydroxy-3-methylglutaryl coenzymeA reductase inhibitors or statins, which are used to reduce risk of cardiovascular events, appear to alter plaque calcification. However, the signaling mechanisms that promote this process and how statins alter these mechanisms are minimally understood. Prior investigation of these mechanisms defined a macrophage Rac (Ras-related C3 botulinum toxin substrate)-IL-1 β (interleukin-1 beta) signaling axis as important in promoting atherosclerotic calcification in *Rac2* deficient mice, which exhibit elevated calcification. This project sought to evaluate the role of Rac-IL-1 β signaling in endogenous atherosclerotic calcification and to investigate the role of statin therapy in this pathway. Both endogenous macrophage Rac1 expression and Rac1-dependent IL-1 β expression are demonstrated to contribute to the development of atherosclerotic calcification. Statins are shown to enhance calcification by disrupting the complex between Rac1 and its inhibitor RhoGDI (Rho GDP-dissociation inhibitor), leading to increased Rac1 activity (GTP bound) in primary monocytes/macrophages. Rac1 regulation of IL-1 β expression appears to be dependent upon NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activity in both endogenous and statin-treated macrophage signaling under inflammasome stimulation. These findings demonstrate that macrophage endogenous Rac1-IL-1 β signaling is important for atherosclerotic calcification and that statin-enhanced calcification is facilitated through this same signaling axis by disrupting the inhibition of Rac1 activity.

Chapter 1:

Introduction to Atherosclerotic Cardiovascular Disease

Historical Perspective of Atherosclerotic Calcification

Coronary artery disease (CAD) was initially thought to be a modern disease born from significant changes to people's lifestyle in the Western society during the 20th century. ¹ However, this disease has been documented in ancient humans. ²⁻⁵ One of the earliest formal reports published in 1852 found "multiple considerably large and calcified plaques" in the preserved aortic arch of an ancient mummified Egyptian woman. ³ More recently, an extensive study of 137 mummies found 34% to have "probable or definitive atherosclerosis" as defined by calcification of aortic tissue.⁴

Despite the influence of modern lifestyle on the rise of cardiovascular disease, the discovery of atherosclerosis in ancient mummies suggests that other risk factors that were more pertinent to ancient times may explain the development of CAD. Some of these proposed ancient risk factors that may have induced chronic inflammation include chronic microbial infections and smoke inhalation. ⁶ Historically, poor hygiene and a lack of medicine are attributed to chronic infections and people would frequently be exposed to smoke from daily cooking over an open fire. ⁷⁻¹¹

Throughout most of human history autopsies were outlawed or scarcely conducted until the Renaissance Period (14th century). ¹ Leonardo da Vinci, was among the first individuals to describe atherosclerosis, “vessels in the elderly restrict the transit of blood through thickening of the tunics.” ¹² By the 19th and 20th century our understanding of CAD became clearer and myocardial infarction was understood to be a result of a sudden obstruction of the coronary artery by a thrombus generated by a ruptured atherosclerotic plaque. ¹

Introduction to Atherosclerotic Calcification

Vascular calcification presents both a clinical challenge and opportunity. In the United States (U.S.) alone, the population of adults equal to or over the age of 65 years will more than double from 46 million to 98 million by the year 2060. ¹³ Among these older adults, coronary artery disease (CAD), caused by atherosclerosis is the leading cause of death in the world. The prevalence in the U.S. for men and women between the ages of 60-79 is 24% and 15%, respectively, and increases to 36% of men and 24% of women for those over 80. ¹⁴ Atherosclerosis is a process by which lipids accumulate in arterial tissue leading to plaque formation, which can restrict blood flow and rupture leading to a thrombus (or blood clot). Plaques are sites of lipid accumulation in the intimal layer of aortic tissue. Plaques start with a lipid core but can change overtime and can be heterogeneous in nature, consisting of necrotic cells, infiltrative inflammatory cells, vascular smooth muscle cells, collagen, and calcium deposits. Atherosclerotic plaque can be categorized as soft, mixed, or hard, based on the increasing degrees of calcification present. ¹⁵ Calcification of atherosclerotic plaque in the coronary arteries is relatively

common occurring in about 90% of men and 67% of women over the age of 70 years.¹⁶ Coronary artery calcification is a well-established predictor total atherosclerotic burden and of future cardiovascular events and all-cause mortality.^{17,18} Despite the clinical impact of vascular calcification in CAD, there are currently no treatments directed at calcification to prevent or mitigate the burden of calcific atherosclerosis.

CAD develops from the progression of atherosclerotic plaques. Plaques can be broadly defined as regions within arterial tissue that have abnormally accumulated material that can include but not be limited to lipids, fibrotic material, cellular debris, and calcium deposits.¹⁹ Atherosclerosis in very general terms is the process by which these materials accumulate to form plaque and how plaque develops over time.¹⁹ Atherosclerotic plaques build up over time, resulting in chronic blockage of blood flow leading to heart failure. As plaques build up in the arteries they can rupture, forming a blood clot that acutely blocks blood flow as observed in myocardial infarction. Arteries are comprised of three layers: intima, media, and adventitia.²⁰ All three layers are separated by a boundary of elastic lamina (internal and external). The intima is the innermost layer of arterial tissue that consists of an endothelial monolayer that faces the arterial lumen. The media is found between the intima and adventitia and is composed primarily of vascular smooth muscle cells (VSMCs), elastin, and collagen type I, III, IV.²¹ The VSMCs in the media serve the function of contracting the artery to regulate blood pressure.²² The adventitia is the outermost layer of the artery. It is defined primarily by the presence of fibroblast cells, collagen type I and III, and elastin.²¹ Atherosclerotic plaque develops

within the intimal region, which faces the aortic lumen where lipids in the blood can enter through the permeable endothelium of the intima. ²³

In early plaque development, extracellular lipid accumulation occurs in the intima beneath the endothelial monolayer. ²⁴ These lipids primarily consist of oxidized low-density lipoproteins (LDL), which package cholesterol. ²⁵ The presence of oxidized LDL in the intima promotes endothelial recruitment of circulating monocytes into the plaque, which is driven by endothelial expression of pro-inflammatory signaling proteins, intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule (VCAM-1), and chemokine C-C motif ligand 2 (CCL2). ²⁶⁻²⁹ The presence of oxidized LDL also promotes endothelial expression of both macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF), which trigger differentiation of recruited monocytes into macrophages and promotes their local proliferation and survival within the plaque. ^{26,30-32} The macrophages phagocytose the lipid-buildup forming what are called “foam cells.” ³³⁻³⁵ Foam cells are primarily macrophages that have become highly enriched in LDL, which is stored as cytoplasmic liquid droplets generating a “foamy” appearance — hence the name. ³⁶ While macrophages possess LDL receptors, which have a negative feedback loop in terms of lipid uptake, LDL receptors do not recognize the oxidized LDL that accumulates in the intima. ^{37,38} When intracellular cholesterol levels are high, LDL receptor’s transcription factors (e.g. sterol regulatory element binding protein, SREBP-1 and -2) localize to the endoplasmic reticulum, where they cannot be cleaved into their activated state in the Golgi apparatus, and thus LDL receptor transcription is downregulated. ³⁹ However, macrophage scavenger receptors do

recognize oxidized LDL, including scavenger receptor class A 1 and scavenger receptor class B 3 (also known as CD36).⁴⁰⁻⁴³ Unlike the macrophage LDL receptors, scavenger receptors lack a negative feedback mechanism, which causes macrophages to consume unlimited amounts of oxidized LDL, leading to the foam cell formation.⁴⁴⁻⁴⁶

Foam cells experience dysregulation of lipid metabolism, resulting in the precipitation of cholesterol microcrystals, which activates the inflammasome to promote the cleavage and secretion of the mature proinflammatory cytokine interleukin-1 β (IL-1 β) into the extracellular plaque environment.⁴⁷⁻⁴⁹ Foam cells eventually undergo apoptosis or necrosis, contributing to build-up of cellular debris and expansion of the atherosclerotic plaque.⁵⁰ In addition to macrophages, T cells and mast cells infiltrate the plaque and contribute to propagation of the pro-inflammatory signaling.^{51,52} Vascular smooth muscle cells (VSMCs) can also migrate into the plaque, traveling from the media to the intima.⁵³ The vast majority of plaques are composed of monoclonal VSMCs, meaning that they proliferate from one VSMC progenitor that migrates into the intima.⁵⁴ VSMCs contribute to plaque growth (through proliferation and apoptosis), promote fibrosis, and can adopt new phenotypes that resemble that of macrophage or osteoblast cells that promote inflammation and calcification, respectively.⁵⁵⁻⁵⁸ This cycle of plaque growth and inflammatory responses leads to complex plaque evolution as observed by the heterogeneity of plaque content.

Atherosclerotic plaques exhibit tremendous heterogeneity not only between patients but also within a single patient.^{59,60} Some of the ways plaque composition is

known to vary includes: the degree of matrix metalloproteinases (MMPs) expression, the degree of macrophages present, plaque temperature, pH, and calcification.

Matrix metalloproteinases (MMPs) are proteases that are known for their role in degrading extracellular matrix proteins for remodeling of the extracellular matrix. Variations in the expression of MMPs in plaque among apolipoprotein-deficient mice *in vivo* is independent of plaque burden (indicated by Oil Red O staining).⁶¹ Variance in the mRNA expression of macrophage marker, CD68, in plaque and a correlation between the presence of CD68 and MMPs has been observed.⁶² *In vitro* studies support increased MMP expression in human macrophages stimulated under atherosclerotic-like conditions to be dependent on the key inflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B).⁶³ Macrophage density is positively correlated with plaque surface temperature in humans, suggesting a relation between inflammation and temperature in plaques.⁶⁴ Heterogeneity of plaque temperature *in vivo* has been observed in both humans and animal models.^{65,66} Local increases in temperature from inflammation is the result of increased blood flow being directed to the site of inflammation. This increase in temperature helps to mediated the inflammatory response by increasing the activity of transcription factor, NF- κ B.⁶⁷ Increased MMP-9 activity in plaques with higher surface temperatures has been observed *ex vivo* in rabbit aortas.⁶⁸ Macrophage density is positively correlated with plaque surface temperature in humans, suggesting a relation between inflammation and temperature in plaques.⁶⁴ This has also been observed in rabbit models for atherosclerosis.⁶⁶ In both humans and animal models, atherosclerotic plaques with higher temperatures were associated with

lower plaque pH.⁶⁴ This acidic environment in plaque is promoted by macrophages and VSMCs that preferentially manage energy production through glycolysis, which elevates proton concentrations in the extracellular environment of the intima.^{69,70} *In vitro* studies demonstrate that a more acidic pH environment promotes macrophage proinflammatory signaling through NF- κ B activation and enhanced phagocytic activity.^{71,72} Elevated plaque temperature among patients' coronary arterial plaques has been shown to be physiologically important as it is a significant risk factor for future cardiovascular events.⁷³

Mechanisms for Vascular Calcification

Vascular calcification was previously thought to be a passive process of serum calcium naturally precipitating over time.⁷⁴ More recently, growing evidence indicates that vascular calcification is an active biological process that requires the participation of multiple cell types, including macrophages and VSMCs, in a concerted and carefully orchestrated process. Macrophages appear to play an important early role in promoting the pro-inflammatory plaque environment that is critical for calcification. In a study using the apolipoprotein E deficient (*ApoE*^{-/-}), hyperlipidemia experimental mouse model of atherosclerosis, *ex vivo* imaging showed the localization of macrophage infiltration throughout the aorta during the early stages of atherosclerosis prior to and during calcification.⁷⁵ Aortas harvested during more advanced stages of atherosclerosis revealed intense calcification in regions of the aorta that paralleled the regions of intense macrophage infiltration. *In vitro* studies suggest that various macrophage-derived cytokines, such as TNF- α and IL-1 β , promote calcifying phenotypes among vascular

cells. ⁷⁶⁻⁷⁸ Recombinant human TNF- α and TNF- α from macrophage-conditioned cell media enhances the expression of osteogenic factors, bone morphogenetic protein 2 (BMP-2) and Msh homeobox 2 (MSX2), in human VSMCs and enhances calcium deposition. ^{76,77} BMP-2 is well known to play an important role in bone development by inducing osteogenic differentiation of mesenchymal stem cells but the exact mechanism remains to be elucidated. ^{79,80} MSX-2 is a transcription factor and also promotes osteogenic differentiation among mesenchymal stem cells. ⁸¹ Recombinant IL-1 β promotes the transcription of several osteogenic factors among primary *ApoE*^{-/-} VSMCs and enhances calcium deposition in a dose-dependent manner. ⁷⁸ These osteogenic factors include *RUNX2*, *MSX2*, *SRY-Box 9 (SOX9)*, and *osterix (OSX)*. ⁷⁸ Both *SOX9* and *OSX* are transcription factors that contribute to the differentiation of immature to mature osteoblasts. ^{82,83} Key mechanisms that regulate vascular calcification include: calcifying extracellular vesicles (EVs) derived from osteoblast-like cells or macrophages, calcification inhibitor proteins, and apoptotic bodies.

Calcifying Extracellular Vesicles (EVs)

Calcifying extracellular vesicles (EVs) are an important contributor to vascular calcification that can be generated by osteoblast-like VMSCs and macrophages in the plaque environment. ^{84,85} Calcifying EVs have also been observed in calcifying human aortic valves and medial tissue. ^{86,87} EVs are composed of distinct lipids, protein, ions, and small non-coding RNAs, which dictate their function and participate in a form of intercellular signaling and communication. ^{88,89} Calcifying EVs share some similarities to matrix vesicles (30-300nm), which are extracellular vesicles that participate in bone

mineralization by first initiating calcium-phosphate crystal formation internally and then exposing the crystal outside of its membrane for the crystal to grow and mature into hydroxyapatite, which is the key building block of bone.⁹⁰ Matrix vesicles contribute to normal bone development and are secreted by osteoblasts, chondrocytes, odontoblasts, tenocytes, and cementoblasts.^{91,92}

Both calcifying EVs from VMSCs or macrophages and matrix vesicles from osteoblasts are enriched in phosphatidylserine (PS), a calcium-binding negatively charged phospholipid, which serves as an anchor to secure annexins on the internal-facing surface, external-facing surface, or within the hydrophobic interior of the EV's membrane bilayer.^{85,92-95} Annexins are a multigene family of calcium sensitive PS-anchored membrane binding proteins and can bind to and regulate the influx of calcium.⁹⁶ Osteoblast matrix vesicles are abundant in annexins (I, II, IV, V, VI, and VII).⁹¹ In calcifying EVs annexins II, V, and VI are enriched.⁹⁷ They can function calcium ion channels located in the hydrophobic region of the EV's bilayer membrane to import external calcium and are similar with a predominately alpha helical structure with a hydrophilic pore.⁹⁸ In addition to EV's regulation of its internal calcium concentration, sodium-dependent phosphate transporters are used to gather phosphate ions into the lumen, which is commonly considered for calcifying EVs to acquire enough phosphate for hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) crystal formation to begin. Within calcifying EVs calcium and phosphate ions become concentrated and it is thought that mineralization may be initiated internally—similarly to matrix vesicles.⁹⁹ After nucleation, the orderly arrangement of

calcium and phosphate into a crystalline structure is ultimately held together by van der Waals attractive force. ¹⁰⁰

It is important to note that the localization of mineralization in or on the surface of EVs remains to be fully elucidated. Nano-sized crystals of hydroxyapatite have been observed within EVs found in atherosclerotic plaque of *ApoE* deficient mice. ⁷⁵ Furthermore, the formation of calcium deposits from EVs to plaque tissue, is directed to collagen through annexins to aggregate microcalcifications in the fibrous cap of plaque, which can increase the risk of plaque rupture. In time, the development of macrocalcifications of plaque can potentially serve as a plaque stabilizing force. ¹⁰¹ Different types of collagen regulate the rate of EV-mediated mineralization in normal bone tissue and in pathogenic vascular calcification in an annexin-dependent manner. ^{102,103}

Annexins are a class of 12 mammalian calcium-dependent, phospholipid-binding proteins. ¹⁰⁴ Annexins translocate bilayer membranes in a calcium ion dependent manner and can be found facing the lumen, inside the hydrophobic region of the bilayer, or on the external surface of the membrane. ^{105,106} The sensitivity to calcium concentrations differs among annexins. ¹⁰⁵ Annexin binding to PS enriched membranes on vesicles permits the formation of calcium channels that are capable of both calcium influx and efflux. ^{94,107} They also play a functional role in the regulation of coagulation, inflammation, exocytosis, membrane fusion, and the mineralization of cartilage and bone. ¹⁰⁸⁻¹¹³ Annexins II, V, and VI, which are abundant in calcifying EVs, will be discussed next. ⁹⁷

Annexin II contributes to actin-mediated vesicle trafficking, and it is important for the formation of matrix vesicles. ^{114,115} Human annexin II can also contribute to in osteoblast differentiation, proliferation, and responsiveness to cytokine (IL-4) signaling *in vitro*. ¹⁰⁴ However, its role in calcifying EVs has not been extensively investigated.

Annexin V in matrix vesicles promotes mineral nucleation of calcium and phosphate ions. ¹⁰³ However, it is not required for mineralization. ¹¹⁶ It is thought that the annexins locally concentrate the positively charged calcium on the vesicle membrane to help initiate the nucleation of hydroxyapatite with negatively charged free phosphate ions.¹⁰³ Studies of annexin localization in calcifying extracellular vesicles are limited. However, one study of annexin V suggests that annexin-mediate nucleation occurs on the external surface of the vesicle membrane facing the extracellular environment. ⁸⁵ Annexin V can bind to collagen type II and X and the uptake of calcium is dependent on the presence of collagen type II and X. ¹¹⁷ Annexin V derived from VSMCs show high affinity to binding collagen type II (although not exclusively). ¹¹⁸

Alkaline phosphatase (ALP) is a ubiquitous membrane-bound homodimeric glycoprotein that catalyzes the hydrolysis of pyrophosphate (PPi), an inhibitor of calcification, and converts it into inorganic phosphate that can then potentially bind to calcium for mineralization to take place. ^{119,120} Localization of ALP with annexin V on the vesicle membrane disrupted V's binding to collagen, and ALP cleavage is required before annexin V interacts with collagen to allow calcium deposition. ¹¹⁸ One clinical study demonstrated that annexin V enriched circulating matrix vesicles derived from endothelial

cells are associated with coronary artery disease and calcification compared to healthy controls. ¹²¹ Perhaps circulating matrix vesicles enriched with annexin V, which is known to promote osteogenic differentiation, could participate in the development of osteogenic-like cells in plaque. ¹²¹

Annexin VI is abundant in calcifying EVs derived from valvular interstitial cells in calcific aortic valve disease. ¹²² Annexin VI may potentially interact with collagen type I to promote mineral deposition. ⁹⁵ Annexin VI preferentially locates to the internal membrane layer of EVs at high internal calcium concentrations and binds PS on the membrane and thought to initiate the nucleation core for hydroxyapatite formation internally. ⁹⁵ Conversely, PS-anchored annexin VI is primarily located on the EV's external membrane surface when stimulated with high calcium concentrations and is also considered to have the potential to initiate crystal nucleation externally. ^{94,123} The exact localization of crystal nucleation in or outside of the calcifying EVs membrane remains unclear.

For the process of internal crystal formation, it is thought a high concentration of calcium ions are imported via annexin VI's function as a calcium ion channel. For this to occur EVs accumulate internal proton concentrations (H^+) and protonates annexin VI's carboxylate residues, which causes a conformation change from a helix-loop-helix motif to an exclusively helical motif that makes the protein hydrophobic and facilitates its translocation within the hydrophobic vesicle bilayer to form an ion channel. ^{106,124} Annexin VI's carboxylate residues can be deprotonated and relocate. When located to the membrane's external surface, annexin VI serve another function by binding to collagen

fibers (independently of calcium levels), which is thought to facilitated EV-mediated hydroxyapatite crystal formation preferentially at the collagen-rich regions of plaque. ¹⁰⁶

Macrophage Calcifying EVs

Transformed murine macrophages are capable of secreting calcifying EVs under tissue culture conditions involving calcifying media and an influx of external calcium ions into their intracellular compartment. These macrophages then released EVs with elevated calcium levels and ALP activity. ⁸⁵ This observation was also recapitulated using primary human macrophages derived from peripheral blood monocytes. ⁸⁵ Aikawa and colleagues observed that macrophages calcifying EVs were enriched with S100A9 and annexin V. ⁸⁵ S100A9 is a proinflammatory calcium binding protein. In macrophages, it is involved in the regulation of phosphorylation, nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) activity, and fatty acid transport. It is highly expressed in calcifying atherosclerotic plaques and enriched in EVs in humans and *ApoE* deficient mice. ¹²⁵ S100A9 can trigger RAGE- (receptor for advanced-glycation-end-products) and TLR4-mediated proinflammatory pathways. ¹²⁶⁻¹²⁸ Macrophage secreted calcifying EVs enriched with annexin V and S100A9 grew in size over time, which suggests that they may be potentially aggregating together. ⁸⁵ These macrophages exhibited a proinflammatory M1 phenotype when stimulated with pro-calcifying media as indicated by increased mRNA levels of nitric oxide synthase and interleukin-6. Annexin V and S100A9 were co-localized in EVs and pull-down of annexin V demonstrated that S100A9 interacts with annexin V under calcifying conditions. ⁸⁵ Phosphatidylserine (PS), which annexin V binds to, was found to be concentrated on the external surface of EVs suggesting that a

phosphatidylserine-annexin-V-S100A9 complex may form externally on these EVs and potentially act as nucleation sites.⁸⁵ Reducing or increasing S100A9 signaling correlated with EV calcium levels and the number of EVs that macrophages released *in vitro*.⁸⁵ Another study observed calcifying EVs from human serum had PS concentrated on the external surface of the membrane.¹²⁹

Macrophage-derived EVs can promote valvular interstitial cell calcification *in vitro*. These EVs promoted ALP activity and osteogenic expression (i.e. osteocalcin) in valvular interstitial cells (VICs). One study found that macrophage EVs containing miRNA-214 were able to modified VIC calcification *in vitro* by downregulating twist-related protein 1 (*Twist1*) in VICs. *Twist1* is a transcription factor and is thought to play a role in osteogenic differentiation.¹³⁰ This finding suggests that miRNAs may play an important role in the calcifying effect of macrophage EVs.¹³¹

Another investigation of macrophage-derived EVs found that cytokine stimulation via High Mobility Group Box 1 (HMGB1) can promote the release of calcifying EVs *in vitro*.¹⁰⁷ This pathway was mediated by Receptor for Advanced Glycation End-products (RAGE), p38 mitogen-activated protein kinases (p38 MAPK), and neutral sphingomyelinase 2 (nSMase2) signaling.¹⁰⁷ The secreted EVs exhibited elevated ALP activity and subsequently formed hydroxyapatite (a calcium-phosphate complex) internally.¹⁰⁷ As discussed S100A9, a protein known to trigger RAGE-mediate pathways also promoted calcifying EV secretion among macrophages *in vitro*.¹²⁹ Therefore, other

factors involved in RAGE-signaling may elucidate novel regulators of macrophage-derived calcifying EVs.

Osteoblast-like Cells & Calcifying EVs

Runt-related transcription factor 2 (*RUNX2*) is a bone-tissue specific master transcription factor that has been implicated in osteogenesis in the context of atherosclerotic plaque calcification in both *in vitro* and *in vivo* studies.¹³²⁻¹³⁴ The Runt family utilizes its runt domain at the promoter sites of target genes and consists of *RUNX1*, *RUNX2*, and *RUNX3*, which all participate in the stimulation of osteogenic genes.¹³⁵ In healthy bone tissues, it is a well-studied regulator that is required for multipotent mesenchymal stem cells to differentiate into immature osteoblast-like cells and is also important in the function of mature osteoblasts.^{83,136} Osteoblasts are the primary cell responsible for bone formation through the secretion of bone matrix proteins, such as type I collagen, and matrix vesicles, which resemble calcifying EVs.¹³⁷ The collagen acts as a scaffold for osteoblast-mediated mineral deposition of hydroxyapatite crystals.¹³⁸ Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a calcium-phosphate complex with a hexagonal structure that composes 65-70% of dry weight in bone—the remainder is composed of type I collagen, a variety of non-collagenous proteins, and lipids.^{139,140} Osteoblasts also express alkaline phosphatase (ALP), which hydrolyzes extracellular pyrophosphate (PPi) to generate inorganic phosphate (Pi) that can then bind to extracellular calcium ions to form hydroxyapatite.¹⁴¹ The supply of extracellular PPi and Pi, which are critical in regulating hydroxyapatite formation, are governed by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), progressive ankylosis protein (ANK),

ALP.¹⁴² While ALP converts extracellular PPi to Pi, ENPP1 converts extracellular adenosine triphosphate (ATP) into PPi and ANK acts as a membrane channel protein for intracellular PPi to be exported into the extracellular environment.¹⁴²

RUNX2-mediated associated osteogenesis of vascular calcifying tissue is observed in chronic kidney disease (hyperphosphatemia), valve disease, and atherosclerosis plaques.¹⁴³⁻¹⁴⁵ The exact source of these osteoblast-like cells is unclear, but there is evidence that supports several potential sources: (1) circulating bone-marrow-derived progenitor cells (2) VSMC-to-osteoblasts (3) endothelial-to-mesenchymal transition and osteoblast differentiation. In addition to calcifying tissue, these osteoblast-like cells also appear to promote hematopoiesis as indicated by the development of fully formed bone tissue containing marrow in the atherosclerotic plaques of patients.¹⁴⁶ Further research is needed to establish the degree to which each of these sources contribute to osteoblast-like cell formation in calcifying plaque.

Circulating Progenitors

In support of the hypothesis that circulating progenitors are supplying osteoblast-like cells in plaque, circulating osteocalcin-positive cells will be reviewed. Osteocalcin is an osteoblast-specific secreted protein found in bone and contributes to the organization of hydroxyapatite crystals into the bone matrix.^{147,148} A clinical study showed that circulating osteocalcin-positive cells correlated with bone growth. When osteocalcin-positive cells were cultured, they expressed ALP and formed calcium deposits in fibronectin-coated plates.¹⁴⁹ Another study identified that circulation CD34⁺ endothelial

progenitor cells expressing osteocalcin were significantly higher among patients with atherosclerosis compared to controls.¹⁵⁰ These osteocalcin-positive endothelial progenitor cells exhibited upregulation of RUNX2 and were also capable of mineralizing hydroxyapatite in culture. The study suggests that endothelial progenitors respond to distressed endothelia overlying the plaque to promote repair. CD34 is commonly accepted as a marker of cells derived from hematopoietic stem cells found in bone marrow.¹⁵¹ CD34⁺ endothelial progenitor cells have been shown *in vivo* to localize at sites of vascular distress.¹⁵²

Circulating levels of exclusively osteocalcin-positive endothelial progenitor cells are significantly elevated in coronary artery patients with a history of myocardial infarction.¹⁵³ However, when analyzing the total population of circulating endothelial progenitor cells (regardless of whether or not osteocalcin is expressed), the total population of progenitors decreases among patients with increasing risk factors for cardiovascular disease—demonstrating an inverse relationship between the total progenitor population and cardiovascular risk factors.^{154,155} This inverse relationship has been observed in both healthy patients and patients with a history of myocardial infarction with various risk factors.^{154,155} Collectively, these studies suggest that the total circulating endothelial progenitor population may be exhausted over time from responding to a continuously distressed plaque endothelium and that the remaining progenitor population becomes predominately osteocalcin positive, which could hypothetically contribute to increased vascular calcification *in vivo*.¹⁵³⁻¹⁵⁵ In contrast to this concept of progenitor exhaustion, increased exercise or pharmaceutical interventions among patients with coronary artery

disease has been shown to increase circulating progenitor cells.¹⁵⁶ However, it remains unclear if these interventions have any effect on osteocalcin expression in the progenitor cells or provides any clinical benefit by increasing total progenitor levels.¹⁵⁶

In *ApoE*^{-/-} mice, bone marrow from mice with advanced atherosclerosis exhibit reduced endothelial progenitor populations (CD34⁺) compared to controls while the total stem cell population remains similar.¹⁵⁷ The authors also used bone marrow transplant experiments demonstrating that donor marrow with reduced progenitor cells exacerbated atherosclerosis in the recipient. This finding suggesting that the exhaustion of these cell may be important in the progression of disease. Further investigation should establish if circulating progenitor populations with osteogenic potential—as indicated by osteocalcin expression—are being exhausted or down-regulated in patients with atherosclerosis and to what extent they contribute to osteoblast-like cells found in calcifying plaques.

Further investigation is important to clarify if the reduction in circulation endothelial progenitor cells is a result of exhausting this population or through some down-regulation mechanism mediated from atherosclerosis. More research is needed to establish if the total progenitor population is decreasing or only specific subsets of osteocalcin-negative progenitors. If only osteocalcin-negative progenitors are declining then it explains how osteocalcin-positive progenitors can be correlated with atherosclerosis but still have the total progenitor population expanded through exercise or pharmaceutical intervention. Finally, the degree to which circulating endothelial progenitor cells contribution to the osteoblast-like cell population in plaque, if at all, needs to be evaluated.

VSMC-Osteogenic Differentiation

VSMCs in the neointima of plaque have been observed to deviate from their normal contractile phenotype. The contractile phenotype of VSMCs observed in the medial layer is characterized by limited proliferation, migration, and an elongated spindle morphology.^{158,159} However, VSMCs in the neointima of plaque develop a synthetic phenotype characterized by enhanced proliferation, migration, hypertrophic morphology, and the ability to synthesize extracellular matrix components.^{158,159} VSMCs derived from both animal models and humans have been shown to deposit mineralized calcium in a manner similar to bone-forming osteoblast cells in the extracellular matrix of in vitro cellular cultures, and moreover, they upregulate expression of osteoblast-associated genes like *RUNX2* and *ALP*.^{78,160,161} Studies of calcifying plaque observed increased BMP-2, MSX2, and Wnt signaling (*Wnt3a*, *Wnt7a*).^{162,163} Wnt signaling pathways are an evolutionarily conserved group of paracrine or autocrine signal transduction pathways expressed in many cell types that is primarily known for its involvement in the regulation of stem cell fate during development, .¹⁶⁴⁻¹⁶⁶ Both *BMP-2* and *Wnt* signaling have been shown to be important in the regulation of *RUNX2* in osteoblast-like VSMCs in culture.^{143,167} These observations mimic the regulation of *RUNX2* in normal osteoblast-differentiating cells found in bone tissue.^{27,168,169} Significant up- or down-regulation of miRNA expression in calcifying plaques have also been observed, including miRNA-21, -27, -34a, -126, -146a, -155, -210). Several miRNAs have been observed in healthy bone tissue as critical to the down-regulation of *RUNX2* protein expression in mature osteoblasts and are capable of inhibiting osteoblast differentiation among mesenchymal

stem cells. (These miRNAs including miRNA-23a, -30c, -34c, -133a, -135a, -137, -204, -205, -217, and -338.)^{170,171} Additional investigation of plaque miRNAs is warranted to test if miRNA regulation of RUNX in calcifying plaque recapitulates the regulation of RUNX2 observed in healthy bone tissue.

Lipoproteins, proinflammatory cytokines, and oxidative stress which are all present in plaque, have been implicated in the upregulation of osteogenic gene expression in VSMCs. Oxidized LDL promotes ALP activity among aortic VSMCs through the upregulation of MSX2 in a Erk1/2-dependent manner (independently of RUNX2).¹⁷² Macrophage-derived cytokines, TNF- α and IL-1 β , have been shown to promote an osteogenic phenotype and calcification among VSMCs in culture.^{78,173} The proinflammatory cytokine, TNF- α promotes osteogenic-markers among calcifying human aortic VSMCs including BMP2, MSX2, and RUNX2.¹⁷⁴ TNF- α appears to regulated osteogenesis through a NF- κ B and MSX2-depedent manner.⁷⁷ These *in vitro* findings involving TNF- α have been supported *in vivo* using *LDLR*^{-/-} mice and further implicated Wnt signaling (Wnt3a/7a).¹⁶³ Another proinflammatory cytokine, IL-1 β , has been shown to promote calcification and osteogenic expression in primary murine VSMCs.⁷⁸ A major source of proinflammatory cytokines in plaques is thought to be from macrophages which are associated with calcifying plaque.⁷⁵

Primary aortic murine VSMCs exposed to elevated oxidative stress (via hydrogen peroxide) demonstrated increased expression of RUNX2, ALK, and osteocalcin, and calcium mineralization in an AKT-signaling dependent manner.¹⁷⁵ Oxidative stress can

induce an osteogenic phenotype in VSMCs of *ApoE*^{-/-} mice, which was RUNX2 dependent.¹³² Smooth muscle cell-specific *RUNX2* deficient *ApoE*^{-/-} mice (using a Cre recombinase under the control of mouse smooth muscle protein 22-alpha promoter), which demonstrated reduced calcification and macrophage infiltration of atherosclerotic plaque. In another study using *LDLR*^{-/-} mice, smooth muscle cell-specific *RUNX2* deficiency also resulted in reduced plaque calcification.¹³³ Oxidative stress is defined as an imbalance between the accumulation of reactive oxygen species (ROS) and a cell's ability to neutralize ROS and repair ROS-induced damage.¹⁷⁶ The accumulation of oxidized lipoproteins in the neointima are a major cause of oxidative stress in plaque. Therefore, oxidative stress may be a downstream effect of oxidized LDL that accumulates in the neointima.

The upregulation of osteogenic genes (e.g. RUNX2, MSX2) in VSMCs is associated with the generation of EVs and the downregulation of endogenous calcification inhibitors (including pyrophosphate and matrix Gla protein).¹⁷⁷ Osteoblast-like VSMCs generate calcifying EVs.¹⁰⁴ These calcifying EVs have increased ALP activity, increased annexin content, and calcium concentration.¹⁰² Mineralization requires EV formation and EV-binding to collagen for calcification, both processes require annexins.¹⁰² Another protein, sortilin (SORT1) regulates ALP levels in VSMC-derived calcifying EVs and modulates the degree of mineralization. SORT1 is a type I membrane glycoprotein that is ubiquitously expressed and functions as a receptor, co-receptor, and sorting protein to the plasma membrane and/or lysosomes.^{178,179} Phosphorylation of SORT1's C-terminus is critical to regulating ALP levels in EVs.¹⁸⁰ In *LDLR*^{-/-} *SORT1*^{-/-} mice, plaque (but not

bone) mineralization was reduced. When *LDLR*^{-/-} (*SORT1*^{+/+}) mice had their bone marrow replaced with bone marrow from *SORT1*^{-/-} mice, plaque calcification was not affected. Note that macrophages are hematopoietic lineage cells derived from bone marrow. Because hematopoietic derived cells deficient in *SORT1* did not affect vascular calcification *in vivo*, the authors hypothesized that macrophage *SORT1* signaling is not important in calcification.¹⁸⁰ Moreover, siRNA silencing of *SORT1* in human VSMCs reduced calcium deposition in culture and suggests that loss of *SORT1* may modulate calcification *in vivo* in a VSMC-specific manner.¹⁸⁰

Endothelial Mesenchymal Transition

Vascular endothelial cells have the ability to undergo a phenotypic transition to mesenchymal cells in a process termed endothelial to mesenchymal transition (EndoMT). Animal models have demonstrated this process in tissue under pathological conditions like atherosclerosis as well as during maintenance and function of healthy tissue.¹⁸¹ Mesenchymal stem cells *in vitro* differentiate into fibroblasts, VSMCs, chondrocytes, adipocytes, and osteoblasts.¹⁸¹ Because mesenchymal stem cells have the potential differentiated into osteoblasts, EndoMT may be a critical source for osteoblast-like cells observed in calcifying plaque.¹⁸² This transition is generally defined by reduced expression of endothelial protein markers like VE-cadherin and CD31 and increased expression of mesenchymal markers, including alpha-smooth muscle actin (SMA), N-cadherin, and calponin. Transcription factors involved in EndoMT, such as zinc finger protein SNAI2 (SLUG), have been well documented to upregulate the expression of alpha-smooth muscle actin (SMA), N-cadherin, and calponin.¹⁸³ In humans and animal

models, atherosclerotic plaques exhibit cells with markers for both endothelial and mesenchymal stem cell types. ^{181,184-186} Some of the signaling factors observed in promoting endothelial to mesenchymal transition include TGF- β 1, TGF- β 2, BMP-4, and oxidative stress induced by oxidized LDL. ^{182,187} Both BMP-6 and oxidative stress promote osteogenic differentiation of endothelial cells, leading to upregulation of RUNX2, MSX2, osterix, osteopontin, and calcification *in vitro*.¹⁸⁸ Shear stress generated from blood flow in the lumen of arteries can induce EndoMT by reducing the expression of fibroblast growth factor receptor 1 (FGFR1) and enhancing the expression of transcription factor, SLUG. ^{184,185} *In vivo* studies of atherosclerosis have not demonstrated a direct link between endothelial-to-mesenchymal transition and plaque calcification. ^{182,185,189}

During valvular calcification, a process that shares many similarities to atherosclerotic plaque calcification, valvular endothelial cells in culture have been observed to undergo mesenchymal transition, and when these cells are incubated in osteogenic media, the endothelial cells first transition into a mesenchymal phenotype and then into osteoblast-like cells with high expression of osteocalcin, osteopontin, and RUNX2. ¹⁸⁷ Valvular interstitial cells, which resemble VSMCs, appeared to have no effect on mesenchymal transition but inhibited expression of osteogenic markers. *ApoE*^{-/-} bone-marrow derived macrophages have also been shown to participate in the regulation of EndoMT in co-culture via the up-regulation of SNAIL mRNA. ^{190,191} Moreover, when macrophages are co-cultured with human endothelial cells, endothelial cells express significantly higher levels of pro-inflammatory cytokines, interleukin-6 and interleukin-8, in an IL-1 β dependent manner. ¹⁹² These findings suggest that in vascular tissue there is

potential coordination of multiple cell types in the regulation of osteoblast-like cell formation. Further studies of endothelial to mesenchymal transition in atherosclerosis are required to establish if it has any effect on vascular calcification and if so, further research will be important to answer if mesenchymal cells are differentiating into osteoblast-like cells in plaque.

Apoptotic Bodies

Apoptosis is enhanced in the plaque environment and is thought to play a role in the early stages of nucleation of calcium crystals.¹⁹³ The accumulation of apoptotic bodies and failure of efficient efferocytosis can lead to secondary necrosis of these apoptotic bodies. Efferocytosis is defined as the process by which apoptotic bodies are removed by phagocytosis. Secondary necrosis can then contribute to the formation of a necrotic core that adds to plaque growth.¹⁹⁴ Calcification is known to form at the site of the necrotic core.^{195,196} Secondary necrosis of apoptotic bodies releases cytokines and damage-associated molecular patterns (DAMPs) such as oxidized LDL that recruit macrophages and promote an inflammatory response in plaque.^{197,198}

Apoptosis is programmed cell death that results in the organized fracturing and disassembly of cellular components into small bilayer membrane vesicles formed from the plasma membrane. Unlike necrotic cell death, apoptosis is an organized process that retains the plasma membrane's integrity and avoids triggering a default inflammatory response.¹⁹⁹ The size of the apoptotic bodies can vary and have been reported to range from 50 nm to 5,000 nm in diameter.^{92,200} Apoptotic bodies can vary in their content,

containing distinct DNA, microRNAs, proteins, and lipids, which can selectively regulate the activities of other cells in the environment. ^{84,198,201,202} Apoptotic bodies play a role in regulating immune cell expression, antigen presentation, recruitment of phagocytes, and promoting phagocytic activity. ²⁰³

Triggers for apoptosis in plaque include death ligand and receptor signaling (TNF-Receptor), hypoxia (HIF-1 α), growth factor activation (IGF-1), cytokines (IL-4), and ER stress. ²⁰⁴⁻²¹⁰ ER stress mediated by the unfolded protein response (via CHOP) appears to be the primary cause of apoptosis in both humans and animal models of atherosclerosis. ^{211,212} While ER stress can be detected in early human plaque formation, robust apoptosis develops in more mature and unstable plaques. ²¹² Other stimuli such as oxidized LDL or inflammatory cytokines, which become more abundant over time, may help promote apoptosis among vulnerable cells undergoing ER stress via scavenger receptor or TLR2 signaling. ²¹³ In animal models of vascular calcification, ER stress-induced apoptotic signals (caspase-12 and C/EBP) are upregulated in aortic tissue and pharmaceutical inhibition of ER stress can reduce calcification both *in vivo* and *in vitro*.^{214,215} In diabetic patients, who have elevated risk for plaque calcification, high glycemic variability correlated with vascular calcification. Cultured VSMCs in diabetic media conditions demonstrated elevated ER stress-related apoptosis. ²¹⁶ In patients with chronic kidney disease, another risk factor for vascular calcification, ER stress was associated with calcification and interruption of ER stress effector, CHOP, inhibited vascular calcification. ²¹⁷ Collectively, these studies suggest that ER stress plays a role in apoptosis and may serve as an upstream mechanism for vascular calcification.

Apoptotic bodies are thought to directly contribute to vascular calcification by concentrating calcium and acting as a nidus for hydroxyapatite crystal deposition. ^{193,218,219} In human and animal models of atherosclerosis, nano-sized hydroxyapatite crystals appear to form in plaque apoptotic bodies. ^{75,220} The underlying mechanism by which apoptotic bodies promote mineralization is distinct from calcification induced by cell-mediated EVs. Similar to calcifying EVs, PS is primarily located on the exterior of apoptotic bodies facing the lumen. ^{85,95,129} Unlike calcifying EVs, apoptotic bodies can promote calcification without ALP activity and calcium ion channel-forming annexins. ¹²³ Apoptotic bodies contain less annexins relative to calcifying EVs and inhibition of the most abundant annexin, annexin V, has no effect on apoptotic body-mediated calcification while it inhibits EV-mediated calcification. ¹²³ Annexin V may still play a role in the local organization of calcification in plaque. In healthy cells, ~90% of PS in the plasma membrane faces the cytoplasmic side of the bilayer. ²²¹ However, during apoptosis PS is enriched in the extracellular facing side of the plasma membrane. ²²¹ The translocation of PS to the exterior via ATP-dependent scramblase activity also facilitates the translocation of PS-anchored annexin V to the extracellular surface of apoptotic bodies. ^{85,92,95 221} Annexin V has a high affinity for binding to collagen type II and X. ^{95,117} Therefore, apoptotic bodies may potentially direct calcification preferentially to the fibrous cap of plaque.

Apoptotic bodies contain both calcium and phosphate ions. These ions are concentrated into the cytoplasm that are contained within apoptotic bodies and are not

imported from their extracellular environment through annexin-mediated ions channels as observed in calcifying EVs.¹²³ This difference in calcium internalization also helps explain the lack of ion channel forming annexin proteins in apoptotic bodies and why inhibition of annexins doesn't affect apoptotic body mediated calcification.¹²³ The exact molecular mechanism for this nucleation of calcium and phosphate ions into orderly crystal structures within apoptotic bodies remains unclear.

In plaque, apoptosis can occur among VMSCs, macrophages, and endothelial cells.²²² In culture, human VSMC apoptosis occurs before calcification is observed and is important in modulating the degree mineralization. VSMC-derived apoptotic bodies can accumulate calcium and act as nucleating factors of calcium crystal formation.¹⁹³ Plaque-derived VSMCs from the intima exhibit increased sensitivity to TNF- α -induced apoptosis compared to controls, which may be a result of chronic ER stress that primes VMSCs to undergo apoptosis.²²³ In *ApoE* deficient mice with VSMC-specific inducible chronic apoptosis, significantly increased plaque calcification compared to controls. The rate of chronic apoptosis in these mice mimicked that observed in human plaques of ~1% turnover.²²⁴ Calcification was enhanced only in the intima and not the media, suggesting that VSMC apoptosis may be a mechanism specific to intimal calcification.²²⁵ In addition to the role of VSMC apoptosis in calcification, the loss of VSMCs through apoptosis occurs primarily near the fibrotic cap and disrupts VSMC-mediated stabilization of the fibrotic cap and promotes plaque rupture.^{226,227} VSMC apoptosis has been observed *in vivo* to release CCL2 and interleukin 8 (IL-8), which further promotes the infiltration of macrophages, leading to plaque growth and inflammation.²²⁸ CCL2 and IL-8 are well

known to drive the chemotaxis of myeloid cells (e.g. macrophages and neutrophils).²²⁹⁻
²³¹ This further infiltration of macrophages and inflammation can cause a cycle of repeated inflammation and apoptosis that feeds the plaque's necrotic core, promotes microcalcifications, and destabilizes the plaque's structure, which can lead to plaque rupture and thrombosis.

One clinical study of patients with coronary artery disease found that macrophages are the primary source of apoptotic cells in unstable plaques.²³² Both the rate of apoptosis and the total cellular population in unstable plaques was determined by macrophages.²³² In stable plaques, VSMCs exceeded the macrophage population. However, the rate of macrophage apoptosis was still greater than VMSCs but significantly reduced compared to the rate observed in unstable plaques.²³² This intrinsically heightened apoptotic activity among macrophages may be driven by foam cell formation. The phagocytosis of oxidized LDL can lead to dysregulation of cholesterol metabolism and promote ER stress, which induces apoptosis among macrophages in plaque.^{233,234}

The timing of macrophage-specific apoptosis also has an impact on the progression of atherosclerotic plaques. Several studies of both *ApoE* and *LDLR* deficient mouse models of atherosclerosis have demonstrated that macrophage-specific apoptosis mitigates early plaque size but promotes plaque size in advanced plaques.²³⁵⁻²³⁸ These results suggest that macrophages in early plaque slow disease progression, through the effective removal of lipids and apoptotic bodies in the intima. In contrast, mature plaque macrophages become foam cells, are ineffective in the removal of lipids and apoptotic

bodies, and ultimately undergo apoptosis, which contributes to necrotic core growth, further recruiting macrophages in a cycle of inflammation.

Calcification Inhibitors

Circulating EVs collected from the serum of healthy humans are capable of calcium deposition in cell-free cultures over time. Given that these EVs are from healthy individuals with no known calcifying disorders and are capable of calcium deposition may suggest that the loss of anti-calcifying factors may play a critical role in pathogenic calcification.¹²⁹ Several important inhibitors of calcification have been identified and play a role in vascular calcification associated diseases: pyrophosphate, matrix Gla protein, and fetuin-A.

Pyrophosphate

EVs possess ALP which generates free phosphate ions from pyrophosphate (PPi) in the extracellular environment.^{239,240} PPi in the extracellular environment is primarily generated from extracellular ATP that is hydrolyzed by ecto-nucleotide pyrophosphatase/phosphodiesterases (eNPPs), which are a type II transmembrane protein.²⁴¹ Mouse models for *eNPP* deficiency or dysfunction have increased vascular calcification.²⁴² VSMC intracellular pyrophosphate can be released into the extracellular environment through the membrane channel, progressive ankylosis protein (ANK).^{142,243} Ectonucleotide pyrophosphatase/phosphodiesterase family member 1 (*eNPP1*) globally gene-deficient mice have demonstrated reduced calcification and PPi levels.²⁴⁴ Outside of the plaque environment, PPi is found systemically and reduced levels of circulating PPi

are observed during vascular calcification.²⁴⁵ PPI not only inhibits the mineralization but also the degradation of hydroxyapatite, which may contribute to the development of osteoporosis in patients with vascular calcification.^{246,247} A major source of ATP for circulating PPI production is derived from hepatocytes via the ATP-binding cassette subfamily C member 6 (ABCC6).²⁴⁸ Global *ABCC6*-deficient mice have reduced circulating PPI and exhibit enhanced vascular calcification.²⁴⁹ In patients and rat models of chronic kidney disease, aortic calcification is associated with an initial increase in ALK, followed by a compensatory increase in the expression of local ANK and eNPP.²⁴² PPI is also a direct inhibitor of hydroxyapatite formation as seen both *in vitro* and *in vivo*.²⁵⁰ An extracellular environment enriched with free calcium and phosphate ions is not sufficient for mineralization without inhibitors such as PPI being downregulated.²⁵¹ Therefore, PPI does not inhibit calcification through the reduction of free phosphate ions but by directly inhibiting mineralization of calcium and phosphate ions. However, ALP activity may influence calcification two-fold by (1) increasing the availability of organic phosphate (2) eliminating the inhibitor effects of PPI.

Matrix Gla Protein

Matrix γ -carboxylated glutamic (Gla) acid protein (MGP) is a small (11 kD) vitamin K-dependent extracellular matrix protein.²⁵² It can be phosphorylated on three serine residues and undergoes carboxylation on five glutamic (Glu) acid residues, which fully activates MGP.^{253,254} Both phosphorylation and then the vitamin K-dependent carboxylation steps occur in the endoplasmic reticulum.²⁵⁵ Active extracellular MGP has high affinity binding to calcium, which is facilitated through its Gla residues.^{256,257} The

phosphorylation of MGP is primarily important for its release into the extracellular environment. ²⁵⁸ *In vitro* studies of MGP have indicated that it is capable of binding to hydroxyapatite and that its binding is mediated by both phosphate and Gla residues in a calcium ion dose-dependent manner. ^{259,260} MGP can inhibit hydroxyapatite nucleation and crystal growth. ²⁶¹

In healthy tissue, MGP is constitutively expressed by VSMCs and chondrocytes.^{262,263} MGP mRNA is also highly expressed in lung, heart, and kidney tissue, but its protein expression is much lower than that found in bone and vascular tissue. ²⁶⁴ In humans MGP mutations are rarely associated with vascular calcification but dysregulation of cartilage calcification has been observed. ²⁴² Patients with chronic kidney disease demonstrated elevated serum levels of the inactive form of MGP, dephosphorylated uncarboxylated MGPs (dp-ucMGP), which is associated with aortic calcification. ^{265,266} MGP is elevated in human atherosclerotic plaques, the primary sources appear to be plaque VSMCs and plaque macrophages. ^{267,268} VSMCs have a G protein-coupled cation sensing mechanism that can promote the expression of MGP whenever exposed to increased extracellular calcium levels. ²⁶⁹ Despite elevated expression of MGP protein, oxidized LDL-induced ER stress can contribute to reduced MGP activity by interrupting its post-translational modifications while not impairing its expression. ^{270,271} In the intima of plaque uncarboxylated MGP appears to be increased in EVs and associated with vascular calcification. ²⁷² Another study reported that non-calcifying EVs are loaded with MGP, while calcifying EVs lack MGP and have elevated calcium concentrations. ²⁷³ In culture, elevated intracellular calcium levels can promote

human VSMC-derived calcifying EVs with reduced MGP loading into these vesicles.^{84,94} It is important to note that MGP may potentially not be detected through antibody-mediated assays that are specific to the carboxylated conformation of MGP and should be considered in future studies.²⁷²

MGP has also demonstrated a role in the differentiation of pluripotent stem cells into chondrocytes and osteoblasts.^{274,275} In tissue culture, *MGP*-deficient endothelial cells undergo EndoMT and are more susceptible to BMP-induced osteoblast differentiation.^{274,276,277} MGP-based promotion of osteogenic differentiation can be mediated by Wnt/ β -catenin signaling pathway.²⁷⁸ Both Wnt and BMP signaling contribute to osteoblast differentiation through RUNX2.^{278,279} BMP-2 promotes the phosphorylation of SMADs 1, 5, and 8, which act as downstream signal transducers or transcription factors that directly promote RUNX2 expression.²⁷⁹⁻²⁸¹ *In vivo*, the MGP has also been demonstrated to inhibit BMP-4 using MGP-overexpressing transgenic mice.²⁸² MGP inhibition is facilitated through the direct binding of BMP-2 as indicated by immunoprecipitation.²⁸³ *MGP*-deficient mice die within 2 months due to arterial calcification that induces blood vessel rupture.²⁶³

MGP deficient macrophages undergo increased intracellular calcium flux.²⁸⁴ Increased macrophage influx of calcium is associated with the release of calcifying EVs.⁸⁵ Therefore, loss of MGP expression may be important for the regulation of macrophage-specific calcifying EVs.

Vascular endothelial cells have also been found to express MGP and inhibit BMP-2 and BMP-4. ^{283,285,286} Serum levels of BMP-2 among diabetic patients with coronary artery disease is positively associated with atherosclerotic calcification. ²⁸⁷ Also, aortic BMP-4 expression is increased in diabetic, *ApoE*-deficient mice. ²⁸⁸ In *LDLR*-deficient mice, small molecule inhibition of BMP signal reduces vascular calcification. ²⁸⁹

Fetuin-A

Alpha-2-Heremans-Schmid glycoprotein, also known as Fetuin A (48kDa), is a circulating hepatic-derived inhibitor of calcification. Among circulating anti-calcifying protein, fetuin-A is the most expressed—ranging from 0.5 to 1.5 mg/mL. ²⁹⁰ Fetuin-A undergoes a series of post-translational modifications including proteolytic processing, glycosylation, sulfurylation, and phosphorylation. ²⁹¹ However, unlike some calcification inhibitors fetuin-A does not require phosphorylation to effectively inhibit mineralization.²⁹² Fetuin-A inhibits mineralization by binding to both calcium and phosphate ions and preventing them from aggregating. ²⁹³

In patients with diabetes, serum fetuin-A is negatively correlated with many proinflammatory cytokines, and in hepatic cell line (HepG2) cultures treated with either TNF- α or IL-1 β , fetuin-A expression was significantly reduced. ²⁹⁴ As mentioned above, both of these cytokines have been implicated in promoting vascular calcification via a number of pathways. ^{78,173,295,296} Among patients with chronic kidney disease, serum fetuin-A levels are reduced and negatively associated with coronary artery calcification scores. ²⁹⁷

Along with this global reduction in systemic fetuin-A, the remaining supply of fetuin-A in patients is found to be preferentially concentrated in calcifying human and murine arterial tissue.^{297,298} The effectiveness of fetuin-A's calcification inhibition effects may be limited due to overwhelming pro-calcifying forces or due to potential changes to fetuin-A's structure via posttranslational modifications, includes proteolytic processing, glycosylation, phosphorylation, and sulfation.²⁹⁹ *In vitro*, the inhibitory effects of fetuin-A within a calcium-phosphate rich environment are time sensitive, suggesting that a chronically elevated levels of calcium and phosphate can overcome fetuin-A inhibition to eventually form crystal structures.²⁹² Fetuin-A can be internalized in VSMCs and found in the cytoplasm or packaged into EVs *in vitro*.²⁹⁸ Fetuin-A is associated with reduced vesicle calcium concentration in both EVs and apoptotic bodies.²⁹⁸ EVs containing fetuin-A enhanced their internalization by VSMCs.²⁹⁸ Endocytosis of fetuin-A by VSMCs is mediated by annexins and extracellular calcium concentrations and can modify the packaging of fetuin-A in EVs, which consequently effects their calcifying potential.^{300,301}

Fetuin-A inhibits VSMC-mediated calcification in a dose-dependent manner.²⁹⁸ Fetuin-A also reduces VSMCs apoptosis in a calcium-phosphate rich media.²⁹⁸ Reduced VSMC apoptosis may contribute to the reduced calcification.¹⁹³ This anti-apoptotic effect may be a result from fetuin-A mediated formation of protein-mineral complexes called calciprotein particles. Primary calciprotein particles help to package and isolate amorphous calcium-phosphate complexes (50-100nm) from aggregating into hydroxyapatite crystals.^{242,302} However, over time the primary calciprotein particles can

form secondary calcioprotein particles (>100nm), which contains hydroxyapatite but crystal growth remains impaired by fetuin-A.²⁴² These extracellular calcioprotein particles can be phagocytosed by macrophages via scavenger receptor signaling. However, plaque macrophages also phagocytose oxidized LDL via scavenger receptor signaling becoming foam cells and eventually undergo apoptosis.^{40,47,50} Therefore, it is possible that in atherosclerotic plaque these calcioprotein particles may not be effectively cleared by macrophages and partially explain the elevated levels of fetuin-A observed in calcifying arteries.³⁰³

Free hydroxyapatite crystals in cell culture can stimulate apoptosis of VSMCs by inducing excessive calcium influx.³⁰⁴ These fetuin-A containing calcioprotein particles can also slow down VSMC uptake of calcium/phosphate and allow more time for VSMCs to maintain intracellular homeostasis to mitigate apoptosis.³⁰⁵ Human VSMC cell cultures treated with synthetic calcium-phosphate complexes (30-60nm) induced IL-1 β maturation and secretion.³⁰⁶ Fetuin-A may act as an effective inhibitor of vascular calcification in the early stages of atherosclerosis, but as systemic pro-inflammatory activity is induced by plaque progression, cytokines like IL-1 β downregulate hepatic fetuin-A expression. The reduced fetuin-A, which preferentially localizes in plaque, is less effective at preventing mineralization. Then crystal formation occurs, promoting further inflammation and apoptosis and enhancing calcification. Therefore, a potential negative feedback loop may exist between systemic inflammatory signaling and the liver to downregulate anti-calcifying fetuin-A while further promoting a cycle of inflammation that enhances vascular calcification.

Macrophages incubated with fetuin-A-containing calciprotein particles can stimulate cytokine expression (TNF- α and IL-1 β) and apoptosis.³⁰² Calciprotein particles exhibit significantly less cytokine expression and apoptosis compared to hydroxyapatite-stimulated cells *in vitro*.³⁰² Therefore, fetuin-A also functions as an anti-inflammatory factor in atherosclerosis.³⁰²

Summary

Atherosclerotic calcification has been observed since ancient times and continues to significantly impact human health today. Many advances within the 20th century has led to a greater understanding of atherosclerosis as a pathogenic process initially driven by an accumulation of lipids within the intima of arteries. More recent investigation has also led to a new appreciation for the role of inflammation in plaque development. Inflammation appears to contribute to plaque instability and the development of calcification, which is associated with greater risk of mortality. The mechanisms by which inflammation and atherosclerotic calcification appear to intersect is through the development of osteogenic-like cells and macrophages that are capable of releasing calcifying extracellular vesicles (EVs) that directly contribute to the formation of hydroxyapatite. Also, apoptotic bodies that are primarily generated from macrophage “foam” cells can also contribute to mineralization but through a passive process rather than an actively regulated process observed with calcifying EVs. Another factor that is critical for plaque calcification includes the dysregulation of calcification inhibitors such as pyrophosphate, matrix Gla protein (MGP), and fetuin-A. Today atherosclerotic plaque

calcification is now understood to be driven by both an accumulation of lipids in the intima and chronic inflammation. These factors ultimately lead to plaque calcification through the development of calcifying EVs, apoptotic bodies, and dysregulation of calcification inhibitors. Despite this general understanding of what drives plaque calcification, the role of specific cell types and the signaling pathways that contribute to rise of these pro-calcifying mechanisms in plaque remains to be understood.

Chapter 2:

Introduction to Rac1 and Previous Findings on Rac2's effects on Vascular Calcification

Introduction to Rac1

Rac1, also known as Ras-related C3 botulinum toxin substrate 1, is a protein that is ubiquitously expressed in both human and mouse cells. ³⁰⁷⁻³⁰⁹ Rac1 is a small GTPase consisting of 192 amino acids (~21 kDa). ³¹⁰ Rac1 is a member of the Rac subfamily (4 proteins in mammals) of the larger Rho family of GTPases (20 proteins in mammals). The Rho family of GTPases belongs to the superfamily of Ras proteins, consisting of over 150 proteins in mammals).³¹¹ The Rac family consists of Rac1, Rac2, Rac3, and RhoG. ³¹² While Rac1 is ubiquitously expressed, Rac2 and Rac3 expression is more tissues specific. ³⁰⁹ Rac2 is primarily found in hematopoietic-derived cells, including macrophages, while Rac3 is primarily found to be expressed in neural tissue. ³¹³⁻³¹⁵ Rac1 are in an activated state when bound to guanosine-5'-triphosphate (GTP) and an inactivated state when bound to guanosine diphosphate (GDP). Activation is regulated by guanine nucleotide exchange factors (GEFs) or GTPase-accelerating proteins (GAPs). ^{309,316} Rac1 binding to GTP or GDP can also be regulated by accessory proteins such as Rho GDP-dissociation inhibitor 1 (RhoGDI1), which consequently controls Rac activity. ³¹⁷ The vast majority of Rac1 in most cellular contexts can be found in its inactive state in the cytosol where it is bound to RhoGDI1. ³⁰⁹ Rac1 has been shown to play a critical role in numerous cellular functions and in disease. ³¹⁸

Regulation of Rac1 Activity

Rac1 can be activated by interacting with a number of different GEFs, which exchange GDP for GTP, including but not limited to T-cell lymphoma invasion and metastasis 1 (Tiam1), triple functional domain protein (Trio), proto-oncogene vav (Vav1), guanine nucleotide exchange factor vav2 (Vav2), Rho guanine nucleotide exchange factor 2 (ArhGEF2), phosphatidylinositol 3,4,5,-trisphosphate-dependent Rac exchanger 1 protein (PRex1), and protein ECT2 (ECT2).³¹⁹⁻³²⁵ Each GEF has the ability to direct Rac1 activity along different downstream signaling pathways, which may in part help to explain how Rac1 can exercise such diverse functionality within the cell. Typically, downstream signaling is triggered by an effector protein's high affinity for the activated conformation of Rac1 bound to GTP. Rac1 activation by GEFs has been observed to occur at the plasma membrane and it is thought that Rac1 localization to the plasma membrane is required for GEF-mediated activation.³²⁶ Rac1's localization to the interior side of the plasma membrane is mediated by its post-translational modification by C-terminal isoprenyl group.³²⁶ Please see a visual depiction of the regulatory mechanisms of Rac1 activity in **Figure 2.1**.

GAPs inactivate Rac1 helping to catalyze the hydrolysis of GTP, which results in the products, GDP and inorganic phosphate (Pi). Common GAPs of Rac1 include Rho GTPase-activating protein 1 (RhoGAP1), Rac GTPase-activating protein 1 (RacGAP1), active breakpoint cluster region-related protein (ABR), and breakpoint cluster region protein (BCR).³¹⁸ In the inactive state Rac1 will then bind to RhoGDI1, which inhibits the intrinsic exchange of GDP for GTP to maintain Rac1's inactivity and shuttles Rac1 to and

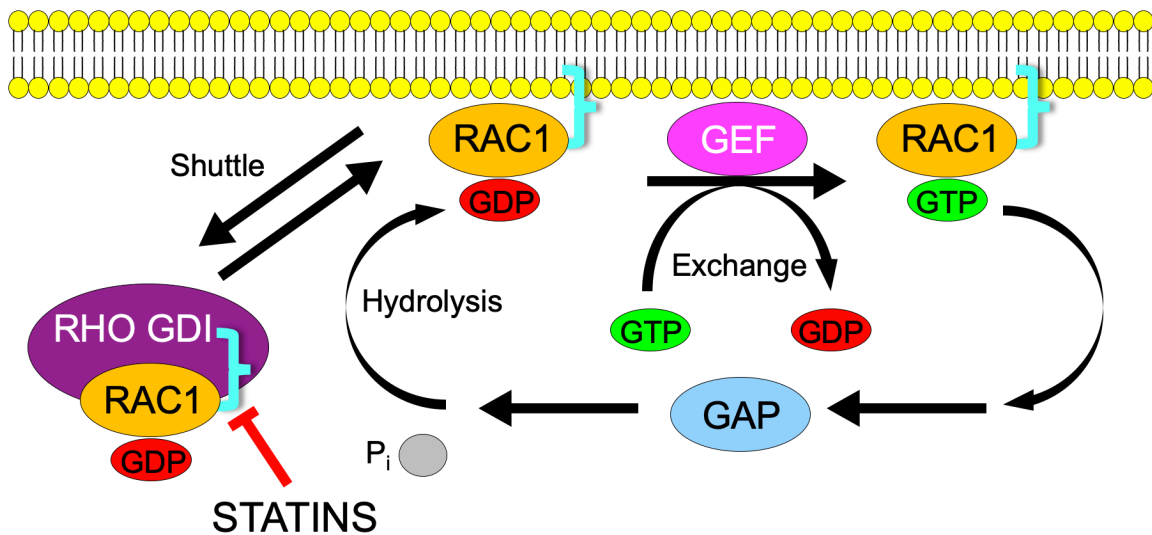


Figure 2.1 GTPase Regulation

The small GTPase Rac1 is activated when bound to GTP and inactive when bound to GDP. GEFs promote Rac1 activation at the plasma membrane, where Rac1 is anchored by its posttranslational isoprenyl group. GAPs promote inactivation by the hydrolyzing GTP to GDP and inorganic phosphate. Regulation of Rac1 includes binding by RHO GDI, which can prevent GDP release. Rac1 is primarily found in the cytosol in its inactive state and bound to RHO GDI. Additionally, RHO GDI regulates activation by trafficking of Rac1 to/from the plasma membrane where Rac1 can interact with GEFs.

from the cytoplasm.^{327,328} For Rac1 to be re-activated it is thought that RhoGDI1 shuttles Rac1 back to the plasma membrane where it can disassociate from RhoGDI1 and then interact with a GEF.³²⁸ Structural studies of Rac1 and RhoGDI1 suggests that binding is mediated through a hydrophobic pocket within RhoGDI1 that serves to contain Rac1's isoprenyl group.³²⁸ However, the role of Rac1 isoprenylation with regard to its association to RhoGDI1 in the cellular context has not been fully studied.

Isoprenylation of Racs has been shown to modify their activity.^{329,330} The regulation of Rac1 activity by changes to its post-translational isoprenyl modification has also been implicated in studies involving the use of statins.²⁹⁵ Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors and are a part of the current standard of care for preventing cardiovascular events.³³¹⁻³³³ Statin inhibition of HMG-CoA reductase disrupts cholesterol synthesis resulting in lower serum levels of cholesterol in patients.^{334,335} However, statins also disrupt the formation of precursors that are required for Ras and Rac protein's to be post-translationally isoprenylated.³³⁶⁻³³⁸ Please see **Figure 2.2**, which depicts that pathway by which statins disrupt both cholesterol synthesis and Rac isoprenylation. Studies of statin efficacy at preventing cardiovascular events suggests that lipid-lowering effects alone do not explain their efficacy and that there may be an additional, pleiotropic mechanism at play.³³⁹ The effects of statin-disrupted isoprenylation of Racs in the context of cardiovascular disease has been minimally investigated and may play an unknown role in impact of statin treatment. More specifically, its potential role in atherosclerotic calcification is also unknown.

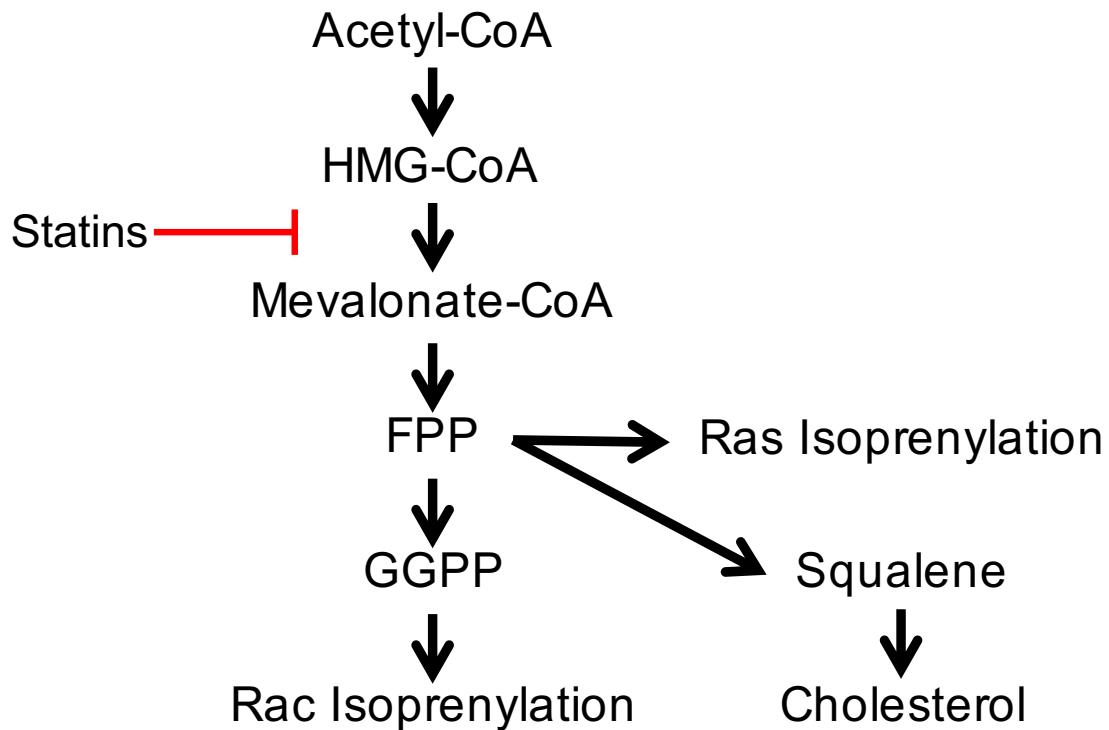


Figure 2.2. Statins disrupt Rac Isoprenylation

HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) is inhibited by statins and this disrupts the synthesis of both cholesterol and isoprenyl precursors. These isoprenyl precursors include farnesyl diphosphate (FPP) and geranyl-geranyl diphosphate (GGPP) synthesis. The geranyl-geranyl transferase enzyme is responsible for covalently attaching the isoprenyl group from GGPP to Rac1 postranslationally. Downstream of FPP, the cholesterol precursor squalene is generated but has no relation to Rac isoprenylation. Statin inhibition of HMG-CoA reductase disrupts both cholesterol synthesis and Rac isoprenylation.

Other post-translational regulation of Rac1 activity includes the ubiquitination, leading to proteasome degradation Rac1, which decreases both Rac1 activity and Rac1 protein concentration. ³⁴⁰⁻³⁴³ HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (also called *HACE1*) is a major regulator of Rac1 poly-ubiquitination at lysine 147 and it preferentially ubiquitinates Rac1-GTP. ³⁴²⁻³⁴⁴

Rac1 in Normal Cell Function

Rac1 plays numerous roles in normal cell functioning, including cell migration, cytoskeletal remodeling, cell cycling, cell signaling, and ROS production.³⁰⁹ Rac1 localization is thought to play a role in the regulation of its activity and may also be important in regulating Rac1's role in cellular functions. Rac1 has a polybasic region at the C terminus that acts as both a plasma membrane targeting motif and a nuclear localization sequence. ³⁴⁵ It has been found with in concentrated zones of the plasma membrane, diffused in the cytosol, and also within the nucleus. ³⁴⁶⁻³⁴⁸ While RhoGDI1 shuttles Rac1 to and from the plasma membrane and the cytosol, the mechanism for Rac1's nuclear localization is unknown. Rac1 is small enough (21kDa) to passively diffuse through nuclear pores. ³⁴⁷ However, Rac1's nuclear localization sequence is thought to be required to enter the nucleus if it were interacting with other proteins. ³⁴⁷ Nuclear import receptor, Karyopherin- α 2 (KPNA2), has been reported to bind to Rac1 and is dependent upon Rac1's nuclear localization sequence. ³⁴⁹ Furthermore, siRNA inhibition of KPNA2 expression in HeLa cells is sufficient to reduce the presence of Rac1 in the nucleus. ³⁴⁹

Rac1's role in cell migration and cytoskeletal remodeling is the result of Rac1's ability to help facilitate reorganization of actin. ^{350,351} Activated Rac1 is critical for many cell types to generate lamellipodia protrusions important for migration. ^{352,353} Rac1 localization to the plasma membrane facilitates its role in cell mobility. High concentrations of active Rac1 have been observed to appear at the plasma membrane where lamellipodia are generated and polarized in direction the cell is traveling towards. ³⁵⁴ Active Rac1 has been shown to localize both to the leading edge of actin where polymerization takes place as well as the trailing edge. Rac1 is critical to the formation of lamellipodia via its regulation of p21-activated kinase mediated actin polymerization. ^{352,355} Interestingly, Rac1 is not necessary for cell migration in some cell types, but it may be critical for others, indicating the possibility of redundant functions with other Rho GTPases. For example, macrophages do not appear to require Rac1 for migration but neutrophils do. ³⁵⁶⁻³⁵⁸ Given that neutrophils migrate up to 20 times faster than macrophages, it is thought that neutrophils may be more dependent on Rac1 to facilitate migration (via rapid actin polymerization) than macrophages. ³⁵⁶ Studies of Rac1 suggest that it may also play an important role in other plasma membrane functions such as Fc γ Receptor-mediated phagocytosis by creating F-actin-rich phagocytic cups and membrane ruffling, where Rac1 initiates actin polymerization at the plasma membrane to prime cells for cell migration activity.^{350,358-360}

Cell cycling has also been implicated in Rac1's functions. Activated Rac1's localization to the cytosol or nucleus during the G₂ phase plays an important role in regulating cytokinesis during mitosis. ^{361,362} In healthy cells activated Rac1 concentrations

have been observed to shift between the cytosol and the nucleus and are in sync with cell's dividing or not. ³⁶¹ *In vitro*, constitutively active Rac1 in the cytosol of fibroblast cells inhibits the transition from G₂ to mitosis while nuclear constitutively active Rac1 enhances cell division. ³⁶¹ This suggests that Rac1 plays a mechanistic role in mitotic cell cycling. Rac1 has also been shown to play an important role in cell cycling during early development. ³⁶³⁻³⁶⁵

Other studies have demonstrated that Rac1 can alter cell signaling. Most notably increased NF- κ B activity in various cancer cells is dependent on Rac1 activity. ^{366,367} Rac1 regulation of NADPH oxidase, which participates in ROS production, can act as downstream effector of Rac1 that can modulate NF- κ B activity to increase transcription of collagenase-1 in fibroblast cells. ³⁶⁸ Rac signaling has also been implicated NF- κ B dependent toll-like receptor 2 (TLR2) assembly and cytokine expression (TNF- α and IL-1 β) among macrophages. ^{78,369,370} Rac1 activity has also been implicated in regulating heat shock factor responses by modulating heat shock factor 1 transcriptional activity by promoting ROS production and to environmental stress, and can regulate NLRP3 inflammasome activation by altering NF- κ B activity. ^{370,371} ³⁷² There is still a limited understanding of Rac1 signaling and its relation to NF- κ B in proinflammatory macrophages. Moreover, *in vivo* studies of Rac1's effects in inflammatory cell signaling and vascular remodeling are limited.

Reactive oxygen species (ROS) production has been shown to be modified by Racs. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a plasma

membrane bound enzymatic complex facing the extracellular side of the bilayer. ³⁷³ NADPH oxidase interacts with extracellular oxygen (O_2) to generate and import superoxide anions (O_2^-) into the intracellular environment. ³⁷³ The assembled and active NADPH oxidase complex consists of a plasma membrane-bound flavocytochrome b558 (gp91_{phox}/p22_{phox}), three phosphorylated cytosolic subunits (p47_{phox}, p40_{phox} and p67_{phox}), and an active Rac-GTPase. ³⁷⁴⁻³⁷⁶ Rac1 is associated with macrophages while Rac2 is associated with neutrophils in NADPH oxidase formation. ^{373,377,378} Expression and activation of the NADPH oxidase is primarily observed among macrophages and neutrophils. Rac-dependent NADPH oxidase mediated ROS production has been implicated in the innate immune response to microbial infections. ³⁷⁹ However, there is still a limited understanding of the full scope of Rac1's functional relationship with ROS production. ³⁸⁰ For example, Rac1's role in ROS production has been demonstrated to modulate NF- κ B signaling, which is a known transcription factor for IL-1 β and the inflammasome protein, NLRP3. ³⁶⁷ Could macrophage ROS production be acting as a downstream effector of Rac1 to regulate inflammatory activity?

Rac1 in Disease

Given that Rac1 has a myriad of critical functions in healthy cells, it should be of no surprise to find that Rac1 has been found to play a role in many diseases. Rac1 has been shown to play a role in pathological conditions, involving inflammation, cancer, and neurological disorders. ³⁸¹⁻³⁸⁶ Regarding Rac1's role in inflammation in the context of disease, it has been found to be important in atherosclerotic plaque, chronic kidney disease, inflammatory bowel disease, and rheumatoid arthritis. ^{78,387-389}

Other studies have shown that Rac1 activity plays an important role in signal transduction of the mineralocorticoid receptor (MR) in models of chronic kidney disease, which is associated with robust vascular calcification. ³⁹⁰ MR translocation to the nucleus and its transcriptional activity is increased by Rac1 activity in kidney cells in culture. In Rho GDP dissociation inhibitor alpha (*Arhgdia*) deficient mice, a mouse model for chronic kidney disease, activated Rac1 levels are elevated in kidney tissue and treatment of these mice with Rac1 inhibitor, NSC23766, reduced signs of renal damage. This reduction in renal damage was associated with reduced MR-mediated downstream inflammatory signaling via reduced transcription of plasminogen activator inhibitor-1 (*Serpine1*) and chemokine C-C motif ligand 2 (CCL2). ³⁹⁰ Therefore, suggesting that Rac1 activity in kidney tissue is important in regulating MR-mediated inflammation, which contributes to tissue injury in chronic kidney disease. The mineralocorticoid receptor pathway has been shown to be upregulated by Rac1 in mouse models of obesity and diabetes. ^{389,391,392}

Given that Rac1 is a key regulator of both cell migration and cell cycling, it is no surprise that it can play a critical role in cancer. Dysregulation of Rac1 expression or activity primarily contributes to the formation and spread of cancer by permitting irregular cell growth and metastasis. ^{383,393,394} Rac1 can also aid cancer in its rapid proliferation by augmenting glucose intake. ^{395,396} This dysregulation of Rac1 has been observed to arise from the overexpression of Rac1, overactivation by Rac1 GEFs, and altered splice variant expression of Rac1. ³⁹⁷⁻³⁹⁹ Rac1 can be alternatively spliced to include exon 3b, which generates a constitutively active Rac1 protein (Rac1b). ³⁹⁷ (Rac1b is known to play a role

in the normal development of neurons. ⁴⁰⁰) Direct Rac1 mutations are rarely observed in cancer, unlike other Ras proteins in their family, but the dysregulation of Rac1 has been found in a variety of cancers but is more commonly observed in ovarian, skin (melanoma), lung, bladder/urinary tract cancers. ³⁹⁹ However, Rac1b-associated cancers are more associated with colon and breast cancer. ³⁹⁹ Small molecule inhibition of Rac1 activity using NSC23766, which exclusively interrupts the interaction between Rac1 and its GEFs, Trio and Tiam1, has been effective at inhibiting cancerous growth and metastasis in cell culture and animal models. ^{399,401,402}

Since Rac1 has been implicated in neural development, it has also been found to play a role in neurodegenerative disease. Rac1 is known to be responsible for neural growth and survival, which appears to be mediated by Rac1's role in cytoskeletal regulation through actin. ⁴⁰³ Abnormal Rac1 activity has been shown to be associated with diseases like Alzheimer's disease, familial ALS (amyotrophic lateral sclerosis), and Huntington's disease. ⁴⁰⁴⁻⁴⁰⁷

In cardiovascular disease, Rac1 has been implicated as a mediator of pathological ROS production, vascular smooth muscle cell proliferation, cardiomyocyte hypertrophy, and endothelial dysfunction. ³⁸⁶ As discussed previously, Rac1 plays an important role in ROS production, cell cycling, cytoskeletal regulation, and cell migration all of which play a role in cardiovascular disease in a variety of cell types. Rac1 activity in atherosclerotic plaques has been shown to promote ROS by enhancing NADPH oxidase activity in vascular smooth muscle cells. ^{408,409} In mouse diabetic mouse models, Rac1's promotion

of ROS has also been implicated in promoting vascular smooth muscle cell proliferation and migration. ⁴¹⁰ Likewise in cardiomyocytes, which are responsible for contracting the heart, Rac1-mediated production of ROS promotes apoptosis and myocyte hypertrophy in diabetic mice leading to progression of cardiomyopathy. ⁴¹¹ In other mouse models of cardiovascular disease, enhanced Rac1 activity in cardiomyocytes has been shown to promote cardiac hypertrophy and consequently myocardial infarctions. ^{412,413}

In vascular tissue, Rac1-dependent dysregulation of endothelial cell function has also been observed. ⁴¹⁴ Shear forces on vascular endothelial cells has been shown to trigger elevated Rac1 activity. ⁴¹⁵ In cardiovascular disease, enhanced shear forces often develop as atherosclerotic plaque disrupts the flow of blood. ⁴¹⁶ These forces have been observed to remodel the endothelial barrier in a Rac1-dependent manner to adapt to pathogenic shear forces in the vasculature. ⁴¹⁵

Rac2 & Rac3

Ras-related C3 botulinum toxin substrate 2 (Rac2) and Ras-related C3 botulinum toxin substrate 3 (Rac3) are also GTPase proteins and are from the same (Rac) subfamily as Rac1, and their activity is also dependent upon their binding of either GTP or GDP. Rac2 and Rac3 share the same GEFs and are thought to compete among each other to interact with a GEF to be activated. However, each GEF may have some preferential selectivity to one Rac. This has been demonstrated *in vitro* with Tiam1, which more strongly interacts with Rac2, compared to Rac1 and Rac3. ⁴¹⁷ Rac2 expression is primarily expressed in hematopoietic cells while Rac3 is somewhat ubiquitously expressed but significantly elevated in brain

tissue. ^{313-315,418,419} Rac1, Rac2, and Rac3 are 92% homologous. Despite these similarities, each Rac serves different roles in regulating cellular functions. A key distinction between Rac1 and Rac2/Rac3 is that Rac2/Rac3 lack a nuclear localization signal. ³⁴⁹ One study demonstrated in neurons that Rac1 and Rac3 exercise opposite effects on cell adhesion and morphology. ⁴¹⁹ These functional differences are attributed to differences in Rac residues (180-192) at the carboxyl terminus, which affects Rac's intracellular localization. In neurons Rac1 is predominately located in the cytoplasm while Rac3 is primarily found at the nuclear envelope. ⁴¹⁹ Substituting residues for Rac3 with the amino acid sequence found in Rac1 at the carboxyl terminus, resulted in Rac3 localization to the cytoplasm in neurons and mimicked the effects of constitutively active Rac1 on cell adhesion and morphology.⁴¹⁹

Because these proteins share many functional similarities, Rac2 and Rac3 have also been associated with a variety of cancers, as observed with Rac1. ⁴²⁰⁻⁴²² They primarily play a role in solid cancer cell invasion and metastasis. ³⁹⁹ In solid tumors, such as colorectal cancer, Rac1 abnormally accumulates in the nucleus and depletes the supply of cytoplasmic Rac1. This accumulation of nuclear Rac1 is responsible for changes to cancer cell morphology that enhance cancer's invasiveness. ⁴²³ It is thought to occur that disrupting the availability of cytoplasmic Rac1, which can then be activated and translocate to the plasma membrane, leads to abnormal regulation of the cytoskeleton in solid cancers that promotes invasion and metastasis . ³⁹⁹

Macrophage Rac2 modulates Atherosclerotic Calcification

Inflammation plays an important role in atherosclerotic plaque progression. Elevated systemic inflammation in patients is linked to risk of cardiovascular events.^{73,424} Coronary artery calcification has also been identified as an independent risk factor for cardiovascular events and mortality.^{17,18,425} Plaque infiltrating macrophages promote inflammation and are associated with plaque calcification *in vivo*.^{50,75} Animal models and cell culture studies have demonstrated that inflammatory signaling can contribute cell-mediated calcification.^{78,163,173} Although inflammation is associated with the formation of vascular calcification, the mechanisms contribute to atherosclerotic calcification are minimally understood.

Knocking down expression of Rac2 has been shown to cause elevated Rac1 activity in pro-inflammatory macrophages.⁷⁸ This balance of different Racs being expressed may intrinsically play an important role in their regulation and ultimately healthy function of tissue by modulating inflammation. We found that the Rac2-dependent elevation of Rac1 activity in macrophages led to enhanced IL-1 β secretion in serum and robust vascular calcification our mouse model for cardiovascular disease.⁷⁸ It is thought that a loss of Rac2 can lead to decreased competition between Rac1 and Rac2 for GEFs that promote the activity of both Rac1 and Rac2. Therefore, the loss of Rac2 would increase the availability of these shared GEFs to interact with Rac1. Thus, resulting in more Rac1 activity in the *Rac2* deleted cells, observed *in vitro* and *in vivo*.⁷⁸ Alternatively, one could argue that Rac2 has some unknown inhibitor effects on Rac1-specific GEFs or

promotes GAP or RhoGDI binding to Rac1 to keep it in its inactive state. More investigation is required to fully understand this dynamic between Rac1 and Rac2 activity.

Rac2-deletion in macrophages generate elevated Rac1 activity.⁴²⁶ Both Rac1 and Rac2 have been implicated in the regulation of pro-inflammatory cytokine expression.^{388,427} Rac1 activity in both macrophages and neutrophils can alter ROS production via NADPH oxidase and Rac1-mediated ROS production can act as a downstream effector which can trigger NF- κ B activity.^{370,380,428} Both ROS (via NADPH oxidase) and NF- κ B are activators of the NACHT, LRR, and PYD domains-containing protein 3 (NALP3 or cryopyrin), which is critical for NLRP3 inflammasome formation.

The NLRP3 inflammasome is a critical component of the innate immune response. It is responsible for activating caspase-1, which cleaves and converts immature cytokines into mature proteins, including IL-1 β , allowing them to be secreted into the extracellular environment.⁴²⁹ Cleavage by caspase-1 occurs either in secretory lysosomes or in the cytosol.⁴³⁰ After maturation, the IL-1 β can enter the extracellular environment by either being shuttled to the plasma membrane by secretory lysosomes or by directly diffusing through the plasma membrane.^{430,431}

The NLRP3 inflammasome is expressed in innate immune cells, primarily macrophages.⁴³² Its formation is driven by a two-step process consisting of priming and then activation. The priming step is driven by stimuli that trigger NF- κ B activity, which upregulates the transcription of NLRP3 and pro-IL-1 β .⁴²⁹ This priming step is necessary

for macrophages to accumulate enough NLRP3 and pro-IL-1 β protein in order for the inflammasome to be assembled and have a readily available substrate to begin processing. In atherosclerotic plaque, macrophages can be stimulated by oxidized LDL, which triggers toll-like receptor 4 (TLR4) signaling to promote NF- κ B translocation to the nucleus for transcriptional activity. ^{433,434}

After the priming step, additional stimuli are required to trigger the, secondary activation step, in which the NLRP3 proteins are assembled into the inflammasome complex. A diverse set of stimuli can trigger this secondary step including mitochondrial dysfunction, (NADPH oxidase independent) ROS production, and lysosomal damage. ⁴²⁹ In atherosclerosis, LDL-induced macrophage foam cell formation is known to enhance mitochondrial dysfunction via ROS-induced mitochondrial DNA damage. ^{435,436} The exact mechanism for mitochondria's relationship to inflammasome activation is not fully understood. However, oxidized mitochondrial DNA has been shown to bind to the NLRP3 protein and in TLR4-induced inflammasome activation oxidized mitochondrial DNA is required for inflammasome activation. ^{436,437} Another notable stimulus for inflammasome activation in atherosclerosis includes lysosomal damage. Macrophage phagocytosis of cholesterol crystals disrupt lysosomal membrane integrity, resulting in leakage of its content into the cytoplasm. ^{438,439} In general lysosomal membranes poor function in the isolation of crystals. The mechanisms connecting lysosomal damage to inflammasome activation remains unclear. ⁴²⁹

Given that Racs can regulate NLRP3 inflammasome stimuli such as ROS production, which can also subsequently regulate NF- κ B activity for priming, the Morrison lab became interested in the relationship between Racs and inflammasome. It was previously reported that *Rac2*-deletion in macrophages generates elevated Rac1 activity, and both Rac1 and Rac2 have been implicated in the regulation of pro-inflammatory cytokine expression. ^{388,426,427}. Given that NLRP3 inflammasome activity is required for IL-1 β maturation, the Morrison laboratory hypothesized that *Rac2* gene-deletion and consequent elevated Rac1 activation may enhance macrophage inflammatory activity through IL-1 β signaling and modulate the course of atherosclerotic plaque development.

The Morrison laboratory found among aortic tissue from both *ApoE*^{-/-} mice and human patients that calcification was associated with reduced Rac2 expression and increased IL-1 β expression. ⁷⁸ Because an inverse association between Rac2 and IL-1 β was identified in calcified atherosclerotic lesions from patients, *Rac2* gene-deleted mice were bred onto an *ApoE*-deficient background to determine whether this relationship was mechanistic and impacted atherosclerotic calcification. *Rac2* null mice on an *ApoE*-deficient background were bred and placed on a HFD. Aortic calcification from these mice were measured using a near-infrared bisphosphonate agent that preferentially binds to the bone forming mineral, hydroxyapatite. (For further details on the near-infrared agent used for imaging calcification, please see Methods: “Near-Infrared Aortic Calcification Imaging.”) Atherosclerotic aortic tissue in the setting of *Rac2*-deletion exhibited enhanced calcification. ⁷⁸ The Morrison laboratory hypothesized that the source of Rac2’s effects on vascular calcification may be derived from macrophages, which are generated from the

hematopoietic cellular population. Using a mouse bone marrow transplant experiment, loss of Rac2 from hematopoietic cells was shown to be responsible for both enhanced aortic calcification and systemic IL-1 β expression.⁷⁸ To investigate if IL-1 signaling is important for calcification *in vivo*, *ApoE*^{-/-} mice were treated with IL-1 receptor antagonist or vehicle control for several weeks on a HFD. Inhibition of IL-1 signaling resulted in significantly less aortic calcification compared to controls.⁷⁸ This finding further suggested that IL-1 β contributes to vascular calcification.

To elucidate the cell type responsible for elevated IL-1 β expression, immunofluorescent histology was conducted and demonstrated co-localization primarily between IL-1 β and a macrophage-specific marker (CD68).⁷⁸ Using inflammasome stimulated BMDMs with or without *Rac2* deletion demonstrated that loss of Rac2 expression enhances IL-1 β expression.⁷⁸ Inflammasome stimulated *Rac2*-deficient BMDMs also exhibited increased ROS production and NF- κ B activity compared to macrophages with endogenous Rac2 expression.⁷⁸

Other studies indicate have indicated that Rac1 activity was implicated in NF- κ B and ROS production, which can both promote IL-1 β expression and maturation.^{370,380,428} Therefore, the role of Rac2 expression on Rac1 activity was then investigated. Reduced Rac2 expression lead to enhanced Rac1 activity in BMDMs. To test the hypothesis that Rac1 activity was enhanced in *Rac2*-deficient macrophage and regulating IL-1 β expression, *ApoE*^{-/-} *Rac2*^{-/-} BMDMs were cultured and treated with increasing dosages of a Rac-inhibitor, EHT1864. IL-1 β protein was expression reduced in a dose dependent

manner by EHT1864, suggesting that in these cells Rac1 activity is important for promoting mature IL-1 β expression.⁷⁸ In order to study if IL-1 β plays a causative role in calcification, primary VSMCs were cultured with varying doses of recombinant IL-1 β protein and calcium deposition was then measured using alizarin red staining. The degree of calcium deposition was strongly associated with the IL-1 β dosage. This result suggested that IL-1 β does contribute to VSMC-mediated calcification.⁷⁸

In summary, this study of Rac2 led to a working model of inflammatory atherosclerotic calcification via a macrophage Rac-IL-1 β signaling pathway. Decreased Rac2 expression did not affect Rac1 expression but led to increased Rac1 activity, which in turn promoted the expression of mature IL-1 β and promoted VSMC-mediated calcification. It remains unclear though if Rac1 activity alone (without the loss of Rac2 expression) is sufficient to modulate vascular calcification *in vivo*. Furthermore, both *in vitro* and *in vivo* studies suggest that macrophage IL-1 β expression is associated with vascular calcification. However, the potential causal relationship between IL-1 β and calcification *in vivo* remains to be elucidated due to limitations of current experimental findings.

Chapter 3:

Methods, Logic behind Experimental Design and Detailed Protocols for Reproducibility

This dedicated chapter to the methods used in this study was created in the spirit of full transparency. The detailed protocols and specific reagents used should allow others to repeat the assays employed in this body of work and to further test the reproducibility of its findings if desired. Moreover, the reasoning for employing the specific methods described here are shared to better allow one to understand the logic behind the experimental design of this project.

Animal Use

The Providence VA Medical Center Institutional Animal Care and Use Committees approved all housing protocols and experimental animal procedures. All animal studies adhered to the guidelines for experimental atherosclerosis studies as described in the recent AHA Scientific Statement.⁶² All animal studies adhered to the guidelines as described in the ATVB Council Statement for considering "sex difference" as a biological variable.⁶³ *ApoE*^{-/-}, *CSF1R*^{mcm}, and *Rac1*^{fl/fl} mice on a C57BL/6 background were purchased at The Jackson Laboratory and have been previously described.^{43, 44, 61} These individual strains were then bred together to develop *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} mice. The Morrison laboratory also generated its own loxP flanked (i.e. floxed) *IL-1β* mouse on a C57BL/6 background with Brown University's Mouse Transgenic and Gene Targeting

Facility and bred it with *ApoE*^{-/-} and *CSF1R*^{mcm} to generate *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1β*^{fl/fl} mice.

Breeding *Rac1*^{fl/fl} and *IL-1β*^{fl/fl} animals with the *CSF1R*^{mcm} allows for tamoxifen-inducible myeloid-specific gene deletion of *Rac1* or *IL-1β*, respectively. *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} and *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1β*^{fl/fl} mice underwent intraperitoneal 0.1mL injection of either tamoxifen (2mg/day) or vehicle control solution daily for 10 consecutive days in order to induce gene deletion prior to being placed on a high fat diet. Tamoxifen solution was prepared in a ratio of 100mg tamoxifen to 1mL of EtOH to 4mL Corn Oil. Vehicle control solution was made the same way with 0mg of tamoxifen. The solutions were thoroughly vortexed before use.

ApoE^{-/-} (B6.129P2-Apoetm1Unc/J, Stock No: 002052) has been previously described.⁴⁴⁰ *ApoE*^{-/-} show a marked increase in total plasma cholesterol levels that are unaffected by age or sex. Fatty streaks in the proximal aorta can be found at 3 months of age on a normal chow diet and when placed on a high fat diet plaque formation is significantly increased.^{441,442} The lesions increase with age and progress to lesions with less lipid but more elongated cells, typical of a more advanced stage of pre-atherosclerotic lesion. Moderately increased triglyceride levels have been reported in mice with this mutation on a mixed C57BL/6 x 129 genetic background. Aged *ApoE*-deficient mice (>17 months) serves as a model for Alzheimer's disease as they have been shown to develop xanthomatous lesions in the brain consisting mostly of crystalline cholesterol clefts, lipid globules, and foam cells.⁴⁴³⁻⁴⁴⁵ Smaller xanthomas were seen in

the choroid plexus and ventral fornix. Additional studies indicate that *ApoE*-deficient mice have altered responses to stress, impaired spatial learning and memory, altered long term potentiation, and synaptic damage. ⁴⁴⁶

CSF1R^{mcm} (FVB-Tg(Csf1r-cre/Esr1*)1Jwp/J, Stock No: 019098) also known as Tg(Csf1r-Mer-iCre-Mer)1Jwp has been previously described. ⁴⁴⁷ They are transgenic mice express a Cre recombinase/mutant murine estrogen receptor double-fusion protein under the direction of the mouse Csf1r, colony stimulating factor 1 receptor, promoter. Tamoxifen-inducible Cre recominase activity is detected in bone-marrow-derived macrophages and yolk sac macrophages. When these Tg(Csf1r-Mer-iCre-Mer)1Jwp mice are bred with mice containing a loxP-flanked sequence of interest, tamoxifen-inducible, cre-mediated recombination will result in deletion of the flanked sequences in Csf1r-expressing cells; making them useful for studying inflammation, metastasis and tracking myeloid lineage cells. The MerCreMer double fusion protein consists of Cre recombinase flanked on each end with a mutated murine estrogen receptor (mer) ligand binding domain (amino acids 281-599, G525R); which does not bind its natural ligand (17'-estradiol) at physiological concentrations but will bind the synthetic estrogen receptor ligands 4-hydroxytamoxifen (OHT or tamoxifen) and, with lesser sensitivity, ICI 182780. Restricted to the cytoplasm, MerCreMer can only gain access to the nuclear compartment after exposure to tamoxifen. To counteract the mixed estrogen agonist effects of tamoxifen injections, which can result in late fetal abortions in pregnant mice, progesterone may be co-administered. ⁴⁴⁸ This study focuses on macrophage signaling, which is of myeloid lineage. One limitation of using the *CSF1R* promoter to knockdown

loxP flanked (i.e. floxed) genes in macrophages is that it also deletes floxed genes in other myeloid cells such as neutrophils. Alternatively, using a *lysozyme 2* promoter for a tamoxifen-independent Cre recombinase (LysM^{cre}) could have been used to knockdown floxed genes in myeloid cells. However, this Cre recombinase system is constitutively expressed in myeloid cells and would delete target genes in early development, which could have many unknown effects that may alter the development of atherosclerosis. In addition, neutrophils are still affected by LysM Cre recombinase. Therefore, an advantage of using this CSF1R tamoxifen-dependent Cre recombinase is that one can knockdown floxed genes after mice mature at the time of adulthood to avoid altering the normal development of the mice, which can act as a confounding factor in one's study. One common limitation to all myeloid Cre recombinase systems at this time is that neutrophils are affected as well as macrophages, limiting cellular selectivity.

Rac1^{fl/fl} (STOCK Rac1tm1Djk/J, Stock No: 005550) has been previously described.⁴⁴⁹ These mice possess loxP sites on either side of exon 1 of the targeted gene. Mice that are homozygous for this allele are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. When used in conjunction with a Cre recombinase-expressing strain, this strain is useful in generating tissue-specific gene-deletion mutants due to the floxed allele.⁴⁵⁰

The *IL-1 β ^{fl/fl}* mice were generated by CRISPR/Cas9 technology by Brown University's Mouse Transgenic and Gene Targeting Facility and were then crossed with a tamoxifen-dependent Cre recombinase under the control of the myeloid-specific CSF1R

promoter to generate a novel myeloid-specific tamoxifen-inducible mouse. LoxP sites on the *IL-1 β* gene were inserted upstream and downstream of exon 4 and designed in such a way that when tamoxifen is introduced, Cre recombinase cleaves these loxP sites and forms a stop codon that also prevents transcription of exon 5 and exon 6. ⁴⁵¹

High Fat Diet

Male and female animals, 8-10 weeks old, were fed a high fat diet (HFD) that was commercially available from ENVIGO. HFD (~20% fat by weight with ~1.5g/Kg cholesterol, ~42% of calories are from fat; ENVIGO TD.88137) was provided ad libitum for up to 24 weeks. *ApoE*^{-/-}, *Rac1*^{fl/fl}, and *CSF1R*^{mcm} or *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1 β* ^{fl/fl} mice underwent intraperitoneal injection of either tamoxifen (2 mg/day) or vehicle control solution daily for 10 days in order to induce myeloid *Rac1* or *IL-1 β* gene deletion prior to HFD treatment. Fat was derived from anhydrous milkfat.

For the statin study, male and female animals, 8-to-10 weeks old, were fed a high-fat diet that was commercially available from ENVIGO. Atherogenic diet (~20% fat by weight with ~12.5g/Kg cholesterol, ~42% of calories are from fat; ENVIGO TD.02028) or Atherogenic diet + atorvastatin (atorvastatin at dry weight concentration of 60mg/kg of HFD chow, ENVIGO TD.150052) was provided ad libitum for 20 weeks. The dosage of statin in the chow appeared to impact the immune response but did not impact chronic lipid metabolism as serum cholesterol and plaque lipid burden were relatively similar between treatment groups. The estimated daily mouse ad libitum dosing of atorvastatin

at 6mg/kg was near the recommended high-intensity statin dosing for humans (1.07mg/kg), and consistent with other studies that suggest a higher dose is required to have a more significant impact on cholesterol and atherosclerosis burden in mice fed this type of HFD for 20 weeks. ⁴⁵²

Aortic Tissue Harvesting

This is a terminal method to acquire mouse aortic tissue. Mice went under anesthesia using 1-3% inhaled isoflurane and were tested for appropriate level of anesthesia by responsiveness to toe pinching. Upon unresponsiveness to toe pinching, the mice were maintained under anesthesia and surgery began. Mouse fur was sterilized and dampened using 70% isopropanol to reduce hairs from spreading into the soft tissue of the mouse during surgery and to reduce the presence of bacteria/fungi from contaminating harvested tissue. Using clean surgical scissors, the skin of the mouse was dissected below the diaphragm/rib cage down to the mid-section of the mouse's stomach. Then, with the diaphragm exposed, a small cut into the diaphragm was made to access the heart. A 2mM EDTA coated syringe was gently penetrated into the heart at its apex and blood was gently extracted (to avoid lysing cells) using negative pressure.

Blood was then transferred to tubes containing 5µL of 2mM EDTA and gently inverted to mix. The blood was centrifuged for 15 minutes at 1,500 RPM at 4°C. The plasma at the top of the tube was collected and stored in -80°C, while the cellular pellet was discarded.

In addition to blood collection, the mouse's aorta was then harvested. Using sterile surgical scissors, the thoracic part of the aorta (from the diaphragm to the heart) was removed by cutting away the aorta's surrounding connective tissue. The aorta was ultimately removed with the heart fully attached to the aorta. To flush out blood within the aorta's lumen 1mL of 1x PBS was injected into the heart, twice. Lastly, 1mL of 4% paraformaldehyde was injected into the heart to spread into the lumen and fix the tissue. The aorta and attached heart were then placed in a tube filled with 4% paraformaldehyde. The heart, adipose tissue, and the aorta were then removed under the microscope using tweezers and again stored in 4% paraformaldehyde at 4°C. Some aortas could then be imaged for calcification as described in Near-Infrared Aortic Calcification Imaging section. After imaging, some aortas were then prepared for en face Oil Red O staining, and others for standard histology.

Near-Infrared Aortic Calcification Imaging

This study used the Licor IRDye® 680RD BoneTag™ Optical Probe to measure vascular calcification *ex-vivo*. This calcium-chelating probe is a bisphosphonate-conjugated near-infrared (NIR) agent, which has a very high affinity for binding to hydroxyapatite (HA).⁴⁵³⁻⁴⁵⁵ More specifically, it will covalently bind the exposed calcium (Ca^{2+}) component of HA through two of its carboxylic groups.^{456,457} HA is a mineral building-block that is commonly found in calcifying vascular tissue and in healthy bone formation.⁴⁵⁸ Bisphosphonates are preferentially incorporated into sites of active bone remodeling and can be used to mark osteogenic activity in vascular calcification (where HA deposition occurs).⁴⁵⁶ Bisphosphonates that are not incorporated are rapidly excreted

from the body through the renal system.⁴⁵⁹ Once incorporated they demonstrate long-term retention, one clinical study of severe osteoporosis detected bisphosphonates in bone for up to 8 years after treatment.⁴⁶⁰

Near-infrared fluorophores have an excitation range between 650-900nm wavelength.⁴⁵⁴ The Licor IRDye® 680RD BoneTag™ Optical Probe's excitation is at 680nm. Because absorption and scattering decrease as wavelength increases, NIR is more effective at penetrating deeper into tissue.^{461,462} The imaging depth for NIR detection of a target in tissue depends on multiple factors including the abundance of the target, the concentration of probe, the location of the target, and the sensitivity of the device used to measure NIR fluorescence.⁴⁶³ Reports of maximal imaging depth using NIR for in various contexts ranges from 3mm to 4cm.^{462,463} Additionally, NIR has significantly reduced tissue autofluorescence compared to visible light probes, which improves the signal-to-noise ratio.^{461,463,464}

The Licor Odyssey CLx Imager used in this study can measure at a depth of 4mm or less, which permits measurements through the full thickness of finely dissected whole murine aortas *ex vivo*. Using this approach, osteogenic calcification activity through the full depth of the whole aortic tissue can be measured by generate a two-dimensional image that shows the localization of HA deposition throughout the length of the aorta.⁴⁵⁴ Compared to other methods of measuring calcification, such as von Kossa (using silver nitrate) or Alizarin red S staining, they are less sensitive at detecting micro-calcification and their detection is limited to fixed tissue sections using paraffin histology.⁴⁶⁵ The NIR

probe described above can quantify calcification of whole murine aortas and binds specifically to HA.⁴⁵⁵ Staining with von Kossa is not necessarily specific to calcium in HA. It will bind to a wide variety of calcium salts beyond HA because it binds to a variety of anions (like phosphates, sulfates, or carbonates) found in calcium salts. Of note, von Kossa staining is speckled black and can often be confused with the black pigmentation of mice on the C57BL/6J background, reducing specificity for calcification. Alizarin red S binds more specifically to calcium deposits relative to von Kossa and is red, making the concern for false positive staining less likely.⁴⁶⁵

In summary, using NIR conjugated bisphosphonate probes that can bind HA deposited by osteoblast-like cells during the active mineralization of calcium in vascular tissue has several advantages. (1) It has high specificity to HA and preferentially binds to regions of active mineralization. (2) It is more sensitive at detecting aortic calcification than traditional staining methods (*i.e.* Alizarin red S) (3) Compared to other approaches this NIR has improved signal-to-noise ratio and tissue penetration. (4) It allows for the quantification of total vascular calcification through the full-depth and length a finely dissected murine aorta, resulting in less selection bias inherent to traditional histology. Where indicated, supportive histology was carried out with Alizarin red S staining rather than von Kossa staining to reduce the incidence of false positive results. False positive results with von Kossa staining can occur when using tissue from C57BL/6L mice because their tissue has a naturally occurring black pigment that is indistinguishable from von Kossa staining.

For quantification of aortic calcification, animals underwent intraperitoneal injection of 2nmol of freshly thawed IRI-Dye 680RD BoneTag Optical Probe (LI-COR) diluted in 100µL sterile phosphate-buffered saline (PBS) under sterile conditions in a tissue culture hood 48 hours prior to euthanasia with. The solution was protected from light using foil and brought to room temperature prior to injecting animals. After 48 hours animals were anesthetized using 1-3% of inhaled isoflurane, then euthanized by exsanguination through left ventricular puncture. After blood collection and euthanasia, residual blood was flushed out of the aorta using PBS via direct injection into the heart followed by intracardiac injection with 4% paraformaldehyde in PBS. After the aorta was gross dissected out of the mouse and fixed with 4% paraformaldehyde. After fixation for at least 24 hours, the aortas were finely dissected to remove primarily adipose tissue. Thoracic aortas were imaged *ex vivo* with the Odyssey Imaging System (LI-COR). Calcium was quantified with Image Studio Lite (LI-COR). Aortas were scanned using the following acquisition settings: 1) 700nm intensity of 3; 2) resolution of 21µm; 3) quality set to high; and 4) focus offset of 0mm. The visual presentation (*i.e.* LUTS thresholds) of the probe's signal in the aorta was set to "Pseudo Color" and the thresholds were arranged as a minimum of 50 and a maximum of 1500, with a kappa of 0.2. These values were previously determined as the dynamic range for the assay after imaging multiple known aorta test samples ranging from normal to severe calcification. Calcification was quantified by outlining the entire aortic area as the region of interest and determining the ratio of signal intensity to aorta area.

ELISA (Serum & Cell Culture Supernatant Samples)

IL-1 β , IL-1 α , and TNF- α were measured in either serum or cell culture media samples using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA), BioLegend's ELISA MAX™ Deluxe Set (catalog numbers: 432604 (IL-1 β), 433404 (IL-1 α), and 430904 (TNF- α)). Briefly, capture mouse antigen-specific monoclonal antibody (IL-1 β : Armenian hamster, IL-1 α : rat, TNF- α : Armenian hamster) is coated on a 96-well plate. Standards and diluted samples are added to the wells, and the target protein binds to the immobilized capture antibody. Next, a biotinylated polyclonal anti-mouse target protein detection antibody is added, producing an antibody-antigen-antibody "sandwich" (IL-1 β : goat, IL-1 α : rat, TNF- α : goat). Avidin-horseradish peroxidase is subsequently added, followed by TMB (3,3',5,5'-Tetramethylbenzidine) Substrate Solution, producing a blue color in proportion to the concentration of the target protein present in the sample. Finally, the Stop Solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450nm and 570nm with a microplate reader.

Samples were diluted 1:32 for serum and cell culture supernatant. The ELISA protocol was executed according to the manufacturer instructions as follows: (Day 1) Capture Antibody was diluted in Coating Buffer (1:200 dilution). Each plate required a solution made of 60 μ L Capture Antibody in 12mL of Coating Buffer. 100 μ L of Capture Antibody/Coating Buffer solution was added to each well in the plate(s). Paraffin was used to seal the plates and they were then incubated overnight at 4°C. (DAY 2) Wash Buffer was generated, 900mL PBS+0.05% Tween, and setup with an automatic washer device. The washer device was primed with water and then connected to a bottle containing the

PBS+0.05%Tween wash solution. The washer device was primed again using wash buffer. Wash settings were set for each plate to be rinsed 4 times using 300 μ L each time. A paper towel was setup near a sink to dry plates after each wash step. After day 1, the plate was taken out of the refrigerator and emptied into sink. The plate was blotted on paper towel to further dry it. The plate was then washed (4 times, 300 μ L each time with PBS+0.05%Tween) & dried plate against the paper towel again. 200 μ L of Assay Diluent was added in PBS to each well. The plate was incubated and put on shaker (Thermo Scientific #11-676-681) at 250 rotations per minutes (RPM) for 1 hour at room temperature to reduce non-specific binding to the target protein and ultimately reduce noise in the final readout of the assay.

During this time, the standard was prepared as follows: 74 μ L of suspended IL-1 β from the stock vial of IL-1 β protein at 27ng/mL in Assay Diluent was added to an Eppendorf tube with 926 μ L of 1x Assay Diluent. This tube contained 1mL of IL-1 β with a concentration of 2000pg/mL. Then, to create a series of dilutions for the standard, additional tubes were setup with 500 μ L of Assay Diluent (1x) in each. 500 μ L from the 2000pg/ μ L solution were transferred and mixed into 1 tube (containing 500 μ L of Assay Diluent) then 500 μ L was removed from that tube and mix into the next tube and so on until you dilute IL-1B to the lowest concentration you want (before zero). After the 1-hour incubation in assay diluent is over and the standard is prepared, the plate was washed (4 time, 300 μ L each time with PBS+0.05%Tween). 100 μ L of the appropriate standard solutions generated were then added to well in triplicate to generate a standard of known

target protein concentrations. This allows for the signal readout from the known protein values in the standard to be used to extrapolate the protein concentrations from the signal of the unknown samples being tested. The samples were then added to wells in triplicate for a total volume of 100 μ L. Note that the serum was diluted as follows in 1x Assay Diluent: Serum, 1:32 = 3.12 μ L Sample + 96.88 μ L Assay Diluent per well. The samples and standard solutions were incubated for 2 hours, sealed and placed on the shaker at room temperature. The plate was washed (4 times, 300 μ L each time with PBS+0.05%Tween). 100 μ L of diluted Detection Antibody was added to each well, seal, and shake for 1 hour at room temperature. (For 1 plate, 12 μ L Detection Antibody solution was diluted in 12mL of Assay Diluent.) The plate was washed again (4 times, 300 μ L each time with PBS+0.05%Tween). 100 μ L of diluted Avidin-Horseradish Peroxidase (HRP) solution to each well, seal, and shake for 30minutes. (For 1 plate, 12 μ L Avidin-HRP was diluted in 12mL of Assay Diluent.)

The plate then received a final extended wash: 5 times, 300 μ L (PBS+0.05%Tween), with 1 minute of incubation between each wash (and finally dry). Allowing 1-minute incubations between washes reduces noise by allowing time for unbound Avidin-HRP to diffuse into the wash solution. 100 μ L of TMB Substrate Solution (D) was added to each well—12mL for 1 plate. The plate was then cover in foil to avoid light from disrupting the enzymatic reaction and the plate was incubated for 15-30 minutes at room temperature for the reaction to generate blue products that are visible to the eye. The reaction was stopped by adding 100 μ L of 1M Sulfuric Acid (H₂SO₄) to each well—

12mL for 1 plate. The plate was analyzed by the plate reader within 15-30 minutes. Absorbance was measured at 450nm and at 570nm. The absorbance at 570nm was subtracted from the absorbance at 450nm to reduce noise. Using the absorbance values from the known protein concentrations that form the standard curve and multiplying by the dilution factor (1:32), the protein concentrations of the samples were quantified.

Serum Lipid Assays

Serum samples were analyzed at the Yale Center for Clinical Investigation (YCCI) for lipid concentrations, including total cholesterol, LDL, HDL, and triglycerides. All analyses of samples were conducted using an Alfa Wassermann Ace Alera chemistry analyzer and respective reagent kits.

Paraffin Embedded Histology and Brightfield Microscopy

As mentioned, harvested aortas were fixed and finely dissected to remove the surrounding adipose. A ~3mm segment of the tissue from the aortic root to the aortic arch was cut and prepared for paraffin embedded histology. Embedding and staining services were provided by Brown's Molecular Pathology Core. Traditional hematoxylin and eosin (H&E), Masson's trichrome, and Alizarin red staining protocols were performed on 10µm thick cross sections starting at the level of the aortic sinus, between 1400-1700µm from the most inferior section of the valve tissue facing the left ventricular outflow tract.

Per Brown's Molecular Pathology Core, paraffin embedding of aortas took place as follows: incubation with 70% ethanol for 1.5 hours, followed by incubation in 95%

ethanol for 1.5 hours, and then incubated in 100% ethanol for 1.5 hours. Next, aortas were incubated in xylene for 2 hours and then embedded in paraffin molds at 60°C for 2 hours. Tissue was cut into 10µm thick cross sections, placed on glass slides, and labeled before staining.

Hematoxylin and Eosin (H&E) staining; Tissue section slides were incubated in xylene for 5 minutes, submerged in 100% ethanol ~10 times, submerged in 95% ethanol ~10 times, submerged in 70% ethanol ~10 times, submerged in water repeatedly until water sheets off (a few seconds), and then incubated with hematoxylin II (Fisher #22-050-113) for 2 minutes. Slides were then rinsed with running water until clear for ~1 minute and then submerged in acid alcohol solution 2-3 times (0.5% HCL in 70% EtOH). The slides were then rinsed with running water until clear for ~1 minute and then submerged in ammonium hydroxide solution 10 times (0.2% aqueous solution) The slides were rinsed with running water until clear for ~1 minute and submerged in 95% ethanol ~10 times. This was followed by incubation in eosin Y solution (Fisher #22-050-110) for 4 seconds. Slides were submerged in 95% ethanol ~10 times, and then submerge in 100% ethanol ~10 times, twice. Slides were finally incubated in xylene for 5 minutes and mounting media was applied with coverslips.

Masson's Trichrome staining; Tissue sections on slides were incubated in Bouin's fixative (#26357-01) for 1 hour overnight at room temperature and then washed in running water until yellow color disappears, ~5 minutes. The slides were stained in Weigert's iron hematoxylin working solution for 5 minutes—equal parts Weigert's iron hematoxylin A

(#26367-02) and Weigert's iron hematoxylin B (#263667-03). Slides were washed in running water and incubated in Biebrich Scarlet-Acid Fuchsin (#26367-05) for 15 minutes. This was followed by incubation in aniline blue solution (#26367-06) for 15 minutes and washed again in running water. Afterward slides were incubated in 1% acetic acid (#26367-07) for 4 minutes. Slides were submerged in 95% ethanol ~10 times, and then submerge in 100% ethanol ~10 times, twice. Slides were finally incubated in xylene for 5 minutes and mounting media was applied with coverslips.

Alizarin Red S staining; Tissue sections on slides were incubated with 2% Alizarin Red Solution (2g alizarin red s, 100mL distilled water, adjust pH to 4.1-4.3 with 10% ammonium hydroxide) for ~2 minutes. Excess dye was gently blotted from the slides and then submerged in acetone 20 times. Afterwards slides were submerged in equal parts acetone-xylene solution 20 times and then washed in xylene for 5 minutes. Mounting media and coverslips were applied to each slide.

Brightfield microscopy imaging of stained tissue sections was analyzed using ImageJ. Quantifications of plaque area, plaque collagen area, and calcification area were carried out using ImageJ.⁶⁷ The total area of plaque as revealed by H&E staining was calculated. For Masson's Trichrome and Alizarin red S staining, the percentage of plaque area that was positively stain was calculated. Each individual data point represents the average quantification from the 3 aortic valve sinuses taken from the 3-4 adjacent sections per slide of on aortic sinus.

Aortic En Face Oil Red O Staining

A modified aortic en face Oil Red O staining protocol was used and was based on the work of Lichtman et al.⁴⁶⁶ En face staining involves staining of the intact luminal surface of thoracic aortas to quantify atherosclerotic lesions enriched with lipids. Oil Red O is a neutral lipid (primarily cholesterol). Images are acquired using brightfield microscopy, and conducted quantitative analysis using ImageJ to assess the percentage of the aortic luminal area that stained positive for Oil Red O. Please see detail protocols as follows: Aortas were fixed via 4% paraformaldehyde for 24 hours, and then finely dissected to remove primarily adipose covering the aorta. Each sample was washed overnight in 5mL PBS on a shaker (100 RPM) at 4°C. Samples were then cut using surgical micro-scissors within the lumen of the aorta longitudinally so that the aorta can be split open. Using small pins and a silicon-elastomer plates, the cut aortic tissue was then gently stretched open so that the luminal side of the aortic, where plaque forms, is exposed. The aortas are then incubated in 5mL propylene glycol for 2 minutes at room temperature. This dehydrates the tissue so that the staining solution can be effectively absorbed into the aortic tissue. Aortas were then incubated in 0.5% Oil Red O for 30 seconds. The solution was then removed and fresh 0.5% Oil Red O was added and incubated for 4 hours at 25°C. The aortas were washed three separate times with 85% propylene glycol for 1 minute per wash. Afterwards the aortas were washed three times with 5mL PBS and then incubate overnight in PBS on a shaker (100 RPM) at 4°C. Dissecting pins were then removed and the aortas were transferred from the plate to glass slides with mounting media. The aortas were sealed with a coverslip and nail polish, and properly labeled. Brightfield microscopy was used to image the aortas and a ruler was

used to generate a scale. ImageJ analysis was used to measure the total area of the en face aorta and the percent area that was positively stained for Oil Red O to measure plaque burden. ⁴⁶⁷

Submandibular Blood Collection

This blood collection technique was used exclusively on the *ApoE^{-/-} CSF1R^{mcm} Rac1^{fl/fl}* mice at weeks 0, 12, and 18 on HFD. The submandibular vein runs along the lower jaw near the mouse's cheek. ⁴⁶⁸ Multiple 200µL blood samples can be taken from this vein system overtime by alternating between the two sides of the mouse's face.⁴⁶⁸ Mice went under anesthesia for submandibular bleeding. Anesthesia was conducted by a metered-dose 1-3% inhaled isoflurane. Using a 5mm lancet to puncture the submandibular vein, 200µL of blood was collected from each animal using EDTA coated tubes to prevent clotting. Blood was centrifuged at 1,500 RPM for 15 minutes at 4°C. Plasma was then isolated and used for further analysis (*i.e.* ELISA and lipid analysis). During the study, mice were observed for decreased activity, poor food and water intake, abnormal gait or failure to groom. Significant impairment or evidence of systemic illness resulted in euthanasia according to IACUC protocol.

Bone Marrow Derived Macrophage (BMDM) Isolation and Culture

Bone marrow derived macrophages and cultures were carried out as previously described. ^{295,469} In brief, bilateral femurs and tibias of euthanized mice were dissected and sterilized in 10mL of 70% ethanol three times. Ethanol was then washed away with 25mL of Roswell Park Memorial Institute (RPMI) media three times. Bones were then

grinded using a mortar and pestle in 10mL of RPMI to extract the bone marrow cells. The 10mL of RPMI containing the homogenized bone was then passed through a 70µm filter. The grinding and filtration steps were repeated an additional two times to extract as much of the bone marrow cells as possible. The homogenized bone solution was then centrifuged at 1,500 RPM for 5 minutes at 25°C. The supernatant was removed and the pellet was re-suspended in 10mL of ammonium-chloride potassium (ACK) lysis buffer (150mM at a pH of 7.2-7.4) in order to lyse the red blood cells (RBCs). After 5 minutes of RBC lysing at room temperature, RPMI was added to dilute the ACK buffer and stop the lysis.

Complete-RPMI media was made as follows under sterile conditions: 500mL RPMI, 5mL 1M HEPES buffers solution (pH 7.3), 5mL Penicillin-Streptomycin-Glutamine, 50mL Fetal Bovine Serum, 1.75µL 2-Betamercaptoethanol. Complete L-Cell media was made mixing complete-RPMI media and L-cell conditioned media—described below—in a ratio of 2:1, respectively.

Enteroendocrine cells (or L-cell) conditioned media was generated by thawing cryopreserved living ATCC L-cell fibroblasts (ThermoFisher Scientific #50-188-658FP). L-cells were washed in 9mL of RPMO, pelleted at 1,500 RPM for 5 minutes, and supernatant was removed, This process was repeated for a total of 3 times. 5mL of RPMI was added to L-cells and then transferred to a T25 tissue culture flask and placed in incubator for 2-3 days until confluent. Media was aspirated and 4mL of 0.05% trypsin-EDTA was added and placed in an incubator (set to 37°C) for 5 minutes. 18mL of RPMI

was then added and 10mL was transferred to two T75 tissue culture flasks and incubated for 2-3 days until confluent. Media was aspirated off, 4mL of 0.05% trypsin-EDTA was added, and then placed in the incubator for 5 minutes. 23mL of RPMI was added and 10mL was transferred to four T75 tissue culture flasks. Cells were incubated for 2-3 days until confluent. Cells were removed again using 0.05% trypsin-EDTA and 5mL of suspended cells were added into twenty T150 tissue culture flasks. 4.75mL of RPMI was added to each flask and incubate for roughly 1 week until confluent. The L-cell conditioned media was then collected and passed through a sterile filter (ThermoFisher Scientific #5953320) into a sterile air tight bottle. The L-cell conditioned media was then aliquoted and store at -20°C.

The bone marrow cells were re-centrifuged at 1,500 RPM for 5 minutes at 25°C. The supernatant was removed and the pellet was resuspended in 10mL of complete-RPMI and passed through a 40µm filter. Viable bone marrow cells were then diluted, mixed with trypan blue exclusion solution, and quantified under a brightfield microscope using a hemocytometer. The cell suspension was then diluted to the desired concentration of 3.5 million cells per mL of complete RPMI. 1mL of cell suspension was added to 1 non-treated 100x20mm sterile culture dish (ThermoFisher Scientific #CC7672-3394) and an additional 6mL of L-cell media was added. Cells were cultured in an incubator at 35°C with 5% carbon dioxide for 7 days before harvesting. Note that 4 days after starting the culture, an additional 4mL of L-Cell media is added to each culture.

Cell cultures were derived from the bone marrow of mice that were either treated daily with 2mg tamoxifen or vehicle control via intraperitoneal injection for 10 days. The harvested cells were then treated with either 4-hydroxytamoxifen to induce floxed gene deletion via tamoxifen-dependent Cre-recombinase or vehicle control. 1mL of a 8 μ M 4-hydroxytamoxifen solution was added to each culture designated for gene deletion, resulting in a final concentration of ~1 μ M. For control cultures, 1mL of tamoxifen-free L-Cell media was added. Four days after harvesting the cells, this same process was repeated when additional L-Cell media was added to cultures to ensure gene deletion.

After 7 days, >97% of the cultured cells have differentiated into macrophages as previously described.^{295,469} Cells were removed from culture dishes using a serological pipette after incubating cells in a PBS-EDTA solution (500mL PBS 1x, 1mL EDTA 0.5M) for at least 2 minutes. Cells were collected in a 50mL tube and then centrifuged at 1,500 RPM for 5 minutes at 25°C. Supernatant was removed and cells were resuspended in 10mL of complete-RPMI. Cells were then counted as described above using a hemocytometer and diluted to a desired concentration and plated in complete-RPMI solution. Most experiments were conducted in 12-well non-treated sterile plates, 1.5 million cells were incubated per well in 1mL of complete-RPMI. (For the ROS assay, which involved cell culture on a 96-well plate, 150,000 cells were incubated per well.)

Cholesterol Crystal Inflammasome Assay

The inflammasome activation assay is carried out as previously published and validated.^{295,469} In brief, low-dose lipopolysaccharides (LPS) coupled with cholesterol

crystal exposure were used to mimic the *in vivo* environment of plaque in potentially activating the inflammasome and the consequent secretion of mature IL-1 β protein by macrophages. LPS is commonly found on the surface of gram-negative bacteria and acts as an endotoxin that triggers the innate immune response. The LPS used in this study was commercially acquired (ThermoFisher Scientific #00-4976-03) and derived from *E. coli*.⁴⁷⁰ Low-dose (10ng/mL) LPS was used to prime the macrophages for 2 hours at 37°C by stimulating toll-like receptor signaling. Cholesterol crystals (1,000ug/mL) mimicked the cholesterol crystals that form in advance plaque and are phagocytosed by the macrophages. Macrophages were incubated with the crystals for incubated for 12 hours at 37°C. Because the cholesterol crystals cannot be broken down in the lysosomes, lysosomal destabilization and degradation occurs, resulting in activation of the NLRP3 inflammasome.⁴⁷ In summary, cell culture was treated with or without 10ng/mL of LPS for 2 hours at 37°C and then treated with 1,000ug/mL of cholesterol crystals or vehicle control were added and incubated for 12 hours at 37°C to mimic the inflammatory plaque environment.

Cholesterol crystals were generated using 20mg of non-aggregated cholesterol (Sigma-Aldrich #C3045-5G) and mixing with 10mL of 1-propanol. Then, 15mL of water was added to initiate crystal formation and incubated at room temperature overnight. The solution was then centrifuged at 9,000g for 10 minutes. Supernatant was removed and the pellet was allowed to dry in a heated cabinet at 37°C overnight. The dry pellet is then removed and weighed. 50mg of cholesterol crystals should be acquired and then crushed

by mortar and pestle to an average size of 1-2 μ m. Then, the crystals were suspended 1mL of 0.1% FBS in PBS solution and vortexed until homogenous.

ELISA-Based NF- κ B Activity Assay

NF- κ B (p65) activity was assessed using the Abcam NF- κ B p65 Transcription Factor Assay Kit (ab133112) with the modification of isolation of nuclear protein prior to assessing activity. This approach involves a modified sandwich ELISA format, whereby immobilized double stranded DNA with NF- κ B seed sequences are used in place of a traditional capture antibody. A conceptual depiction of this process is shown in **Figure 3.1**. To acquire nuclear NF- κ B protein prior to activity assessment, a Nuclear Extraction Kit (ab113474) was used.

In brief, primary bone-marrow derived macrophage cells from 8 *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} mice (4 male, 4 female) were used as samples for this study. Protocols on cell culture are described in detail in the Bone Marrow Derived Macrophage (BMDM) Isolation and Culture section. Cells were cultured in the presence of 4-hydroxytamoxifen or vehicle control for 7 days. Cells were then primed with or without lipopolysaccharide (LPS; 10 ng/mL) followed by exposure to cholesterol crystals (1000 μ g/mL) or vehicle control for 12 hours—further details treatment conditions are described in the Cholesterol Crystal Inflammasome Assay section.

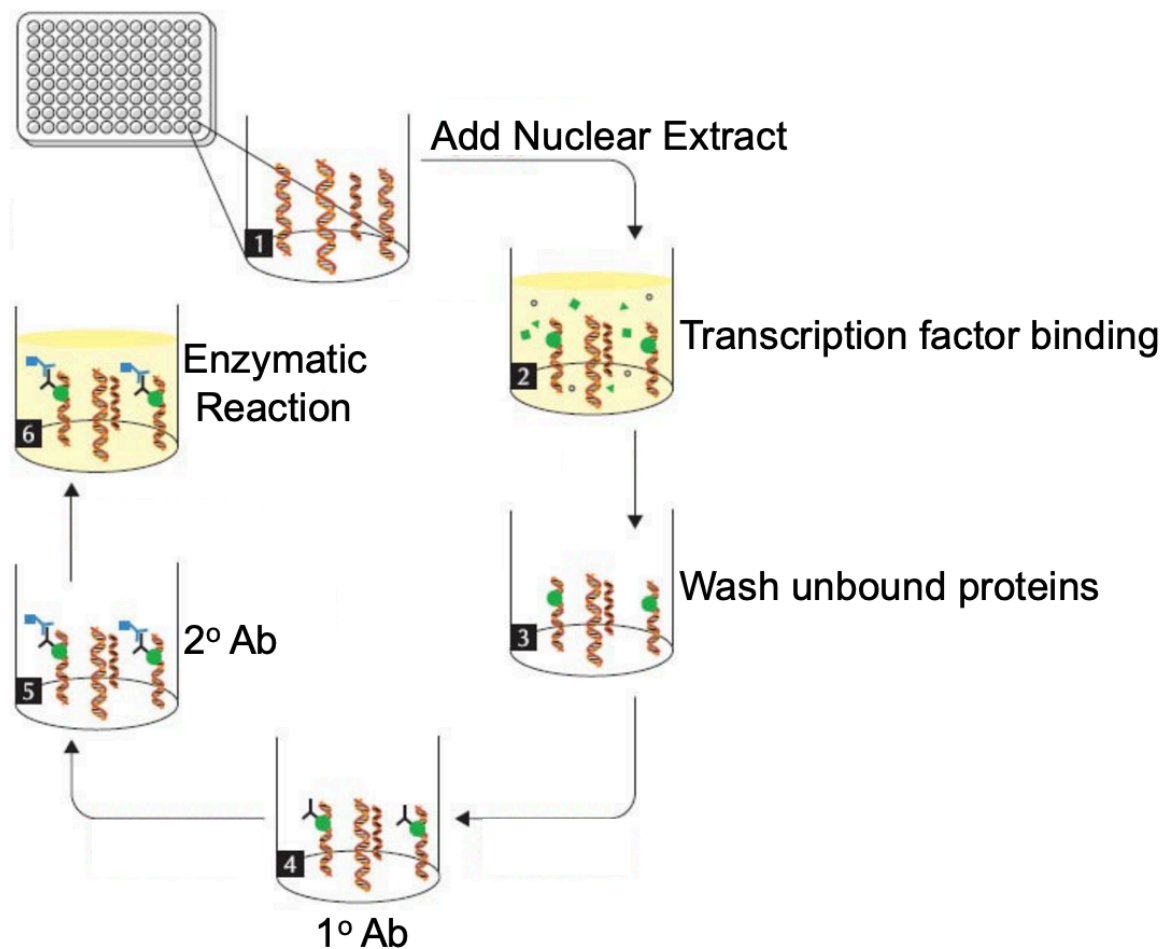


Figure 3.1 Conceptual Depiction of ELISA-Based NF-κB Activity Assay.

(1.) a 96-well plate coated with a double stranded DNA sequence containing the NF-κB response element is incubated with nuclear protein from samples (2.) samples incubate overnight to allow protein binding to the DNA (3.) unbound nuclear proteins are washed away (4.) primary anti-NF-κB antibody is added and binds to DNA-bound NF-κB (5.) horseradish peroxidase (HRP) conjugated secondary antibody is added (6.) a chromogenic substrate is added and is catalyzed by conjugated HRP, which generates a yellow colored product. The intensity of product present can be quantified by a colorimetric readout at 450nm.

To isolate nuclear protein and cytosolic protein separately, macrophages were washed with PBS three times to ensure the removal of protein in the residual media and to remove cholesterol crystals. Cells were then removed from the culture plate using a 1mM EDTA solution diluted in PBS and incubating for at least 2 minutes.

Please see detailed protocols as follows for nuclear protein extraction, protein concentration measurement, and assessing NF- κ B activity: Cells were centrifuge for 5 minutes at 1000 RPM and supernatant was discarded. The cell pellet was resuspended in 100 μ L of 1X Pre-Extraction Buffer per 10⁶ cells, and transferred to a micro-centrifuge vial. Cell were then incubated on ice for 10 minutes, vortexed vigorously for 10 seconds, and then centrifuged for 1 minute at 12,000 RPM. The cytoplasmic extract was gently pipetted away from the nuclear pellet. (The cytoplasmic protein fraction may be quantified and used for separate assays.) Dithiothreitol (DTT) solution and protease inhibitor cocktail (PIC) was added to the Extraction Buffer at a 1:1000 ratio. 900 μ L of Extraction Buffer containing DTT and PIC were then added to the nuclear pellet and incubated on ice for 15 minutes and vortexed every 3 minutes. The extract was further sonicated for 3 x 10 seconds to increase nuclear protein extraction. The suspension was centrifuged for 10 minutes at 14,000 RPM at 4°C. The supernatant was transferred into a new microcentrifuge vial. The protein concentration of the nuclear extract was then measured using Coomassie dye (ab119216). The concentration of nuclear protein for each sample was adjusted such that 10 μ L of solution would have the same concentration of total protein from all samples. To begin testing the nuclear extracts for NF- κ B activity, 96-well plates coated with double stranded DNA sequences containing the NF- κ B response

element was incubated with 90µL of Complete Transcription Factor Binding Assay Buffer (CTFB) solution with 10µL of adjusted/diluted nuclear extracts in duplicates. The plate was then covered and incubated overnight at 4°C without shaking.

Positive controls were generated by adding 90µL of CTFB with 10µL of provided clarified cell lysate (containing human p65). Wells were emptied and washed five times with 200µL of 1X Wash Buffer. The wells were emptied after each wash step. After the final wash, the plate was blotted on a paper towel to remove any residual Wash Buffer. Transcription Factor NF- κ B (p65) Primary Antibody was diluted 1:100 in 1X Antibody Binding Buffer (ABB). 100µL diluted NF- κ B (p65) Primary Antibody was added to each well. The Transcription Factor Goat Anti-Rabbit HRP Conjugate was diluted 1:100 in 1X ABB. Then, 100µL of diluted secondary antibody was added to each well and an additional 100 µl of Transcription Factor Developing Solution was added to each well at room temperature. The plate was then sealed and incubated for 30 minutes at room temperature on a shaker (100 RPM). The plate was protected from light with foil to prevent the enzymatic reaction from being disrupted, which generates a colored product. Afterwards 100µL Transcription Factor Stop Solution was added to each well to stop the enzymatic reaction in order to prevent too much colored product from being generated and exceeding the plate readers maximum colorimetric intensity reading. The solution within the wells changed from blue to yellow after adding the stop solution and absorbance was measured at 450nm. This measurement was taken within five minutes of adding the stop solution.

In brief summary please note that in order to comparably measure NF- κ B activity between samples, total protein levels were measured using the Thermo Scientific Pierce Coomassie (Bradford) Protein Assay (PI23200) and samples were diluted to have equal levels of total protein before measuring NF- κ B activity. The nuclear samples were normalized to have equal total protein concentration and were tested in duplicate. As controls, a positive control of NF- κ B (p65) was used along with several negative controls: blank wells, wells with no sample added, wells with sample plus a NF- κ B competitor, dsDNA. The colorimetric signal from each well was measured on a plate reader at 450nm.

Flow Cytometry

The Flow Cytometry and Sorting Facility at Brown University was used to measure fluorescence emitted by phagocytosed cholesterol crystals among primary *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} macrophage cells with endogenous Rac1 expression (vehicle control) or knockdown (tamoxifen). To generate, the fluorescent cholesterol crystals the same protocol was used as described in the Cholesterol Crystal Inflammasome Assay section with the addition of 5 μ L of TopFluor Cholesterol (Avanti Lipids #810255) to the cholesterol-1-propanol solution for a molecular ratio of 1:4000, TopFluor:Unlabeled cholesterol. TopFluor Cholesterol (or 23-(dipyrrometheneboron difluoride)-24-norcholesterol) is cholesterol attached to boron-dipyrromethene, a fluorescent compound with peak absorbance of 495nm and peak emission of 507nm (comparable to GFP peak emission). It has similar properties to endogenous cholesterol, and is evenly distributed throughout the precipitated cholesterol crystals. 471-473

Cells were pretreated with or without 4-hydroxytamoxifen ($\sim 1\mu\text{M}$) and then primed with or without lipopolysaccharide (LPS; 10 ng/mL) followed by exposure to cholesterol crystals (1000 $\mu\text{g/mL}$) or vehicle control as described in the Cholesterol Crystal Inflammasome Assay section but used the fluorescent cholesterol crystals described above (instead of standard non-fluorescent cholesterol crystals). The negative control group were cells treated with non-fluorescent (standard) cholesterol crystals. For quantitative analysis, the geometric means were generated from the histograms of fluorescent intensity and the number of corresponding cells with various intensities. The geometric means were then normalized to the unstimulated (no LPS and no cholesterol) vehicle control treated cells and analyzed. The geometric mean is used to describe a logarithmic data set (e.g. fluorescence from individual cells) with a normal distribution. The advantage of using the geometric mean compared to the mean is that its value is less sensitive to the effects of outliers that would normally skew the mean. Therefore, the geometric mean acts as an excellent representation of the distribution of the values measured. It is calculated as the root of the product of all values measured in a given data set.

Antibodies, Sources, and Concentrations

The following antibodies were used and their descriptions are listed as such: Target Antigen, Vendor, Catalog Number, and Dilution Used.

- Activated Rac1-GTP, New East Biosciences, #26903, 1:100 (1mg/mL)
- Anti-Actin Alpha-Smooth Muscle-cy3, Mouse Monoclonal, Sigma-Aldrich #C6198, 1:200 (1mg/mL)

- Anti-CD68 Rat Monoclonal, BioLegend, #137002, 1:100 (0.5mg/mL)
- Anti-Alkaline Phosphatase Tissue Non-Specific, Abcam, #Ab65834, 1:500 (0.5mg/mL)
- Anti-Rac1, Abcam, #Ab97732, 1:100 (1mg/mL)
- Goat Anti-Rat IgG H&L (Cy5), Abcam, #Ab6565, 1:500 (1mg/mL)
- Goat Anti-Mouse IgG, IgM (H+L) Alexa Fluor 488, Invitrogen, #A10680, 1:500 (2mg/mL)
- Anti-RUNX2, Abcam, #Ab76956, 1:100 (1mg/mL)
- Goat Anti-Mouse IgG2b Alexa Fluor 488, Invitrogen, #A21141, 1:500 (2mg/mL)
- Goat Anti-Rabbit IgG Alexa Fluor 488, Invitrogen, #A27034, 1:500 (1mg/mL)
- Goat Anti-Mouse IgM Alexa Fluor 488, Invitrogen, #A21042, 1:500 (2mg/mL)

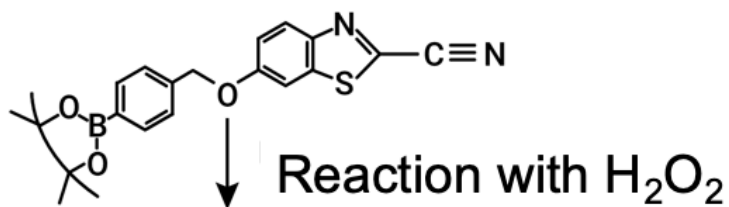
Reactive Oxygen Species (ROS) Assay

The ROS-Glo H₂O₂ Assay by Promega (G8820) was used to quantify hydrogen peroxide (H₂O₂) production, a reactive oxygen species (ROS), directly in cell culture of primary *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} macrophage cells with endogenous Rac1 expression (vehicle control) or (tamoxifen induced) Rac1 knockdown. Protocols on cell culture are described in detail in the Bone Marrow Derived Macrophage (BMDM) Isolation and Culture section. Cells were pretreated with or without 4-hydroxytamoxifen (~1μM). Cells were collected and diluted to a concentration of 1.5 million cells per 1mL. Then, 100μL of suspended cells were added to each well in a 96-well white plate. (Thus, culturing 150,000 cells per well.) Cells were incubated for 2 hours at 37°C. Cells were later treated with LPS (10ng/mL) and incubated for 2 hours. Then, cells were treated with cholesterol crystals

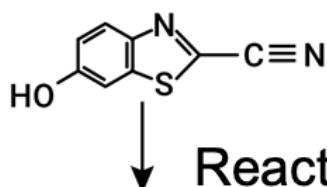
(1000 μ g/mL) and incubated for 30 minutes at 35°C. Each condition/treatment group was done in triplicate (3 wells). A positive control group was formed by treating cells supplementing media with 50 μ M of menadione, which causes oxidative stress and induces ROS production. A negative control group was formed using wells without cells to indicate any potential background signal. Cells in each well were treated with 20 μ L of H₂O₂ substrate solution was added and incubated for 2.5 hours at 35°C. After incubating 100 μ L of detection solution was added and incubated for 20 minutes at room temperature before recording luminescence units using a plate reader to indicate the degree of ROS production.

The H₂O₂ substrate solution reacts directly with H₂O₂ to generate a synthetic luciferin precursor. Detection solution, which contains D-Cysteine and recombinant luciferase, is added. The luciferin precursor that formed in the presence of H₂O₂ is then converted to luciferin via its interaction with D-Cysteine. The luciferin reacts with the recombinant Luciferase to generate a luminescent signal that is proportional to H₂O₂ concentration in each well. Luminescence was quantified using a plate reader. A summary of this approach is illustrated in **Figure 3.2**.

H₂O₂ Substrate



Luciferin Precursor



Luciferin

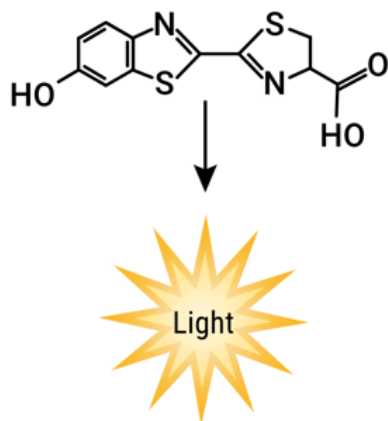


Figure 3.2 Conceptual Depiction of ROS Assay.

In live cell culture, hydrogen peroxide (H₂O₂) is detected by reacting with a H₂O₂-substrate molecule (PBI4472) that reacts with hydrogen peroxide to form a luciferin precursor (CBRT). A detection solution is then added, which converts the luciferin precursor into mature luciferin, which emits light and then luminous intensity can be quantified using a plate reader. (All reagents are propriety by Promega Corporation and the information presented here is the full extent of publicly available information.)

Immunofluorescence Staining & Imaging

In brief, a circular 10mm sterile glass coverslip (0.17mm thick) onto the bottom of each well of 12-well plate. Cultured BMDMs are diluted in Complete RPMI to contain ~500,000 cells/1mL. 1mL of cells in suspension were added to each well. Cells were pretreated with or without 4-hydroxytamoxifen (~1 μ M) and then primed with or without lipopolysaccharide (LPS; 10ng/mL) followed by exposure to cholesterol crystals (1000 μ g/mL) or vehicle control. After incubating, the cell media was aspirated (or collected and stored for ELISA) and cells were washed with PBS 3 times to remove debris and excess cholesterol crystals. To fix the cells in place, 1mL of 4% paraformaldehyde was added to each well and incubated for 15 minutes at room temperature. Each well was washed again 3 times with PBS. 1mL of 0.1% Triton X-100 solution in PBS was added to each well and incubated for 10 minutes at room temperature to permeabilize the cells. Wells were washed 3 times with PBS. 1mL of 5% goat serum (diluted in 0.1% Triton X-100 solution in PBS solution) was added to each well as blocking buffer to reduce non-specific antibody binding. Wells are again washed in 0.1% Triton X-100 solution in PBS, 3 times. Primary antibody solution (New East Biosciences #26903) was diluted 1:100 in 5% goat serum (diluted in 0.1% Triton X-100 solution in PBS solution) and 15 μ L was added to each well, sealed in parafilm, and incubated overnight at 4°C.

The next day, wells were washed in 0.1% Triton X-100 solution in PBS solution, 3 times. The secondary antibody (Invitrogen, #A-10680) was then added in a 1:500 dilution in 5% goat serum (diluted in 0.1% Triton X-100 solution in PBS solution) to each well and incubated at room temperature for 1 hour. Each well was washed in PBS, 3 times, and

aspirated. Using fine surgical tweezers, the coverslip with the adherent fixed and stained macrophages were gently lifted out of their wells. Fluoroshield Mounting Medium with 4,6 diamidino-2-phenylindole (DAPI, Abcam; catalog No. ab104139) was applied and sealed with clear adhesive onto a glass slide. Slides were then stored at 4°C and protected from light. After 24 hours, fluorescent microscopy was used to image the cells. For more details on antibodies used for different experiments for this application, please see Antibodies, Source, and Concentrations section. Imaging was performed on the Nikon Eclipse 80i, Nikon Plan Apochromat microscope using a 20x objective lens (numerical aperture 0.75), and LUT adjustment and exposure times were standardized among all fields of view. Immunofluorescence was quantified using ImageJ. ⁴⁶⁷

Proximity Ligation Assay

To demonstrate a complex between RhoGDI and Rac1 in adherent BMDMs grown on glass coverslips, a proximity ligation assay kit was used as per established protocols.⁴⁷⁴⁻⁴⁷⁶ BMDMs were treated with 10µM atorvastatin or vehicle control (dimethyl sulfoxide, DMSO). Some BMDMs were additionally incubated at 37°C with farnesyl diphosphate (FPP; 5µM), geranylgeranyl diphosphate (GGPP; 5µM), or squalene (5µM) for 24 hours. Both FPP and GGPP are isoprenyl precursors. Squalene is not an isoprenyl precursor but is a precursor for cholesterol synthesis. Primary antibodies to RhoGDI and Rac1 were used, and secondary antibodies were conjugated to oligonucleotides for ligation and subsequent rolling circle amplification. Quantification of proximity ligation signal was performed using ImageJ. ⁴⁶⁷ Each individual data point represents an average

of 4 fields of view from 3 slides with at least 50 cells per field of view. Further details on the protocol are described as follows: BMDM cultures were aspirated and washed with PBS 3 times before being fixed with 4% paraformaldehyde at room temperature for 15 minutes. Cells were washed again in PBS 3 times and then permeabilized using 0.1% (V/V) Triton X in PBS at room temperature for 10 minutes. Cells were washed again 3 times in PBS and then incubated for 1 hour at 4°C in 5% (V/V) fetal bovine serum (in 0.1% Triton-X-PBS solution) to block non-specific antibody binding. Cells were then washed 3 times with PBS.

Next the cells were incubated overnight at 4°C with anti-RhoGDI primary antibody, which was diluted 1:100 in 5% (V/V) fetal bovine serum (in 0.1% Triton-X-PBS solution). Cells were then washed in 0.1% Triton-X-PBS solution, 3 times. Both proximity ligation assay probes were diluted 1:5 in antibody diluent and incubated with the cells in the dark for 1 hour. Cells were then washed in 0.1% Triton-X-PBS solution twice and incubated for 5 minutes each time. Ligase solution diluted 1:40 was then added and incubated for 30 minutes. Cells were then washed in wash buffer A, 2 times and incubated for 2 minutes each time. DNA polymerase in amplification solution was then diluted 1:80 and incubated with the cells for 100 minutes at room temperature. Afterwards, cells were treated with wash buffer B twice and incubated for 10 minutes each time. Cells were finally washed in 0.01x wash buffer B for 1 minute. The glass coverslips the cells adhered to was then removed for the culture wells and treated with mounting media with DAPI and sealed onto a glass slide with nail polish. Slides were stored at 4°C.

Statistical Methods

Laboratory statistical data were analyzed with the use of Prism 7 (GraphPad). Results are presented as mean (standard error) for continuous variables with normal distribution. The t test was used to compare normally distributed continuous variables between 2 independent groups. Differences between multiple groups were assessed by ANOVA followed by Tukey's post hoc multiple comparisons test. A 2-sided $P < 0.05$ was considered statistically significant. For results with a non-normal distribution the Mann-Whitney test was used and a $P < 0.5$ was considered statistically significant.

Clinical statistical data were analyzed with the use of Stata/SE (version 15.1; StataCorp LLC, College Station, TX), or R software, version 3.3.1 (R Core Team). Baseline demographics and clinical characteristics were compared between the cohort that was not taking a statin and cohort taking a statin. Continuous variables were tested for normality and equal variance using Shapiro-Wilk test for normality and 1-way ANOVA to test for equal variance. Results are presented as mean (SE) for continuous variables with normal distribution or equal variance and as median (interquartile range) for continuous variables without normal distribution and as number (percentage) for categorical data.

Chapter 4:

Statins Disrupt Macrophage Rac1 Regulation Leading to Increased Atherosclerotic Plaque Calcification

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Findings and Reasoning leading to the Study of Macrophage Rac1's Role in Statin-Enhanced Atherosclerotic Calcification

The Morrison laboratory previously reported Rac1-dependent pathway for enhanced atherosclerotic calcification in *ApoE*^{-/-} *Rac2*^{-/-} mice.⁷⁸ From the group's review of the literature on vascular calcification, a relationship between statin treatment and enhanced plaque calcification became apparent. Statins are 3-hydroxy-3 methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors and are a part of the current standard of care for preventing cardiovascular events.³³¹⁻³³³ Statin inhibition of HMG-CoA reductase disrupts cholesterol synthesis resulting in lower serum levels of cholesterol in patients.^{334,335} Despite this association between statin treatment and atherosclerotic calcification no molecular mechanisms were identified to explain how statins could be promoting vascular calcification.

Given the laboratory's recent findings of a pathway regulating vascular calcification, it spurred further interest in the potential connection between statins and Rac1-mediated atherosclerotic calcification. Rac1 undergoes post-translational isoprenylation, which has been shown to modify its activity.^{329,330} Statins have been reported to disrupt the formation of precursors that are required for Ras and Rac protein's to be post-translationally isoprenylated.³³⁶⁻³³⁸ Given these reports, it was thought that if statins were to modulate vascular calcification through a Rac-mediated pathway that it may be facilitates through statin's disruption of Rac1 isoprenylation. Therefore, this study was conducted to investigate if statin's role in atherosclerotic calcification was mechanistically linked to its effects on Rac1 activity by disrupting Rac1 isoprenylation.

Abstract

Objective: Calcification of atherosclerotic plaque is traditionally associated with increased cardiovascular event risk; however, recent studies have found increased calcium density to be associated with more stable disease. HMG-CoA reductase inhibitors or “statins” reduce cardiovascular events. Invasive clinical studies have found that statins alter both the lipid and calcium composition of plaque, but the molecular mechanisms of statin-mediated effects on plaque calcium composition remain unclear. We recently defined a macrophage Rac-IL-1 β signaling axis to be a key mechanism in promoting atherosclerotic calcification and sought to define the impact of statin therapy on this pathway.

Approach and Results: Here, we demonstrate that statin therapy is independently associated with elevated coronary calcification in a high-risk patient population and that statins disrupt the complex between Rac1 and its inhibitor, RhoGDI, leading to increased active (GTP-bound) Rac1 in primary monocytes/macrophages. Rac1 activation is prevented by rescue with the isoprenyl precursor geranylgeranyl diphosphate. Statin treated macrophages exhibit increased activation of NF- κ B, increased IL-1 β mRNA, and increased Rac1-dependent IL-1 β protein secretion in response to inflammasome stimulation. Using an animal model of calcific atherosclerosis, inclusion of statin in the atherogenic diet led to a myeloid Rac1-dependent increase in atherosclerotic calcification, which was associated with increased serum IL-1 β expression, increased plaque Rac1-activation, and increased plaque expression of the osteogenic markers, ALP and RUNX2.

Conclusion: Statins are capable of increasing atherosclerotic calcification through disinhibition of a macrophage Rac1-IL-1 β signaling axis.

Introduction

Ischemic heart disease caused by atherosclerosis is the leading cause of morbidity and mortality in the world.⁴⁷⁷ Calcification of atherosclerotic plaques is predictive of total atherosclerotic burden and risk of cardiovascular and all-cause mortality.^{17,18} Rapid progression of vascular calcification is associated with a poor prognosis and increased risk of events.^{478,479} Yet, there has been growing evidence recently that certain types of densely calcified plaques can be associated with more stable disease, highlighting our limited understanding of the determinants of event risk with regard to atherosclerotic calcification.^{18,480-484}

For prevention of cardiovascular events, the current standard of care is pharmacotherapy with 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase inhibitors or “statins”.^{333,485-488} Statins are extremely successful at reducing cardiovascular events, and consequently, guidelines have moved toward more aggressive statin use.^{334,335,489-491} Statins were developed as lipid lowering agents, but multiple studies have demonstrated that statins reduce the risk of myocardial infarction out of proportion to their lipid lowering effects.^{334,335,489,490} These “pleiotropic effects” of statins have been attributed to the possibility that statins affect isoprenyl-based post-translational modification of protein, given isoprenyl groups are derived from precursor molecules within the cholesterol synthesis pathway downstream of mevalonate. The

effects of statin therapy on atherosclerotic calcification have been controversial with initial studies suggesting statins reduce or slow atherosclerotic calcification and later studies indicating the opposite. ⁴⁹²⁻⁴⁹⁵ Moreover, recent studies have identified an association between statin therapy and increased plaque calcium composition relative to lipid composition, yet the mechanism that determines this association remains undefined.^{493,496,497}

Racs are small GTPases and key signal transducers in inflammatory cells, influencing the expression of growth factors and cytokines. ^{369,380,498-500} Racs are post-translationally modified by an isoprenyl lipid group, specifically geranylgeranyl diphosphate (GGPP), which is thought to regulate subcellular localization. ⁵⁰¹ Rac1 expression is ubiquitous, whereas Rac2 is primarily limited to cells of hematopoietic origin. ⁵⁰² Rac1 and Rac2 share significant identity, except for a hypervariable region upstream of their isoprenylation site. ⁴⁹⁹ Though Racs often compensate for each other in terms of cellular function, Racs can also compete with each other for primary regulators, indicating that changes in gene expression of one Rac can simultaneously influence the activation state of the other. ⁵⁰³ Recently, our laboratory identified Rac2 as a major regulatory determinant of the degree of macrophage IL-1 β expression by its regulation of Rac1 activity upstream of NF- κ B and reactive oxygen species production.⁷⁸ Consequently, the *Rac2* gene deletion model led to progressive inflammatory atherosclerotic calcification that was dependent on IL-1 β signaling, and *Rac2*^{-/-} macrophages demonstrated increases in Rac1 activation and Rac1-dependent IL-1 β expression. The activity of Rac1 is regulated by three major enzymes: 1) guanine

nucleotide exchange factors (GEFs), which activate Rac1 by promoting the exchange of GDP for GTP; 2) guanine nucleotide dissociation inhibitors (GDIs), which limit the access of Rac1 to GEFs; and 3) GTPase-activating proteins (GAPs), which lead to inactivation of Rac1 by accelerating its intrinsic GTPase activity.⁵⁰⁴

Statins are known to inhibit mevalonate synthesis upstream of both cholesterol and geranylgeranyl diphosphate synthesis, and thus statins can inhibit the isoprenylation of Rac1 in addition to cholesterol synthesis.^{505,506} Current literature suggests that statin treatment disrupts Rac1 isoprenylation leading to reduced membrane association of Rac1 and consequently reduced membrane-dependent Rac1 functions.⁵⁰⁶ However, the effects of statin treatment on Rac1 activation (GTP-binding) and downstream effector signaling are less clear and somewhat controversial. In fact, recently the genetic knockdown of the geranylgeranyl transferase enzyme that covalently attaches the isoprenyl group to Rac1 demonstrated constitutive GTP-binding of Rac1 and hyperactivation of Rac1-dependent pathways consequent to the loss of isoprenylation.⁴²⁸

In this manuscript, we sought to explore the relationship between statin use and coronary calcification in patients who have undergone noncontrast computed tomography followed by the quantification of Rac1 activity in peripheral blood monocytes of a subset of patients. To define a potential mechanistic relationship, we turned to preclinical models and assessed the effect of statins on macrophage Rac1 activity, Rac1-dependent IL-1 β expression, and consequent atherosclerotic calcification in a mouse model of hypercholesterolemia. We hypothesize that, by inhibiting the isoprenylation of Rac1,

statins may disrupt the regulation of Rac1 activity, leading to increased Rac1 activity, IL-1 β expression, and consequent Rac1-dependent atherosclerotic calcification. Defining potential pleiotropic mechanisms whereby statins modulate plaque phenotype may lead to the development of novel therapeutic strategies for reducing cardiovascular event risk.

Results

Statin treatment is independently associated with increased coronary calcification in patients undergoing noncontrast lung cancer screening chest CT.

Table 1 defines the baseline demographics and clinical characteristics of the patient population. We evaluated a total of 465 patients who had undergone lung cancer screening computed tomography (LCST) between December 1, 2013 and March 31, 2014. All patients were US Veterans with a mean age of 67 (SE: 0.3)-years-old. The majority of the patients were Caucasian, male, and current smokers with an elevated cardiac risk as evidenced by a mean atherosclerotic cardiovascular disease (ASCVD) score of 21.6 (SE: 0.6)%. The mean cholesterol was 177.6 (SE: 2.0) mg/dL and the median body mass index (BMI) was 28.0 (IQR: 24.7, 31.9) kg/m². Approximately 28% of the patients had a history of diabetes and 7% of patients had a family history of early CAD. Eighteen percent of patients had known CAD; approximately 5% had prior myocardial infarction (MI) and 5% had prior CABG. The median coronary artery calcification score (CACS) as determined LCST was 595 (IQR: 125, 1570).

Of the 465 patients, there were 283 (approximately 60%) patients on statin therapy compared to 182 patients who were not taking a statin. Patients on statin therapy had been taking statin for a median of 56 (IQR: 24, 96) months. When comparing those on statin therapy with those not on statin therapy, the two groups were comparable in the percent of patients that were current smokers and that had a family history of early CAD. Patients on a statin were older, Caucasian, and with a higher BMI and had higher rates of hypertension, diabetes, CAD, prior MI, and prior revascularization. Patients in the statin group also had higher ASCVD risk scores with a median score of 23.7 (SE: 0.8) % compared to 18.4 (SE: 0.8) % ($P < 0.0001$).

Patients on statin therapy had significantly higher CACS values with a median of 941 (IQR 276, 2104) compared to 307 (IQR 54, 814) ($P < 0.0001$) (**Table I and Fig. 1A,B**). Coronary artery calcification was significantly associated with statin treatment (β -coefficient = 855.4 ± 141.2 , $P < 0.0001$) and remained significant after adjustment for comorbidities such as age, BMI, DM, HTN, HLD, CAD and active smoking (β -coefficient = 610.1 ± 158.6 , $P < 0.0001$). For those without known CAD and after controlling for ASCVD score, statin use was also significantly associated with coronary artery calcium (β -coefficient = 351.3 ± 121.5 , $P < 0.004$).

	All Patients (N= 465)	No Statin (n=182)	Statin (n= 283)	P Value
Age, years, mean (SE)	67 (0.3)	65 (0.6)	68 (0.4)	0.0004
BMI, median (IQR)	28.0 (24.7, 31.9)	26.8 (24.3, 30.3)	28.8 (25.1, 33.0)	0.0006
Male, n (%)	453 (97.2)	173 (95.0)	280 (98.9)	0.01
Caucasian, n (%)	435 (93.3)	159 (87.3)	276 (97.5)	<0.0001
African American, n (%)	22 (4.7)	17 (9.3)	5 (1.76)	<0.0001
Diabetes Mellitus, n (%)	131 (28.1)	26 (14.2)	105 (37.1)	<0.0001
Hypertension, n (%)	281 (60.3)	91 (50.0)	190 (67.1)	<0.0001
Hyperlipidemia, n (%)	334 (71.6)	77 (42.3)	257 (90.8)	<0.0001
Total Cholesterol, mean (SE)	177.6 (2.0)	187.6 (3.2)	171.1 (2.5)	0.0001
HDL Cholesterol, median (IQR)	42 (26, 50)	43 (37, 55)	41 (36, 48)	0.0071
Statin Use, n (%)	283 (60.8)	---	---	---
Current Smoker, n (%)	221 (47.4)	95 (52.1)	126 (44.5)	0.106
Family History of Early CAD, n (%)	33 (7.0)	14 (7.6)	19 (6.7)	0.688
CAD, n (%)	83 (17.8)	14 (7.6)	69 (24.3)	<0.0001
Prior MI, n (%)	24 (5.1)	4 (2.1)	20 (7.0)	0.021
Prior CABG, n (%)	25 (5.3)	2 (1.0)	23 (8.1)	0.001
ASCVD Risk Score, mean (SE)	21.6 (0.6)	18.4 (0.8)	23.7 (0.8)	<0.0001
CACS, median (IQR)	595 (125, 1570)	307 (54, 814)	941 (276, 2104)	<0.0001

Table I. Baseline Demographics and Clinical Characteristics of Patients.

BMI = body mass index; CAD = coronary artery disease; MI = myocardial infarction; CABG = coronary artery bypass graft surgery; ASCVD = atherosclerotic cardiovascular disease; CACS = coronary artery calcium score.

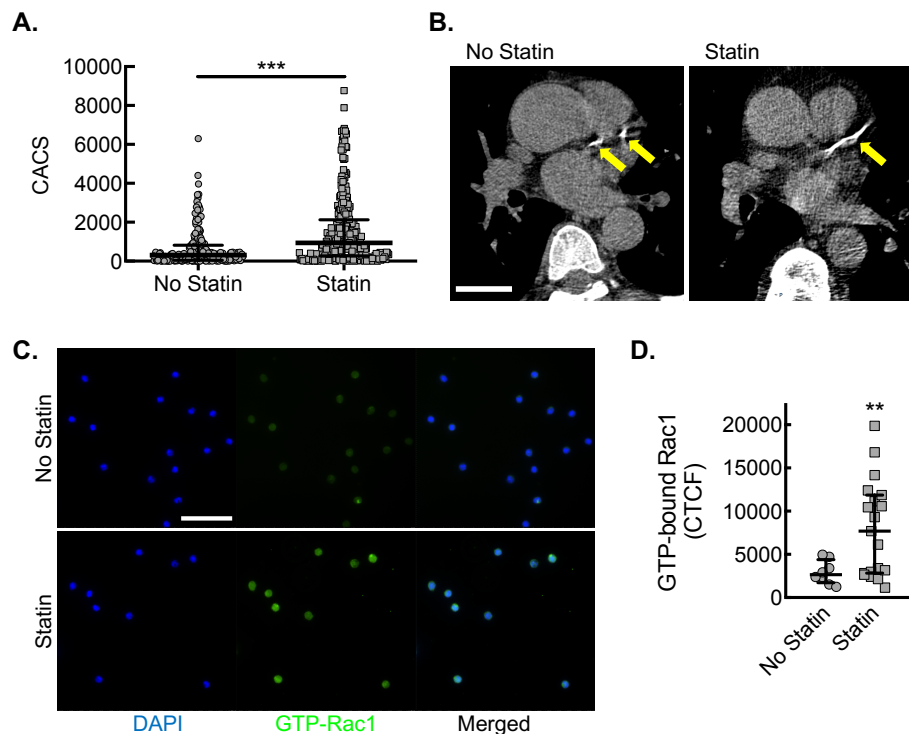


Figure 1. Statin treatment is associated with increased coronary calcification and peripheral blood CD14⁺CD16⁻ monocytes from patients on statin are associated with increased Rac1 activation.

(A) CACS for each patient along with median and interquartile ranges for patients not taking statin (No statin, gray circles) vs. patients taking statin (Statin, gray squares) (***, P=0.0001 Mann-Whitney U; n=182 no statin and n=283 statin). **(B)** Example of differences in coronary calcium from patients of similar age not taking (No statin) or taking statin (Statin), using LCSCCT axial images at the level of proximal left anterior descending coronary artery (LAD, yellow arrows) with total CACS of 283 and 412, respectively. Bar, 3 cm. **(C)** Immunofluorescent micrographs from CD14⁺ CD16⁻ monocytes isolated from patients not taking statin (No statin, gray circles) vs. patients taking statin (Statin, gray squares) stained with antibody specific to activated Rac1 (green). Counterstaining with DAPI (blue). Bar, 50 μ m. **(D)** Quantification of Corrected Total Cell Fluorescence (CTCF) for activated Rac1 (**, P=0.029 Mann-Whitney U; No statin, n=8 patients; Statin, n=16 patients).

Peripheral blood CD14⁺CD16⁻ monocytes from patients on statin are associated with increased Rac1 activation

To assess the impact of systemic statin treatment on monocyte Rac1 activity, we isolated peripheral blood monocytes from a subset of patients either taking or not taking statin. After isolation by negative selection, CD14⁺CD16⁻ monocytes, known as an inflammatory subset of monocytes, demonstrated increased Rac1 activation by immunofluorescence staining with an antibody specific to the GTP-bound (active) state of Rac1 (**Fig. 1C,D**). ⁵⁰⁷

Statin treatment of macrophages disrupted the isoprenyl-dependent complex between RhoGDI and Rac1.

To assess potential molecular mechanisms whereby statins may be permissive of or may promote Rac1 activation and consequently atherosclerotic calcification, and given our access to the relevant genetic strains, we moved to a mouse animal model for further *in vitro* and *in vivo* study. Rac1 is post-translationally modified by geranylgeranylation, which is known to direct Rac1's subcellular localization. ⁵⁰¹ Geranylgeranyl diphosphate (GGPP) is downstream of mevalonate in the cholesterol synthesis pathway, and thus GGPP synthesis can be inhibited by statins. In addition, the primary regulator of Rac1, Rho-specific guanine nucleotide dissociation inhibitor (RhoGDI), maintains Rac1 in the inactive (GDP-bound) state in the cell cytosol until Rac1 can be activated by a GEF. ⁵⁰⁴ Crystal structure analysis demonstrates that the principle binding interaction of Rho-GDI and Rac1 involves a lipophilic pocket that serves to hold the isoprenylation modification

(Fig. 2A).⁵⁰⁸ We first sought to identify whether the complex between Rac1 and RhoGDI can be disrupted by statin treatment. Briefly, bone marrow-derived macrophages (BMDMs) from *ApoE*^{-/-} mice were incubated with or without 10 μ M atorvastatin, followed by proximity ligation assay to assess the molecular complex formation between Rac1 and RhoGDI. Proximity ligation assay demonstrated that the complex between Rac1 and RhoGDI in resting, vehicle control-treated BMDMs was inhibited after a 24-hour atorvastatin treatment (Fig. 2B,C). Moreover, loss of the Rac1-RhoGDI complex by statin treatment was rescued by isoprenyl precursors, farnesyl diphosphate (FPP) and GGPP, but the Rac1-RhoGDI complex was not rescued by the cholesterol synthesis precursor squalene. In summary, the association in macrophages between Rac1 and its inhibitor, RhoGDI, can be inhibited by atorvastatin, and this inhibition can be rescued by supplementation with isoprenyl precursors.

Macrophage treatment with statin led to increased Rac1 activation.

Next, we sought to determine whether treatment of primary mouse *ApoE*^{-/-} BMDMs with atorvastatin would lead to increased Rac1 activation analogous to the human monocytes from patients on statin therapy. Using an activated Rac1 affinity precipitation assay, lysates from BMDMs incubated with atorvastatin for 24 hours demonstrated significantly increased levels of GTP-bound (active) Rac1 in an atorvastatin dose-dependent manner. (Fig. 3A,B). Using the monoclonal antibody specific to activated-Rac1, we confirmed that relative immunofluorescence in *ApoE*^{-/-} BMDMs treated with 10 μ M atorvastatin was also increased. (Fig. 3C,D). The increase in GTP-bound Rac1

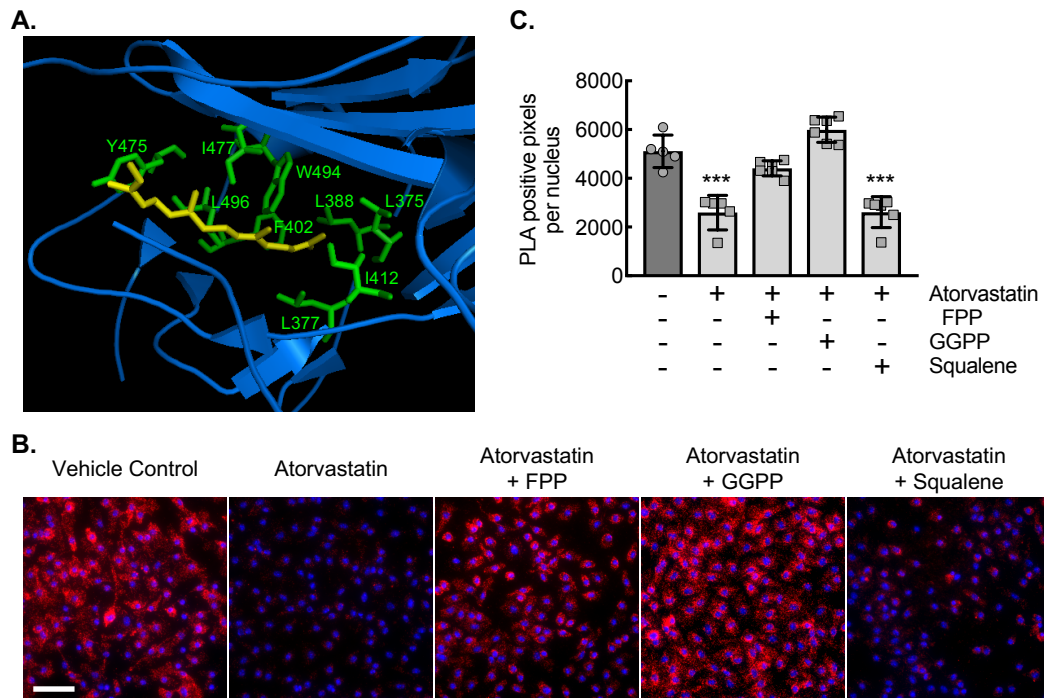


Figure 2. Statin treatment of primary bone derived marrow macrophages (BMDMs) disrupts the isoprenylation-dependent complex between Rho-GDI and Rac1.

(A) Ribbon diagram modeling the interaction between GDP-bound (inactive) Rac1 and Rho-GDI. Yellow arrow denotes the isoprenyl group sitting in a hydrophobic pocket. Hydrophobic amino acids labeled in green. **(B)** Micrographs of *ApoE*^{-/-} BMDMs incubated with vehicle control (DMSO) or with atorvastatin (10 μ M) without or with either FPP (5 μ M), GGPP (5 μ M), or squalene (5 μ M) for 24 hours, followed by proximity ligation assay to detect the Rho-GDI-Rac1 complex (red signal). Counterstaining with DAPI (blue). Bar, 50 μ m. **(C)** Quantification of PLA signal from conditions in panel C (***, $P < 0.0001$ ANOVA; $n = 6$ biological replicates from 3 mice). Quantitative data are displayed as mean $\pm$ SD.

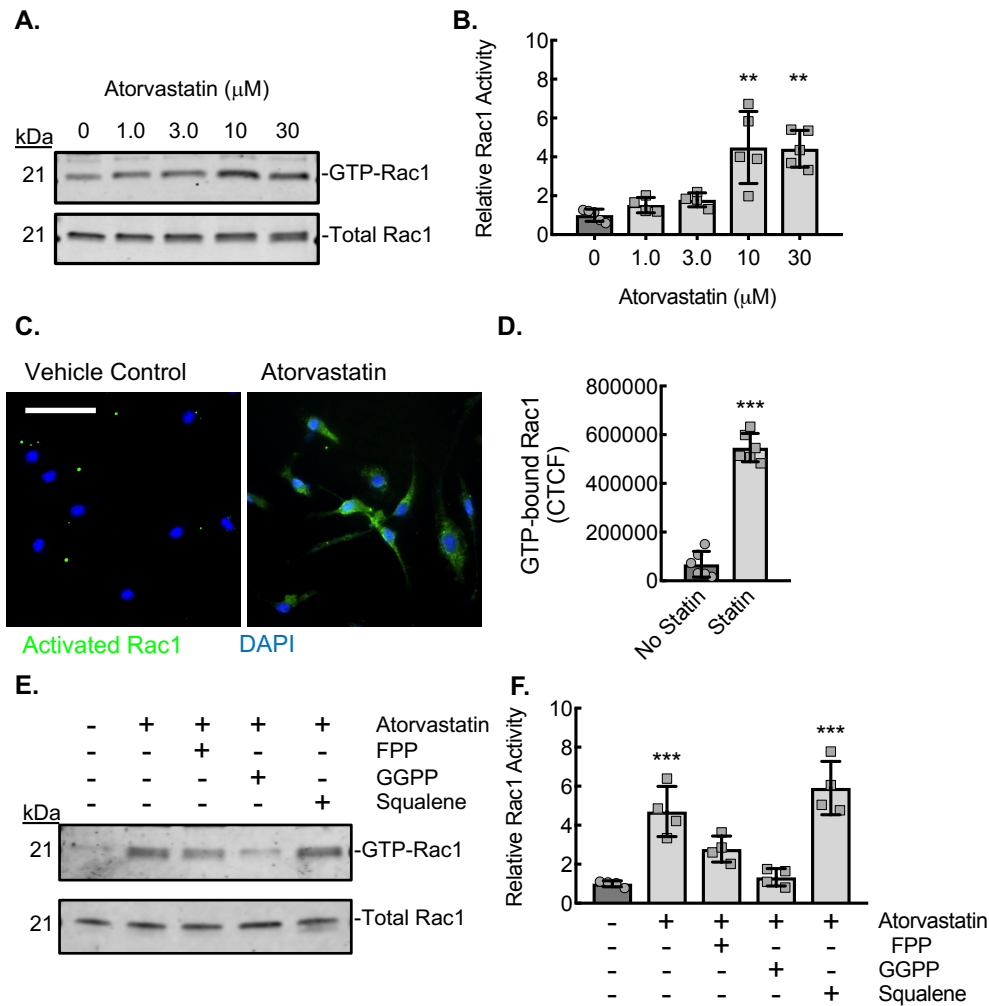


Figure 3. Statin treatment of BMDMs led to an isoprenylation-dependent increase in Rac1 activation.

(A) Rac1 immunoblots from lysates of *ApoE*^{-/-} BMDMs incubated with the indicated concentrations of atorvastatin for 24 hours, after which active (GTP-bound) Rac1 was affinity precipitated by PBD pulldown assay. **(B)** Densitometry quantification of the relative activated (GTP-bound) Rac1 to total Rac1 in lysates from panel A (**, $P < 0.0002$; $n = 5$ biological replicates from 5 mice; 3 male and 2 female). **(C)** Micrographs from BMDMs treated with atorvastatin (10 μM) and stained with antibody specific to activated (GTP-bound) Rac1 (green). Counterstaining with DAPI (blue). Bar, 50 μm . **(D)** Quantification of the Corrected Total Cell Fluorescence (CTCF) for active Rac1 from conditions in panel C (***, $P < 0.0001$ t-test; $n = 6$ biological replicates from 3 mice; 2 male and 1 female). **(E)** Rac1 immunoblots from lysates of *ApoE*^{-/-} BMDMs incubated with vehicle control (DMSO) or atorvastatin (10 μM) without or with either FPP (5 μM), GGPP (5 μM), or squalene (5 μM) for 24 hours, after which GTP-bound Rac1 was affinity precipitated by PBD pulldown. **(F)** Densitometry quantification of the relative active (GTP-bound) Rac1 to total Rac1 in lysates from panel E (**, $P < 0.0001$; $n = 4$ biological replicates from 4 mice; 2 male and 2 female). Quantitative data are displayed as mean $\pm$ SD.

caused by atorvastatin treatment was prevented by isoprenyl precursor rescue, partially by FPP and completely by GGPP (**Fig. 3E,F**). The cholesterol synthesis precursor squalene had no effect on the atorvastatin-induced activation of Rac1.

Macrophage treatment with statin led to an increase in IL-1 β expression that could be prevented by supplementation with isoprenyl precursors in a Rac1-dependent manner.

We previously demonstrated that increased Rac1 activation is associated with increased macrophage IL-1 β expression in the setting of an inflammasome stimulation assay using a combination of LPS-priming and cholesterol crystal exposure.⁷⁸ Having established statin treatment led to increased activation of Rac1, we sought to determine whether statin-induced Rac1 activity led to increased expression of mature IL-1 β protein in the setting of inflammasome stimulation. We evaluated IL-1 β expression from *ApoE*^{-/-} BMDMs cultured under inflammasome stimulation conditions, involving LPS-priming with cholesterol crystal exposure, in the setting or absence of atorvastatin (**Fig. 4A,B**). As expected, we found BMDM IL-1 β mRNA expression and mature IL-1 β protein secretion into the culture media to be increased in a manner specific to the combination of low dose (10 ng/mL) LPS and cholesterol crystal exposure relative to either LPS or cholesterol crystal exposure alone. Moreover, treatment of the *ApoE*^{-/-} BMDMs with 10 μ M atorvastatin during the inflammasome stimulation led to significantly increased BMDM IL-1 β mRNA expression and mature IL-1 β protein secretion. In order to confirm the specificity of this statin-induced increase in IL-1 β expression, we evaluated a second

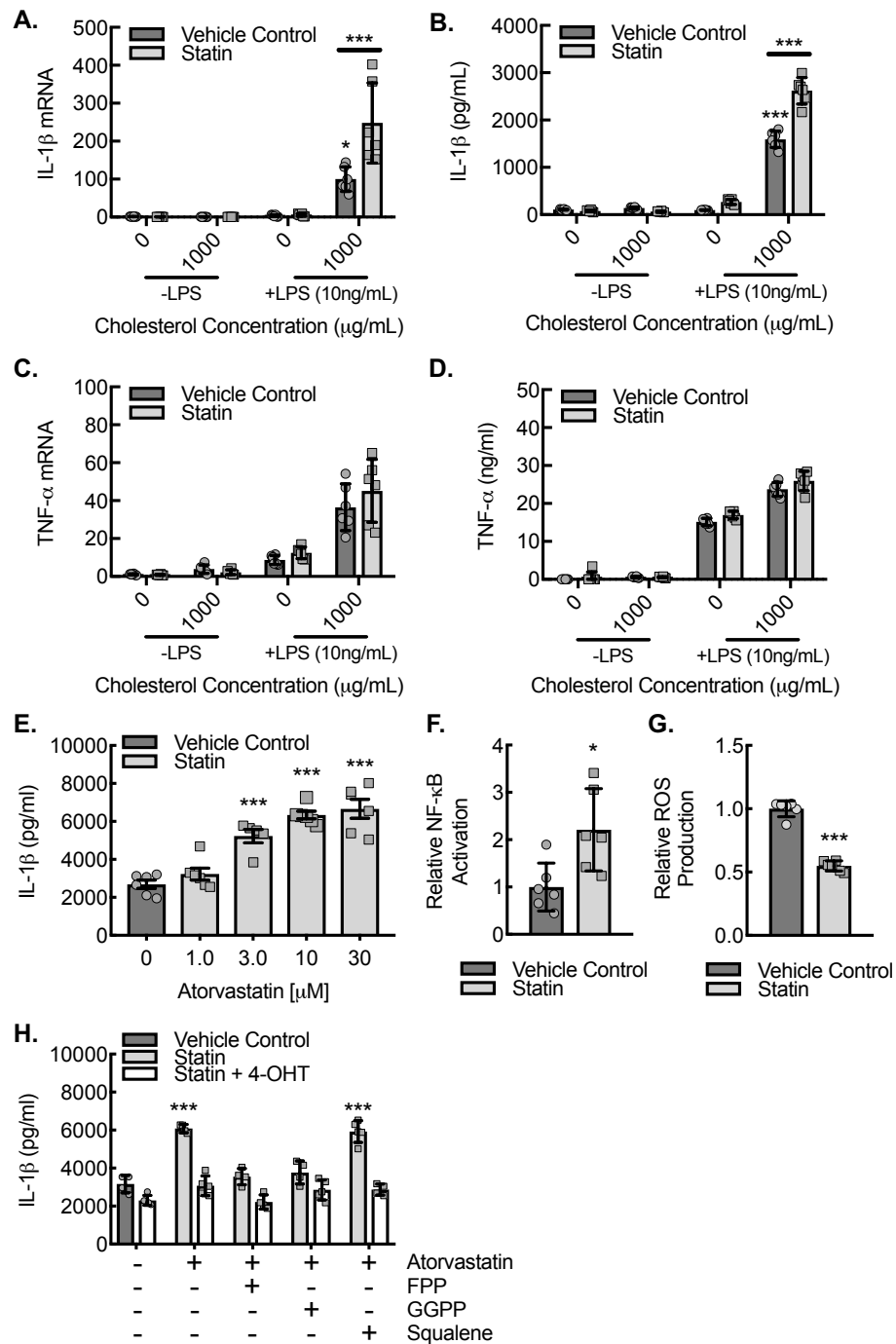


Figure 4.

Figure 4. Statin treatment of BMDMs led to an Rac1 isoprenylation-dependent increase in IL-1 β expression.

(A) *ApoE*^{-/-} BMDMs were pretreated for 24 hours with or without atorvastatin (10 μ M) and then primed with or without LPS (10 ng/ml) followed by exposure to cholesterol crystals (1000 μ g/mL) or vehicle control for another 24 hours, and cell lysates underwent real-time PCR quantification of IL-1 β mRNA (***, $P < 0.0001$ ANOVA; $n = 6$ biological replicates from 6 mice; 3 male and 3 female). (B) *ApoE*^{-/-} BMDMs were treated as in (A) followed by ELISA on culture supernatants for IL-1 β (***, $P < 0.0001$ ANOVA; $n = 6$ biological replicates from 6 mice; 3 male and 3 female). (C) *ApoE*^{-/-} BMDMs were treated as in (A) followed by real-time PCR quantification of TNF- α mRNA from cell lysates. (D) *ApoE*^{-/-} BMDMs were treated as in (A) followed by ELISA on culture supernatants for TNF- α . (E) Resting or LPS-primed (10 ng/ml) *ApoE*^{-/-} BMDMs were treated with or without 1000 mg/mL cholesterol crystals for 24 hours in the setting of indicated atorvastatin concentrations followed by ELISA on culture supernatants for IL-1 β (**, $P < 0.0001$ ANOVA; $n = 6$ biological replicates from 6 mice; 3 male and 3 female). (F) Relative luciferase activity in lysates from BMDMs transfected with a NF- κ B responsive luciferase construct and the treated with vehicle control (DMSO) or atorvastatin (10 μ M) for 24 hours (*, $P < 0.05$ t-test; $n = 6$ biological replicates from 6 mice; 3 male and 3 female). (G) Relative luminescence as a measure of ROS production in BMDMs treated with vehicle control (DMSO) or atorvastatin (10 μ M) for 24 hours (***, $P < 0.0001$ t-test; $n = 6$ biological replicates from 6 mice; 3 male and 3 female). (H) BMDMs from *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} mice were treated with vehicle control or atorvastatin (10 μ M) with or without either FPP (5 μ M), GGPP (5 μ M), or squalene (5 μ M) supplementation for 24 hours with or without 4-hydroxytamoxifen (4-OHT) to induce *Rac1* deletion and then were primed with or without LPS (10 ng/ml) and exposed to cholesterol crystals (1000 μ g/mL) or vehicle control for 24 hours followed by ELISA on culture supernatants for IL-1 β (**, $P = 0.0001$; $n = 6$ biological replicates from 3 mice; 2 male and 1 female). Quantitative data are displayed as mean $\pm$ SD.

marker of general inflammation, TNF- α , and found that TNF- α expression was comparable in the statin treated samples both at the mRNA and secreted protein levels (**Fig. 4C,D**). Finally, under conditions of the combination of low dose LPS and cholesterol crystal exposure, we demonstrated a statin concentration-dependent increase in mature IL-1 β secretion into the culture media (**Fig. 4E**).

NF- κ B activation and reactive oxygen species (ROS) production are downstream effectors of Rac1 activity that promote priming and activation of the NLRP3 inflammasome protein complex that promotes IL-1 β maturation.^{370,380,428} Therefore, we sought to assess the effects of macrophage statin treatment on NF- κ B activation and ROS production. Lysates from statin-treated BMDMs cultured in the setting of inflammasome stimulation with low-dose LPS priming coupled to cholesterol crystal exposure demonstrated increased NF- κ B activation but reduced ROS production (**Fig. 4G,H**).

Given that statin treatment can potentially influence the isoprenylation of multiple small GTPases, we sought to understand whether the statin-induced increases in IL-1 β expression were dependent on Rac1, specifically. We crossed mice that express a tamoxifen inducible Cre recombinase in myeloid cells, *CSF1R_{mcm}*, with *Rac1^{fl/fl}* mice to create mice with inducible macrophage deletion of Rac1 on an *ApoE*^{-/-} background.^{447,449} BMDMs from *ApoE*^{-/-}*CSF1R_{mcm}Rac1^{fl/fl}* mice were treated with or without 4-hydroxytamoxifen (4-OH) and then stimulated with low-dose LPS plus cholesterol crystal exposure in the absence or presence of 10 μ M atorvastatin along with isoprenyl and

cholesterol synthesis precursors (**Fig. 4H,I**). In the vehicle control treated BMDMs, the statin-dependent increase in IL-1 β production could be reduced toward baseline with isoprenyl precursors, FPP and GGPP, but not with cholesterol synthesis precursor, squalene, indicating the statin-induced increase in IL-1 β secretion to be due to a loss of protein isoprenylation. Moreover, genetic deletion of Rac1 abrogated the statin induced increase in IL-1 β secretion and any effects of the isoprenyl precursors at reducing IL-1 β secretion, indicating that the statin induced increases in IL-1 β secretion were in fact dependent on Rac1 and specifically on Rac1's isoprenylation state.

Mice fed a HFD supplemented with atorvastatin revealed comparable cholesterol profile and lipid plaque burden.

We next sought to evaluate the effects of statin treatment on the *ApoE^{-/-}CSF1R^{mcm}Rac1^{fl/fl}* animal model of atherosclerosis. Vehicle control injected animals were fed a high fat diet (20% by fat with 1.25% cholesterol) supplemented without (HFD) or with atorvastatin (HFD+Statin) in the chow at a dose 60 mg/kg chow dry weight. A subgroup of animals on HFD+Statin also underwent tamoxifen injections (2 mg intraperitoneally daily for 10 days) to induce myeloid *Rac1* deletion prior to being fed in order to assess the impact of macrophage *Rac1* expression on the statin-induced phenotype (HFD+Statin+mR1KO). All three groups (HFD, HFD+Statin, and HFD+Statin+mR1KO) were fed for 18 weeks. Animals on HFD+Statin demonstrated similar serum lipid panels, including total cholesterol, triglycerides, HDL, and LDL relative to animals on HFD (**Fig. 5A-D**). Animals on HFD+Statin+mR1KO demonstrated a modest increase in total cholesterol, triglycerides, and LDL. Aortas were harvested from the three groups of mice and demonstrated comparable levels of atherosclerotic lipid burden by

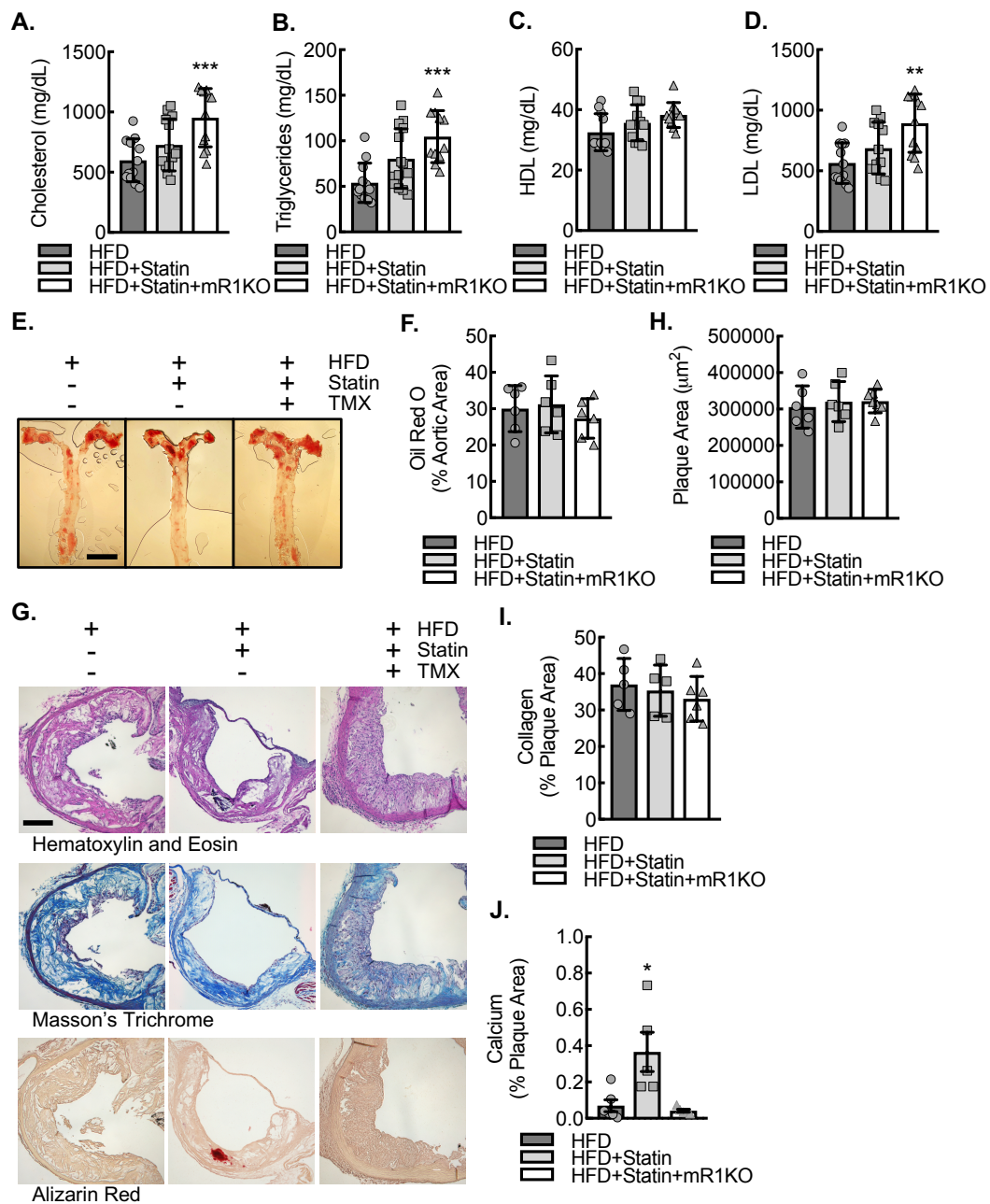


Figure 5

Figure 5. *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} mice fed a HFD supplemented with atorvastatin revealed comparable cholesterol profile and plaque burden relative to those on HFD alone.

Serum total cholesterol **(A)**, triglycerides **(B)**, HDL **(C)**, and LDL **(D)** concentrations after 18 weeks of HFD, HFD+Statin or HFD+Statin+mR1KO (**, $P < 0.002$, ***, $P < 0.001$ relative to HFD by ANOVA; $n = 12$ mice; 6 male and 6 female). **(E)** En face Oil Red O lipid staining of aortic whole mounts after 18 weeks of HFD, HFD+Statin, or HFD+Statin+mR1KO, along with quantification **(F)** of percent aortic area stained positive for Oil Red O. Bar, 3mm. **(G)** Standard histology stained for hematoxylin and eosin, Masson's trichrome, and Alizarin red of adjacent aortic sinus sections at the level of the aortic valve after 18 weeks of HFD, HFD+Statin, or HFD+Statin+mR1KO. Bar, 200 μm . **(H)** Quantification of average aortic plaque area by hematoxylin and eosin. **(I)** Quantification of percent plaque area staining positive for collagen by Masson's trichrome. **(J)** Quantification of percent plaque area staining positive for Alizarin red. (*, $P = 0.012$ by ANOVA; $n = 6$ mice; 3 male and 3 female). Quantitative data are displayed as mean $\pm$ SD.

en face Oil Red O staining (**Fig. 5E,F**). Moreover, traditional histological sectioning and staining at the level of the aortic sinus demonstrated comparable sinus plaque area and sinus plaque collagen area by hematoxylin and eosin and Masson's trichrome staining in all three groups, HFD, HFD+Statin, and HFD+Statin+mR1KO, whereas statin treatment demonstrated a Rac1-dependent increase in sinus plaque calcium area by Alizarin Red staining (**Fig. 5G-J**).

**Mice fed a HFD supplemented with atorvastatin demonstrated
myeloid Rac1-dependent elevations of global atherosclerotic
calcification and of serum IL-1 β protein.**

Despite similarities of serum lipid panel and atherosclerotic lipid burden, there was histological evidence of increased atherosclerotic calcification in the mice on HFD+Statin, using Alizarin Red staining. We next assessed global levels of active atherosclerotic calcification in the aortas of the mice using a commercially available near-infrared conjugated bisphosphonate compound. Near-infrared conjugated bisphosphonate compounds can bind to hydroxyapatite deposited by osteoblast-like cells during the mineralization of calcium, allowing for *ex vivo* molecular imaging and quantification of the calcification process.^{457,461} Overall, the mice fed a HFD+Statin demonstrated increased aortic atherosclerotic calcification relative to animals on HFD, and myeloid *Rac1* deletion abrogated the effect, supporting the statin-induced increase in atherosclerotic calcification to be Rac1-dependent (**Fig. 6A,B**). Moreover, the serum from statin-treated animals demonstrated Rac1-dependent increases in IL-1 β protein levels (**Fig. 6C**). This statin-dependent increase in serum IL-1 β appeared specific, whereas another general

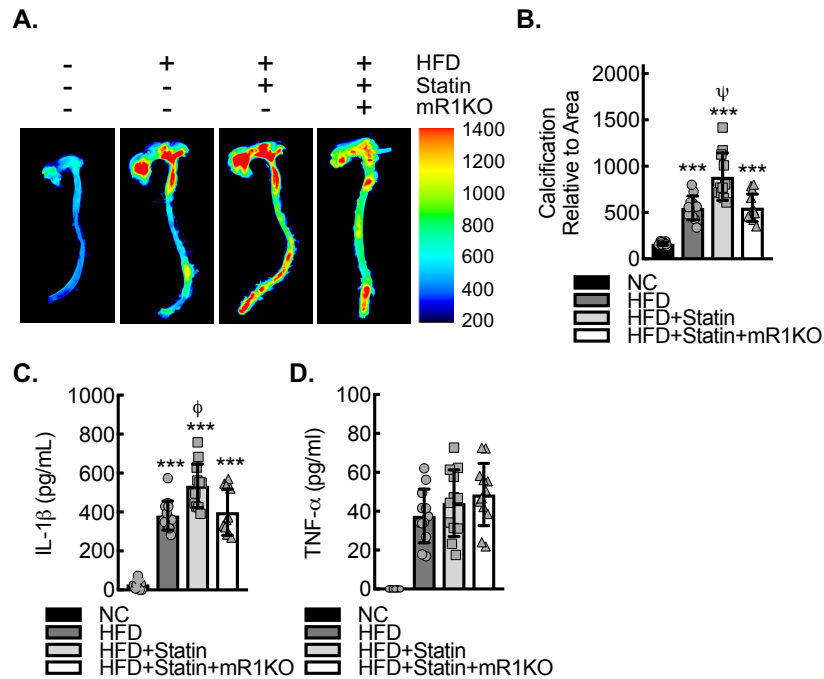


Figure 6. *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} mice fed a HFD supplemented with atorvastatin demonstrate *Rac1* dependent increases in both atherosclerotic calcification and systemic IL-1β expression.

(A) Ex vivo near-infrared calcium imaging from mice on normal chow, HFD, HFD+Statin or HFD+Statin+mR1KO for 18 weeks along with quantification **(B)** of calcification signal relative to total aorta area (***, $P < 0.0001$ relative to NC by ANOVA; ψ $P < 0.0001$ relative to all others by ANOVA; $n = 12$ mice; 6 male and 6 female). **(C)** Serum IL-1β concentrations by ELISA from *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} mice after 18 weeks of normal chow, HFD, HFD+Statin or HFD+Statin+mR1KO (***, $P < 0.0001$ relative to NC by ANOVA; ϕ $P = 0.005$ relative to all others by ANOVA; $n = 12$ mice; 6 male and 6 female). **(D)** Serum TNF-α concentrations by ELISA from *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} mice after 18 weeks of normal chow, HFD, HFD+Statin or HFD+Statin+mR1KO (***, $P < 0.0001$ relative to NC by ANOVA; $n = 12$ mice; 6 male and 6 female). Quantitative data are displayed as mean $\pm$ SD.

inflammatory cytokine, TNF- α , was comparable between HFD, HFD+Statin, and HFD+Statin+mR1KO treated animals (**Fig. 6D**).

Of note, sex was analyzed as a biological variable for lipid panel, serum IL-1 β , and near infrared calcification imaging using ANOVA, and we found no sex-dependent changes in our hands in this preclinical model, indicating that this type of Rac1-dependent increase in IL-1 β and atherosclerotic calcification appears comparably relevant to both sexes (data not shown).

Statin treatment demonstrated increased plaque expression of activated (GTP-bound) Rac1 along with increased expression of osteogenic markers, ALP and RUNX2, in atherosclerotic lesions.

The inner aortic arch neointimal lesions of animals fed a HFD+Statin demonstrated increased expression of activated (GTP-bound) Rac1 which was abrogated by myeloid *Rac1* deletion, consistent with our *in vitro* findings that statin induces activation of Rac1 in macrophages (**Fig. 7A,B**). Finally, neointimal plaque staining for the osteoblast markers, RUNX2 and alkaline phosphatase (ALP), were increased in the mice fed a HFD+Statin, and this increase was abrogated by myeloid *Rac1* deletion, consistent with a myeloid Rac1-dependent expression of these markers (**Fig. 7C-F**).

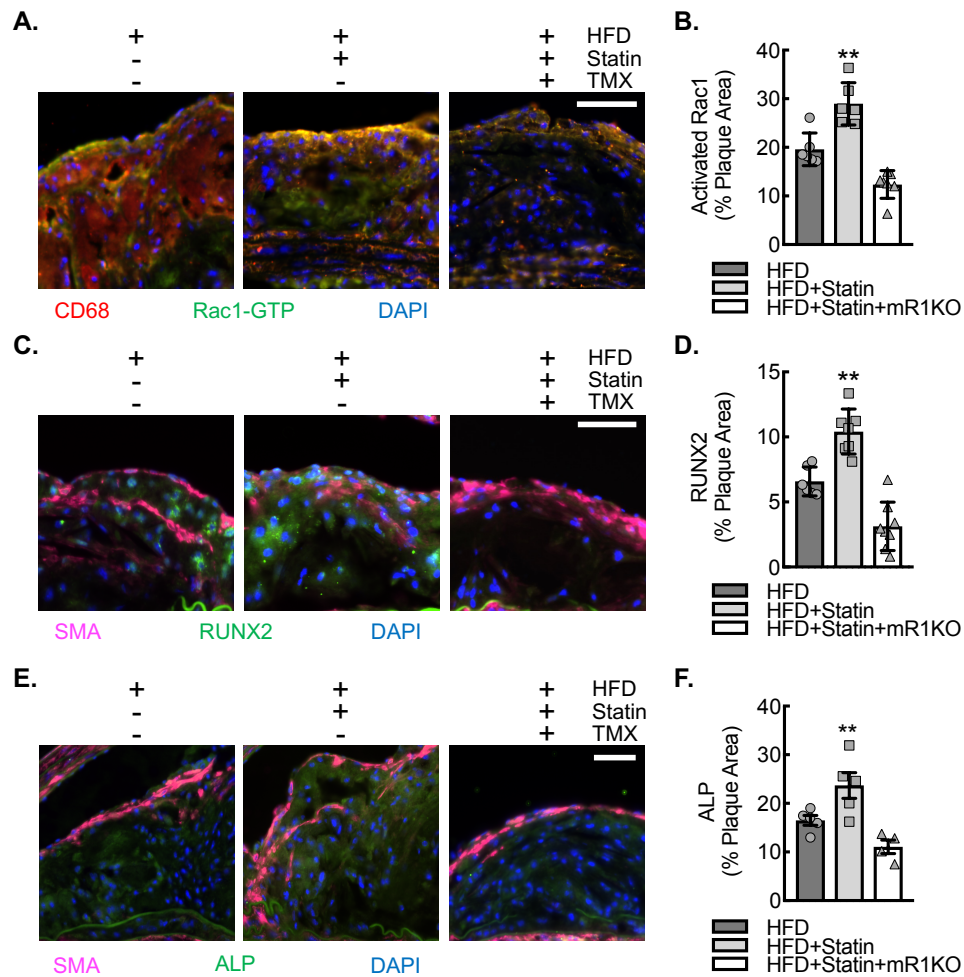


Figure 7. *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} mice fed a HFD supplemented with atorvastatin demonstrate *Rac1* dependent increases activated *Rac1*, ALP, and RUNX2 expression.

(A) Immunofluorescence micrographs of neointimal lesions in the ascending aortic arch lesser curvature of *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} mice fed HFD, HFD+Statin or HFD+Statin+mR1KO for 18 weeks that were stained for GTP-bound (activated) *Rac1* (green) and CD68 (red). Nuclei counterstained with DAPI (blue). Bar, 50 μ m. (B) Quantification of percent plaque area staining positive for activated *Rac1* expression in aortic arch plaques from A (**, $P < 0.005$ relative to others by ANOVA; $n = 6$ animals; 3 male and 3 female). (C) Immunofluorescence micrographs of neointimal lesions derived as described in (A) that were stained for RUNX2 (green) and SMA (magenta). Nuclei counterstained with DAPI (blue). Bar, 50 μ m. (D) Quantification percent plaque area staining positive for RUNX2 expression (**, $P < 0.005$ relative to others by ANOVA; $n = 6$; 3 male and 3 female). (E) Immunofluorescence micrographs of neointimal lesions derived as described in (A) that were stained for ALP (green) and SMA (magenta). Nuclei counterstained with DAPI (blue). Bar, 50 μ m. (F) Quantification percent plaque area staining positive for ALP expression (*, $P < 0.05$ relative to others by ANOVA; $n = 6$; 3 male and 3 female). Quantitative data are displayed as mean $\pm$ SD.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request. Because of the sensitive nature of some data collected for this study, requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to the office of the Associate Chief of Staff for Research at Providence VA Medical Center at Research Services (151), 830 Chalkstone Avenue, Providence, RI 02908, or faxed to 401-457-3305; all data, analytical methods, and study materials are available upon request to researchers, who meet the Institutional Review Board criteria for access to VA confidential research data, for purposes of reproducing the results or replicating the study.

Materials

Anti-Rac1 mouse monoclonal antibody (clone 23A8) was purchased from Abcam (Cat#97732). Anti-activated Rac1 mouse monoclonal IgM antibody was purchased from New East Biosciences (Cat#26903). Anti-Actin, Alpha-smooth muscle-Cy3 antibody was purchased through Sigma Aldrich (Cat#C6198). PAK1 PBD agarose beads were purchased from Cell Biolabs (Cat#STA-411). Alexa Fluor-conjugated secondary antibodies, DAPI (4,6 diamidino-2-phenylindole), and RPMI 1640 culture medium were purchased from Fisher Scientific. Fluoroshield Mounting Medium with DAPI was purchased from Abcam (Cat#ab104139). Cholesterol and endotoxin-free BSA were purchased from Sigma-Aldrich. Primers used in quantitative real-time PCR were all purchased from IDT. Human Monocyte Isolation Kit and EasySep Buffer was purchased

through StemCell (Cat#19359 and #20144, respectively). Sequences for murine primers were as follows:

IL-1 β , 5'- CCCTGCAGCTGGAGAGTGTGGA-3' (sense) and 5'-
CTGAGCGACCTGTCTTGGCCG-3' (antisense);

TNF- α , 5'- CACGTCGTAGCAAACCACCAA -3'(sense) and 5'-
AGCAAATCGGCTGACGGTGT-3' (antisense);

GAPDH, 5'- AAGGCCGGGGCCCACTTGAA-3' (sense) and 5'-
GGACTGTGGTCATGAGCCCTTCCA-3' (antisense);

Rac1, 5'-CTACCCGCAGACAGACGTG-3' (sense) and 5'-
AGATCAAGCTTCGTCCCCAC-3' (antisense);

HPRT, 5'-GACCGGTCCCGTCATGCCGA-3' (sense) and 5'-
TGGCCTCCCATCTCCTTCATGACA-3' (antisense).

Human Subjects Research

The patient studies comply with the Declaration of Helsinki and were approved by the Institutional Review Board of the Providence VA Medical Center. We evaluated a total of 465 US Veteran patients who had undergone LCSCT between December 1, 2013 and March 31, 2014.

Coronary Artery Calcium Scoring

Studies at the Providence VA Medical Center were performed using a 128-slice CT scanner (Siemens SOMATOM Definition AS) with a 128 mm x 0.6 mm collimation,

using a helical acquisition protocol. Rotation time was 0.5 seconds, and pitch was 0.84. Studies were not ECG-gated. Scanning field of view was set to 380 mm. Matrix size was 512 x 512. Based on these parameters, the minimum area required to identify calcium was 0.55 mm². The average tube voltage was 120 kV, and the average tube current was 40 mA. Image reconstruction slice thickness was between 1.0 and 1.25 mm.

Coronary artery calcium scoring (CACS) from lung cancer screening CT (LCST) were calculated using the Agatston method.⁵⁰⁹ CACS was performed using previously described methods for other epidemiologic studies, and images were viewed and scored using a CarestreamVue PACS (Carestream Health) imaging workstation.⁵¹⁰⁻⁵¹² Briefly, the calcium scoring application in the CarestreamVue PACS displays axial image slices for the reader. The reader scrolls through axial slices and identifies coronary arteries with potential calcium to be scored. The reader indicates the appropriate coronary vessel to the program by circling a region of interest. Pixels of attenuation coefficient measurements above 130 HU and an area ≥ 0.55 mm² within the region of interest are identified by the program as calcified and are incorporated into the calcium score. All scans were scored by the preventive cardiology fellow (J.L.C) after being trained on 50 scans by the CCT board-certified cardiologist (A.R.M.). Readers were blinded to patient clinical data. Inter-observer agreement was quantified for total CACS values and the overall kappa was found to be very good at 0.907(0.859-0.956).

Covariates

The VA electronic medical record was searched for patient demographics and cardiovascular covariates including age, sex, race, and BMI. Medical history included smoking status, hypertension, hypertensive medication, cholesterol medication, diabetes, and fasting lipid profile. From the acquired data, the ASCVD risk scores were calculated.

Human CD14⁺CD16⁻ Monocyte Isolation

Peripheral blood CD14⁺CD16⁻ monocytes from a subset of patients either taking or not taking statin medication were isolated from 20 mL of blood using negative selection by the EasySep Human Monocyte Isolation Kit (StemCell Technologies, Cat#19359) per manufacturer protocols.⁵⁰⁷ Briefly, the buffy coat from whole blood was extracted using 1500rpm centrifugation at room temperature and a subsequent Ficoll density gradient to separate out RBCs. Cells were pelleted and washed with EasySep Buffer (StemCell, Cat#20144). EasySep Human Monocyte Isolation Kit was used to further isolate monocytes and eliminate any remaining platelets or extraneous cell types via negative selection and magnet filtration. Purified monocytes were allowed to adhere to glass coverslips in complete RPMI for at least 2 hours. Adherent monocytes were washed with PBS, then fixed with 4% PFA and stored at 4°C for subsequent staining. Fixed monocytes were washed in PBS and incubated in 0.1% (V/V) Triton X in PBS for 10 minutes. After washing, they were incubated in 5% goat serum in 0.1% (V/V) Triton X-PBS for 1 hour. They were then washed and covered with primary antibody, Rac1-GTP (New East Biosciences, Cat#26903), overnight at 4°C. Primary antibody was washed with PBS and secondary antibody, Goat Anti-Mouse IgM AlexaFluor488 (Invitrogen, Cat#A21042), was

added for 1 hour. Cells were washed and coverslips were plated to microscopy slides using Fluoroshield Mounting Medium with DAPI (Abcam, Cat#ab104139), and sealed with clear adhesive. Imaging was performed on the Nikon Eclipse 80i inverted microscope, and LUTs and exposure were standardized among all fields of view. Immunofluorescence was quantified using ImageJ.⁴⁶⁷ Each individual data point represents an average CTCF of 4 fields of view with at least 20 cells per field of view.

Animal Studies

The Providence VA Medical Center Institutional Animal Care and Use Committees approved all housing protocols and experimental animal procedures. *ApoE*^{-/-}, *Rac1*^{fl/fl}, and *CSF1R*^{mcm} mice were purchased at The Jackson Laboratory and have been previously described.^{440,447,449} All animal studies adhered to the guidelines for experimental atherosclerosis studies as described in the recent AHA Scientific Statement.⁵¹³ All animal studies adhered to the guidelines as described in the ATVB Council Statement for considering "sex difference" as a biological variable.⁵¹⁴

High Fat Diet

Male and female animals, 8-10 weeks old, were fed a high fat diet (HFD; 20% fat by weight with 1.25% cholesterol, ENVIGO TD.02028) or HFD+Statin (atorvastatin at dry weight concentration of 60 mg/kg of HFD chow, ENVIGO TD.150052) ad libitum for up to 20 weeks. A subset of *ApoE*^{-/-}, *Rac1*^{fl/fl}, and *CSF1R*^{mcm} mice underwent intraperitoneal injection of tamoxifen at 2 mg daily for 10 days in order to induce myeloid *Rac1* gene deletion prior to HFD+Statin treatment (HFD+Statin+mR1KO).

Macrophage Cell Cultures

Bone marrow-derived macrophages (BMDMs) were obtained by *in vitro* differentiation of primary femur marrow cells with an established protocol that yields >97% CD11b^{hi}F4/80^{hi} cells. ^{469,500} BMDMs were obtained by *in vitro* differentiation of primary femur marrow cells with an established protocol. ^{469,500} Briefly, femurs and tibiae from *ApoE*^{-/-} and *CSF1R^{mcm}Rac1^{fl/fl}ApoE*^{-/-} mice were dissected, cleaned, disinfected in 70% ethanol, and washed with fully supplemented RPMI 1640 medium (10% FBS, 10 mM HEPES pH 7.4, 2 mM L-glutamine, 100 units/ml penicillin, 10 µg/ml streptomycin, and 50 µM 2-ME). Red blood cells were removed by ammonium-chloride-potassium lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, adjusted to pH 7.4) and subsequent centrifugation. Remaining bone marrow cells were then cultured at a density of 3.5 x 10⁶ cells/10 cm petri dish in fully supplemented RPMI with 30% (v/v) L929 cell-conditioned medium. Cells were matured to phenotypic macrophages over 6-8 days. Nonadherent cells were washed away with PBS. Adherent cells were recovered with gentle pipetting in PBS with 1 mM EDTA. Flow cytometric analysis confirmed the recovered cells to be >97% CD11b⁺F4/80⁺. ^{469,500}

Cholesterol Crystal Inflammasome Assay

Cholesterol crystals were generated as previously described. ⁴⁷ Cholesterol (Sigma) was dissolved in 1-propanol at a concentration of 2 mg/ml. Crystals were precipitated overnight at room temperature by dilution to 40% 1-propanol with water. Crystals were pelleted by centrifugation and dried at 70°C. Pellets were re-suspended in

0.1% FBS in PBS at a concentration of 50 mg/ml. Average crystal size of 1-2 μm was confirmed by microscopy. BMDMs (+/- priming with low dose (10 ng/ml) LPS for 2 hours) were then either exposed to 1000 $\mu\text{g/ml}$ cholesterol crystals or vehicle control for 24 hours. Culture supernatants were collected and IL-1 β expression quantified by enzyme-linked immunosorbent assay (ELISA) per manufacturer protocols (BioLegend, Cat#432604 and 430904, respectively).

NF- κ B Assay

BMDMs were transfected with NF- κ B Firefly Luciferase Plasmid (Promega) and pRL-TK Renilla control plasmid (Promega), using the Amaxa Mouse Macrophage Nucleofector Kit (Lonza). 12 hours after transfection, BMDMs were primed with LPS (10 ng/mL) for 2 hours and then exposed to 1000 $\mu\text{g/ml}$ cholesterol crystals for 12 hours. Luciferase activity in cell lysates was measured by Dual-Glo $\text{\textcircled{R}}$ Luciferase Assay System (Promega).

Reactive Oxygen Species (ROS) Assay

BMDMs were primed with 10 ng/mL LPS for 2 hours at 37 $^{\circ}\text{C}$ and then cells were exposed to 1000 $\mu\text{g/ml}$ cholesterol crystals for up to 3 hours at 37 $^{\circ}\text{C}$. ROS detection was carried out using ROS-Glo TM H $_2$ O $_2$ Assay (Promega) per manufacturer protocols.

Rac1 Activation Assay

Rac1 activity was measured by an affinity precipitation assay with Pak1 PBD agarose beads. ⁵¹⁵ BMDMs were lysed in 500 μl lysis buffer (20 mM HEPES, pH 7.4, 150

mM NaCl, 1% Triton X-100, 4 mM EDTA, 4 mM EGTA, 10% glycerol, supplemented with protease inhibitor cocktail [Roche]). Ten percent of the whole cell lysate was set-aside for Rac1 loading control. To the remainder of the sample, 10 µg of Pak1 PBD beads were added, followed by a 45-minute incubation at 4°C. Beads were washed with lysis buffer and subsequently brought up in Laemmli sample buffer. Activated (GTP-bound) Rac1 was detected by immunoblot after affinity precipitation, and whole cell lysates were used as loading controls.

For active Rac1 immunofluorescence staining, human monocytes or BMDMs were adhered, fixed and permeabilized, followed by primary staining with mouse anti-activated Rac1 (New East Biosciences) and secondary staining with Alexa Fluor 488 Goat Anti-Mouse IgM (Invitrogen). Corrected total cell fluorescence was quantified by ImageJ.⁴⁶⁷ Each individual data point represents an average CTCF of 4 fields of view with at least 20 cells per field of view.

Proximity Ligation Assay

To demonstrate a complex between RhoGDI and Rac1 in adhered BMDMs, a proximity ligation assay kit was used as per manufacturer protocols.⁴⁷⁴⁻⁴⁷⁶ Primary antibodies to RhoGDI and Rac1 were used, and secondary antibodies were conjugated to oligonucleotides for ligation and subsequent rolling circle amplification. Quantification of proximity ligation signal was carried out using ImageJ.⁴⁶⁷ Each individual data point represents an average of 4 fields of view from 3 slides with at least 50 cells per field of view. (A more detailed description of this protocol is provided in Chapter 3, Methods.)

Serum Protein and Cholesterol Analysis

Yale Mouse Metabolic Phenotyping Center quantified serum total cholesterol, LDL, and triglyceride concentrations, utilizing the Roche COBAS Mira Plus automated chemistry analyzer. IL-1 β and TNF- α were measured in serum samples using ELISA kits (Biolegend) according to manufacturer's protocols.

RNA Isolation and qRT-PCR

RNA from BMDMS was isolated using RNeasy® Mini Kit (QIAGEN, Cat#74106). RNA was quantified by NanoDrop 2000 (Thermo Scientific) and then reverse transcribed by iScript (Bio-Rad, Cat#1708891) protocol for subsequent qRT-PCR of transcript expression. GAPDH and HPRT expression were used for normalization.

Histology and Microscopy

Animals were euthanized, and tissue was perfusion fixed with 4% paraformaldehyde. Aortas harvested and fixed for 12 hours with paraformaldehyde (4%, buffered neutral) overnight at 4°C. Aortas were then cleaned under dissection microscope. Traditional hematoxylin and eosin (H&E), Masson's trichrome, and Alizarin red staining protocols were performed on 10- μ m thick cross sections at the level of the aortic sinus, between 1400-1700 μ m from the most inferior section of the valve tissue facing the left ventricular outflow tract. Quantifications of plaque area, plaque collagen area, and calcium area were carried out using ImageJ.⁴⁶⁷ Each individual data point represents the average quantification from 3 aortic valve sinuses taken from the 6-8

adjacent sections per slide of on aortic sinus. Some ascending aortas (1-2 mm) at the level of the arch inferior to the brachiocephalic branch were fixed for 12 hours with paraformaldehyde (4%, buffered neutral) overnight at 4°C. Aortas were then cleaned under dissection microscope, placed in 30% Sucrose for up to 1 week, and embedded in Optimal Cutting Temperature (OCT) compound in pre-labeled Cryomold squares (Tissue-Tek®) followed by dry ice for 10-15 minutes. Tissue was sectioned at 10 µm thickness by cryostat. After sectioning, slides were dried at room temperature for 1-2 hours before immunostaining.

Sections (10 µm thickness) were immunostained Cy3-conjugated anti- α -smooth muscle actin (Sigma-Aldrich, Cat#C6198), anti-alkaline phosphatase (Abcam, Cat#Ab65834), anti-RUNX2 (Abcam, Cat#Ab76956), rat anti-mouse CD68 (BioLegend #137002), or mouse anti-activated Rac1 (New East Biosciences, Cat#26903). Unconjugated primary antibody staining was visualized with Goat Anti-Rat IgG H&L Cy5 (Abcam, Cat#Ab6565) and Goat Anti-Mouse IgG, IgM (H+L) AlexaFluor488 (Invitrogen, Cat#A10680). All sections were counterstained with DAPI. Bright field and immunofluorescence microscopy of sectioned tissue were carried out on a Nikon Eclipse 80i inverted microscope. Images were taken with Nikon Plan Apochromat 2X (numerical aperture 0.1), 4X (numerical aperture 0.2), 10x (numerical aperture 0.3), 20x (numerical aperture 0.75) objective lenses. Quantifications of percent area positive activated Rac1 staining, RUNX2 staining, or ALP staining per plaque were carried out using ImageJ.⁴⁶⁷ Each individual data point represents the average quantification of each animal using the average area of images captured from 4-6 adjacent sections per slide.

Plaque Calcium and Lipid Quantification

One day prior to euthanasia, some animals underwent intraperitoneal injection with 2 nmol of IRDye 680RD BoneTag Optical Probe (LI-COR, Cat#926-09374). Animals were euthanized, and tissue perfusion fixed with 4% paraformaldehyde (buffered neutral). Thoracic aortas were Aortas underwent microdissection and subsequently imaged *ex vivo* with the Odyssey Imaging System (LI-COR). Calcification was quantified with Image Studio Lite (LI-COR). Aortas were scanned using the following acquisition settings: 1) 700 nm intensity of 3; 2) resolution of 21 microns; and 3) focus offset of 0 mm. Image LUTS scale was set from a minimum of 50 to a maximum of 1500, with a k of 0.2. Calcification was quantified by outlining the entire aorta as the region of interest and determining the ratio of signal intensity to aorta area.

Plaque lipid burden was quantified in a subset of aortas by *en face* staining with Oil Red O (Sigma) as previously described.⁵¹⁶ Aortas were fixed overnight at 4°C in neutral buffered 4% paraformaldehyde in PBS, dissected luminal surface is exposed, and pinned to a silicon plate. Aortas were then washed in PBS and then covered in propylene glycol for 2 minutes at room temperature for dehydration. Aortas were covered by 0.5% Oil Red O in isopropanol for 2 hours at room temperature and then washed in 85% propylene glycol and then in PBS until background staining is cleared. Percent area positive for Oil Red O staining was quantified using Image J.⁴⁶⁷

Statistical analysis

All statistical data were analyzed with the use of Stata/SE (version 15.1; StataCorp LLC, College Station, TX), Prism 7 (GraphPad) or R software version 3.3.1 (R Core Team). Baseline demographics and clinical characteristics were compared between the cohort that was not taking a statin and cohort taking a statin. Continuous variables were tested for normality and equal variance using Shapiro-Wilk test for normality and one-way ANOVA to test for equal variance. Results are presented as mean (standard error) for continuous variables with normal distribution or equal variance and as median (interquartile range) for continuous variables without normal distribution, and as number (percentage) for categorical data. The *t* test was used to compare normally distributed continuous variables between 2 independent groups. The Mann-Whitney U test was used for continuous variables not normally distributed, and Chi-square test was used for categorical variables. Adjustment for cardiovascular covariates was carried out using Stata. A 2-sided $P < 0.05$ was considered statistically significant.

Conclusions & Limitations leading to Future Study

In summary, these findings suggest that statins can promote atherosclerotic calcification and that the mechanism by which this is facilitated is through statins' disruption of macrophage Rac1 isoprenylation. Statin inhibition of 3-hydroxy-3-methylglutaryl coenzymeA reductase disrupts the synthesis of isoprenyl lipid groups that are required for Rac1 to acquire its post-translational isoprenyl-based modification. This causes Rac1 binding to its inhibitor, RhoGDI, to be diminished and results in elevated Rac1 activity in macrophages. Enhanced atherosclerotic calcification observed in *ApoE*-

deficient animals was dependent upon myeloid Rac1 expression and associated with elevated macrophage Rac1 activity in plaque. Moreover, statin treatment of macrophages *in vitro* is associated with the expression of Rac1-dependent IL-1 β (mRNA and protein) in a dose-dependent manner. Elevated systemic IL-1 β expression was also associated with statin-enhanced aortic calcification *in vivo* and its expression was dependent upon myeloid Rac1 expression in statin treated animals.

While these findings show that statins are capable of contributing to vascular calcification by enhancing macrophage Rac1 activity, it is unclear if endogenous Rac1 activity is sufficient to modulate calcification. Therefore, the next phase of investigation led to the study of aortic calcification among animals with or without myeloid-specific Rac1 basal expression. Also, from these findings regarding statin, IL-1 β appears to be a potential downstream effector of macrophage Rac1 activity that may contribute to calcification. This concept is further supported by prior investigation that has demonstrated that disruption of IL-1 signaling via IL-1receptor antagonist can inhibit the progression of aortic calcification. However, these recent findings regarding statin only demonstrate an association with calcification--not causation--and prior use of IL-1receptor antagonist disrupted both IL-1 β and IL-1 α signaling systemically. Given these limitations, one could alternatively argue that IL-1 β may merely be associated with Rac1 activity and not contribute to calcification *in vivo*. Also, if IL-1 β were to presumably play a mechanistic role in calcification, the specific cell type responsible for IL-1 β -mediated calcification is unclear. Therefore, this gap in knowledge led to the study of macrophage IL-1 β 's relationship to atherosclerotic calcification *in vivo*.

Chapter 5:

Endogenous Macrophage Rac1 contributes to Plaque Calcification and NF- κ B-dependent IL-1 β Expression

Joshua M. Berus, Cadence Lee, Willie Dong, Chris Mantsounga, and Alan R. Morrison

Author contributions: A.R.M conceived the study. J.M.B., C.L. performed the *in vitro* and animal experiments. J.M.B., C.L., W.D., C.M., and A.R.M. analyzed the data. All data presented in Figures 5.1, 5.2, 5.3, 5.4, 5.7, 5.8, and 5.9 are a result of experiments conducted solely by J.M.B., whereas Figure 5.5 and 5.6 are from a collaboration between J.M.B. and C.L.

Findings and Reasoning leading to the Study of Macrophage Rac1's Role in Atherosclerotic Calcification

The Morrison laboratory previously published that *Rac2* gene-deleted macrophages had enhanced Rac1 activity and Rac1-dependent IL-1 β expression, which can induce VSMC-mediated calcification *in vitro*.⁷⁸ Hematopoietic-specific *Rac2-deletion* (via bone marrow transplant) was sufficient to enhance vascular calcification in *Apolipoprotein E* (*ApoE*) deficient mice and was associated with enhanced systemic IL-1 β expression.⁷⁸ In the previous chapter, *ApoE*-deficient mice on a statin supplemented diet were shown to exhibit increased Rac1 activity, which was also associated with increased systemic IL-1 β expression and consequent vascular calcification. The increased atherosclerotic calcification was dependent on myeloid Rac1 expression.²⁹⁵ These findings collectively suggest that enhancement of macrophage Rac1 activity is sufficient to increase the degree of atherosclerotic plaque calcification. However, these

studies are under somewhat contrived conditions to enhance Rac1 activation. The global *Rac2*-deletion study and statin study have additional systemic effects that extend beyond macrophages. And these studies that artificially enhanced Rac1 activity do not elucidate whether endogenous macrophage Rac1 activity contributes to the development of vascular calcification during the natural progression of atherosclerotic plaque.

To evaluate the effects of endogenous macrophage Rac1 activity in a live animal model of atherosclerosis, *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} mice were bred and placed on a HFD ad libitum for up to 24 weeks. The *ApoE*^{-/-} mice are a well-established model for atherosclerosis, which is accelerated with the addition of a HFD. Studying atherosclerosis in the *ApoE*^{-/-} model was chosen because it is a well-established model for hypercholesterolemia-induced atherosclerosis. Moreover, common *ApoE* mutations among humans are associated with hyperlipidemic disorders that predispose individuals to atherosclerosis.^{517,518} *CSF1R*^{mcm} *Rac1*^{fl/fl} allows for Rac1 to be conditionally knocked-down permanently in myeloid-specific manner with a brief pulse tamoxifen treatment of 2mg per day for 10 days. Therefore, Rac1 can be downregulated when the mice are young adults at 10-12 weeks of age without affecting Rac1's developmental functions. We used the only myeloid inducible Cre recombinase system available to modulate Rac1 in macrophages but this system also affects other myeloid cells such as neutrophils.⁵¹⁹

Endogenous Myeloid Rac1 Expression Contributes to Female LDL Levels

Dysregulation of serum levels of cholesterol, a biosynthetic lipid, can act as a risk factor for atherosclerosis. Elevated serum low-density lipoprotein (LDL) is associated with

higher risk for increased plaque burden and instability leading to rupture or myocardial infarction.⁵²⁰⁻⁵²² In order to assess if Rac1 expression could be potentially altering well-established mechanisms involved in atherosclerotic disease progression, which could potentially modify the development of calcification, serum cholesterol levels were evaluated. Previously statin-induced and Rac2 knockdown-induced Rac1 activity did not affect serum lipid levels in *ApoE*-deficient mice.^{78,295} However, these findings focused on enhanced Rac1 activity and exerted systemic effects that could confound the relationship between endogenous macrophage Rac1 and cholesterol. Therefore, systemic lipid levels were studied to establish if myeloid Rac1 had any effects that could alter the development of atherosclerotic calcification.

Systemic lipid levels were assessed as follows: total cholesterol, LDL, high-density lipoprotein (HDL), and triglycerides. The Yale Center for Clinical Investigation analyzed serum samples collected at week 18 on HFD from *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl}. The hypothesis was that myeloid Rac1 modulates atherosclerotic plaque development exclusively through downstream pro-inflammatory signaling and not through elevating serum lipid levels. Myeloid *Rac1* gene-deleted animals demonstrated similar total cholesterol, LDL, HDL, and triglycerides (**Figure 5.1A**). Further analysis of these results revealed that loss of Rac1 expression modulated serum total cholesterol and LDL levels exclusively in female animals (**Figure 5.1B**).

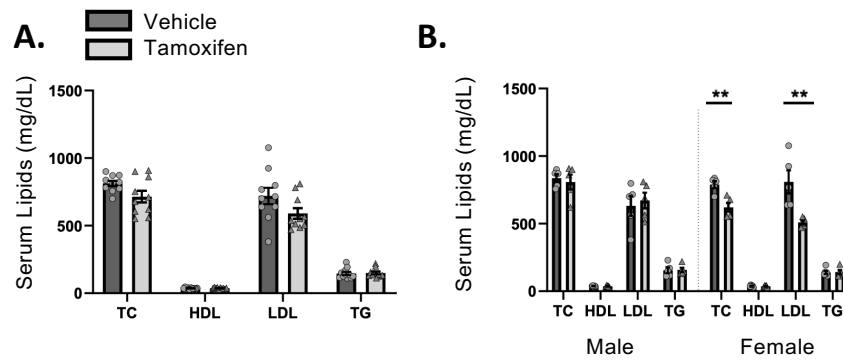


Figure 5.1 Myeloid Rac1 modulates female cholesterol levels.

Lipid panel analysis of serum from *ApoE^{-/-} CSF1R^{mcm} Rac1^{fl/fl}* vehicle control or tamoxifen treated mice fed a high-fat diet (HFD) for 24 weeks. TC = Total Cholesterol, HDL = High-Density Lipoprotein, LDL = Low-Density Lipoprotein, TG = Triglycerides. **A.** Analysis by condition (not significant by ANOVA; n=20 mice; 10 males and 10 females) **B.** Analysis by sex. (Females; TC, **, P<0.05 ANOVA; LDL **, P<0.05 ANOVA; n=10 mice; 5 vehicle and 5 tamoxifen)

Myeloid Rac1 does not affect Aortic Plaque Lipid Burden

Myeloid Rac1 was observed to modulate LDL levels in females. Serum LDL levels are a risk factor for atherosclerotic plaque burden in-part because they contribute to the accumulation of lipids in the intima. Therefore, Rac1's relationship to plaque formation was investigated. Previously, Rac2 knockout mice and statin fed mice, which exhibited enhanced Rac1 activity, showed comparable total plaque burden. The global *Rac2* gene deletion and the systemic effects of statin treatment on all cell types distort our ability to directly evaluate the role of endogenous myeloid Rac1 expression on plaque burden. Therefore, to understand if endogenous Rac1 effects was related to plaque burden, Rac1's effects on aortic plaque formation were directly investigated.

To do this, myeloid Rac1's effect on total plaque burden were evaluated in our animal model. Therefore, en face Oil Red O staining of aortas from *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} after 18 weeks on HFD was used. This method is a common strategy for identifying the presence of atherosclerotic plaques by staining the dense accumulation of lipids—primarily cholesterol—within the intima of plaques. Based on Rac1's contribution to systemic LDL levels in female animals, a difference in plaque burden between vehicle control and myeloid Rac1 knockdown mice was expected in females (but not males). Comparable levels of Oil Red O staining were observed between vehicle and tamoxifen treated animals and no sex-specific effects occurred (**Figure 5.2A and 5.2B**).

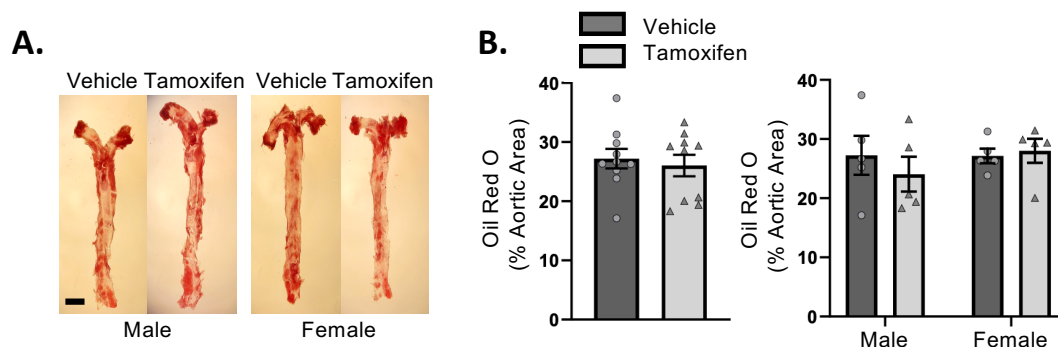


Figure 5.2 Myeloid Rac1 does not affect plaque lipid burden.

A. En face Oil Red O lipid staining of aortic whole mounts from *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} vehicle control or tamoxifen treated mice fed a high-fat diet (HFD) for 18 weeks of HFD, **(B)** along with quantification of percent aortic area stained positive for Oil Red O by condition and sex. Bar=0.5 cm. (Not significant by ANOVA; n=20 mice; 10 males and 10 females.)

Myeloid Rac1 contributes to Plaque Calcification but neither Plaque Size nor Fibrosis

To determine the impact of myeloid *Rac1* gene-knockdown on the phenotypic features of atherosclerotic plaque, traditional histological sectioning and staining at the level of the aortic sinus were utilized. Brightfield microscopy and ImageJ analysis was used to assess plaque size (Hematoxylin & Eosin), fibrosis (Masson's Trichrome), and calcification (Alizarin Red). Paraffin embedded histology was used because it allows one to investigate features of individual plaques among multiple animals with microscopic resolution imaging. Additionally, different stains were applied to the next 6-8 adjacent tissue sections (each section 10µm thick), which allows one to better assess fibrosis and calcification in the same areas of interest. Comparable sinus plaque area and sinus plaque collagen area were observed by Hematoxylin & Eosin and Masson's Trichrome staining, respectively, between vehicle and tamoxifen treated mice (**Figure 5.3A and 5.3B**). Sinus plaque area with calcification, indicated by Alizarin Red staining, exhibited a Rac1-dependent decline among tamoxifen treated animals. (**Figure 5.3A and 5.3B**).

Myeloid Rac1 is critical for Aortic Atherosclerotic Calcification

In order to measure potential changes to global aortic calcification, a calcium-chelating bisphosphonate-conjugated near-infrared agent that has a high affinity for hydroxyapatite at sites of active mineralization was used. Treating the mice with this agent (100µL, 0.02nmol/µL) prior to tissue harvesting allows for *ex-vivo* molecular imaging and quantification of aortic calcification. ^{453,454,457,461} An advantage of this approach is that its near-infrared signal is more sensitive and more specific to hydroxyapatite than other

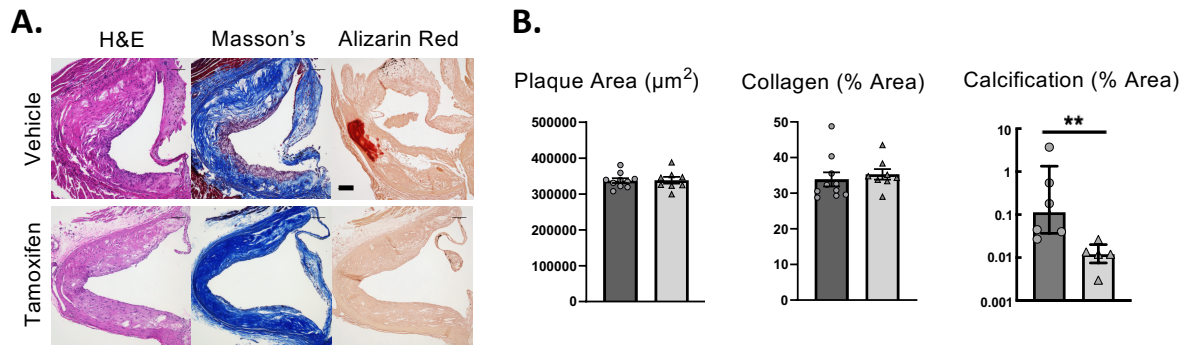


Figure 5.3 Calcified plaque is associated with myeloid *Rac1* expression.

A. Hematoxylin and eosin (H&E) staining, Masson's trichrome staining, and Alizarin Red staining of adjacent aortic sinus sections at the level of the aortic valve from *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} vehicle control or tamoxifen mice fed a high-fat diet (HFD) for 24 weeks. Bar, 100 μm . **B.** Quantification of aortic plaque area (not significant by ANOVA; n=20 mice; 10 male and 10 female), the percentage of collagen within the plaque area (as indicated by positive Masson's trichrome staining) (not significant by ANOVA; n=20 mice; 10 male and 10 female), and the percentage of calcification within the plaque area (as indicated by positive Alizarin Red staining) (**, $P < 0.05$ Mann-Whitney test; n=12 mice; 6 male and 6 female).

histological staining strategies (i.e. von Kossa staining).^{453,465} Tamoxifen induced myeloid-specific Rac1 knockdown (in *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} mice on a HFD) was hypothesized to result in decreased atherosclerotic plaque calcification compared to vehicle control treated mice. Myeloid Rac1 knockdown resulted in significantly less in atherosclerotic calcification compared to vehicle control animals with endogenous myeloid Rac1 expression (**Figure 5.4A and 5.4B**). Of note, sex was considered as a biological variable for aortic calcification using ANOVA analysis, and no sex-dependent differences were observed.

Myeloid Rac1 expression is associated with Systemic IL-1 β Serum Levels

Next, to assess if myeloid *Rac1* gene-deletion affects systemic markers of inflammation, serum cytokine levels of IL-1 β , IL-1 α , and TNF- α were tested. The Morrison laboratory has shown in the global *Rac2*-deletion study a correlation between enhanced macrophage Rac1 activity and IL-1 β expression, without affecting the expression of other cytokines. This same relationship was observed in the statin study, which also exhibited enhanced macrophage Rac1 activity associated with elevated IL-1 β expression *in vitro*.^{78,295}

Beyond Rac1's association with IL-1 β , inhibition of systemic IL-1 β signaling through the use of IL-1receptor antagonist has been shown to be sufficient to inhibit the progression of aortic calcification in *ApoE* deficient mice.⁷⁸ Serum IL-1 β is also associated with cardiovascular death in patients with high coronary calcium burden and can promote VSMC-mediated calcification *in vitro*.⁷⁸ Collectively, the association between

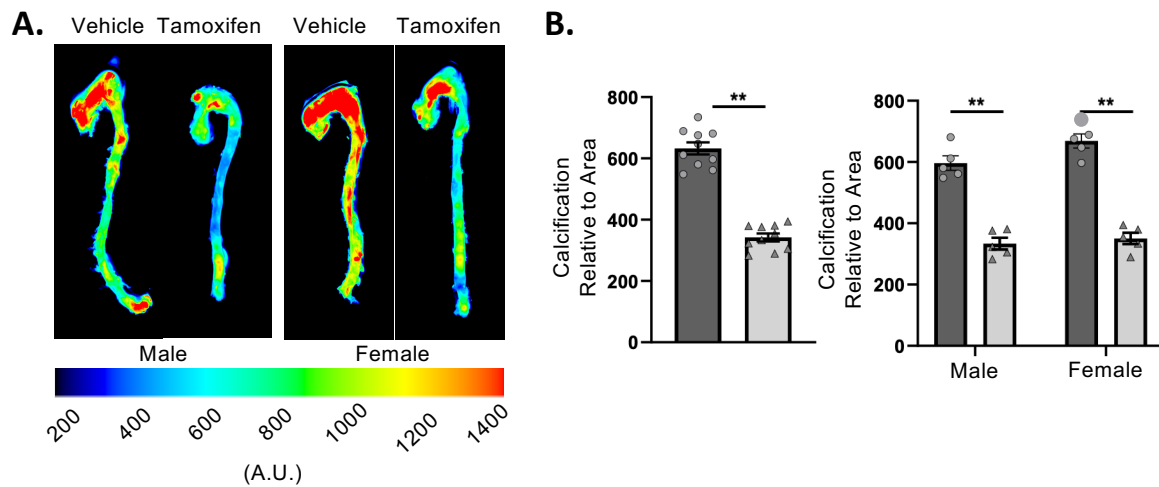


Figure 5.4 Global aortic calcification is associated with myeloid *Rac1* expression.

A. *Ex vivo* near-infrared calcium imaging from *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} vehicle control or tamoxifen treated mice fed a high-fat diet (HFD) over 24 weeks along with **(B)** quantification of calcification signal relative to the area of the aorta by condition (**, $P < 0.0001$ ANOVA; $n = 20$; 10 males and 10 females) and by sex (Males; **, $P < 0.0001$ ANOVA; $n = 10$, 5 vehicle, 5 tamoxifen; Females; **, $P < 0.0001$ ANOVA; $n = 10$, 5 vehicle, 5 tamoxifen).

IL-1 β and calcification in both laboratory and clinical studies suggests that IL-1 β may play a causal role in calcification. Given these findings, it was thought that macrophage Rac1 activity enhances systemic IL-1 β expression, which promotes vascular calcification downstream. Therefore, it was hypothesized that if endogenous myeloid Rac1 expression is modulating calcification through IL-1 β expression, then one should see a decline in IL-1 β among myeloid *Rac1*-deleted mice. To test this, serum IL-1 β levels from myeloid *Rac1* wild-type or gene-deleted mice were measured.

Serum IL-1 α and TNF- α were also assessed to determine the specificity of myeloid *Rac1*-deletion to determine whether Rac1 signaling exerts a broad impact on systemic inflammatory signaling or whether it is more specific to IL-1 β , as suggested from past findings. TNF- α was also studied because it has been implicated in calcification. TNF- α can regulate osteogenic signaling (via MSX2) and consequent calcification both in *LDLR* deficient mice *in vivo* and bovine aortic SMCs *in vitro*.^{163,173} Regarding IL-1 α , it is important to note that both IL-1 α and IL-1 β signaling are mediated through the same IL-1 receptor. The Morrison laboratory previously demonstrated that inhibition of IL-1 receptor signaling via IL-1ra can impair the progression of aortic calcification.^{78,295}

Cytokine levels from serum samples collected at week 0, 12, 18, and 24 on HFD from *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} were measured using ELISA. This method was chosen because of its high protein sensitivity and quantitative ability. Moreover, protein levels of circulating IL-1 β were measured to evaluate the mature secreted protein as opposed to its intracellular, inactive form, pro- IL-1 β . Other assays such as quantitative RT-PCR

cannot distinguish between these forms of IL-1 β . Though Western Blot can distinguish between pro- and mature IL-1 β by molecular weight, it is less sensitive compared to ELISA and densitometry is somewhat semi-quantitative relative to ELISA.

Based on prior preliminary data from the Morrison laboratory, myeloid *Rac1*-deletion mice were anticipated to demonstrate reduced serum IL-1 β levels but similar IL-1 α or TNF- α levels relative to vehicle control samples when animals were placed on a HFD. Myeloid *Rac1* gene-deletion mice demonstrated reduced serum IL-1 β upon HFD for 18 weeks. (**Figure 5.5A**). Also, serum IL-1 β among vehicle control treated animals was observed to be significantly elevated by week 12 and then peaked at week 18, after which it declined (**Figure 5.5A**). Through the course of the study myeloid *Rac1*-deletion was sufficient to significantly reduce serum levels of IL-1 β expression in the mice. Serum concentrations of IL-1 α and TNF- α were comparable between vehicle control and tamoxifen-treated (induced *Rac1*-deletion) mice (**Figure 5.5B and 5.5C**). Of note, sex was analyzed as a biological variable using ANOVA, and no sex-dependent differences were observed. (**Figure 5.5A, 5.5B, and 5.5C**).

Macrophage *Rac1* expression regulates IL-1 β expression

The Morrison laboratory identified that myeloid *Rac1* expression was responsible for the enhanced systemic IL-1 β expression found in the serum of *Rac2*-deleted mice, which exhibited enhanced *Rac1* activity.⁷⁸ Statin-enhanced myeloid *Rac1* activity was also associated with increased serum IL-1 β expression.²⁹⁵ In both cases, *Rac1* activity was enhanced through genetic or pharmaceutical intervention. These new studies

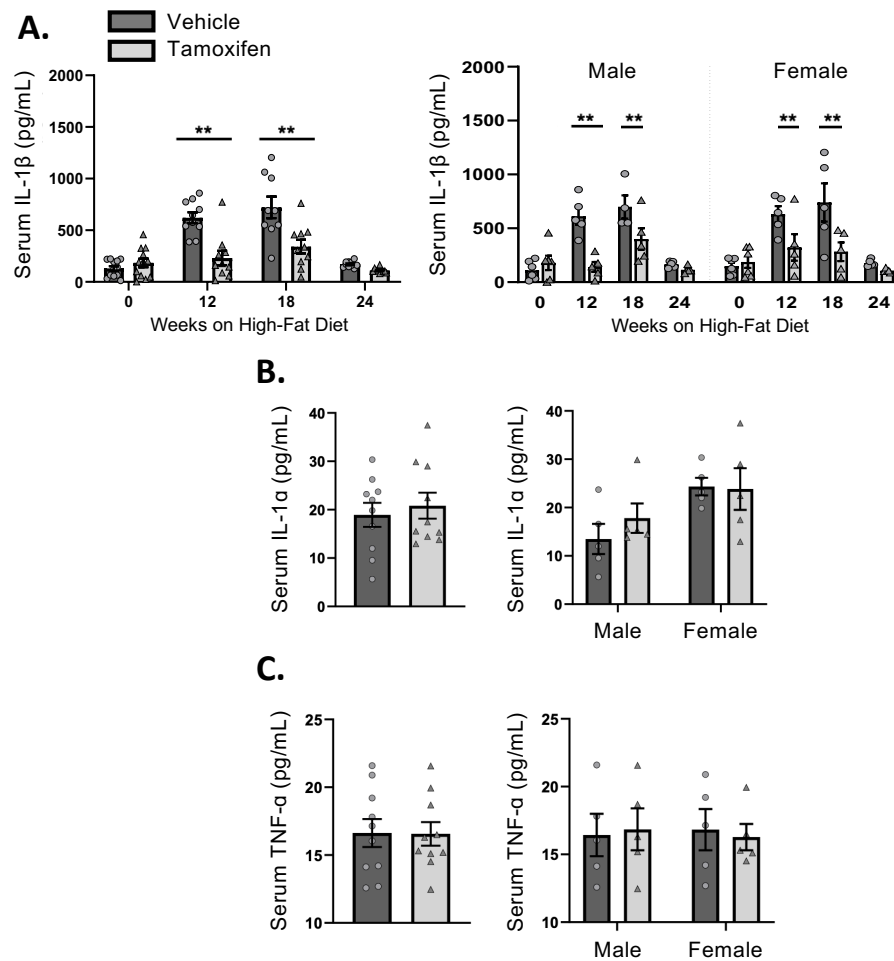


Figure 5.5 Myeloid Rac1 modulates systemic IL-1 β expression.

A. Serum IL-1 β protein concentrations by Enzyme-linked immunosorbent assay (ELISA) from *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} vehicle control or tamoxifen treated mice fed a high-fat diet (HFD). Serum was collected at weeks 0, 12, 18, and 24 on HFD. Measurements were analyzed by condition (**, Week 12 $P < 0.001$ ANOVA; $n = 20$; 10 males and 10 females, **, Week 18 $P < 0.001$ ANOVA; $n = 20$; 10 males and 10 females) and by sex, (**, Males Week 12 $P < 0.001$ ANOVA; $n = 10$; 5 vehicle and 5 tamoxifen, **, Males Week 18 $P < 0.001$ ANOVA; $n = 10$; 5 vehicle and 5 tamoxifen, **, Females Week 12 $P < 0.001$ ANOVA; $n = 10$; 5 vehicle and 5 tamoxifen, **, Females Week 18 $P < 0.001$ ANOVA; $n = 10$; 5 vehicle and 5 tamoxifen).

B. Serum IL-1 α protein concentrations at week 18 analyzed by condition and sex (not significant by ANOVA; $n = 20$ mice; 10 males and 10 females). **C.** Serum TNF- α protein concentrations at week 18 analyzed by condition and sex (not significant by ANOVA; $n = 20$ mice; 10 males and 10 females).

demonstrate that the loss of endogenous myeloid Rac1 expression *in vivo* lead to a reduction in systemic IL-1 β expression but no change in IL-1 α nor TNF- α expression. This further supports a direct relationship between macrophage Rac1 activity and IL-1 β expression. However, the molecular mechanism by which macrophage Rac1 regulates IL-1 β is unknown.

It is important to note that the observed association between Rac1 and IL-1 β *in vivo* has been primarily contributed to macrophages, though myeloid knockdown of *Rac1* may include neutrophils. The relationship between Rac1 and IL-1 β was tested using primary bone-marrow derived macrophages (BMDM) in cell culture. Rac1's relationship to IL-1 α and TNF- α expression was also evaluated to test macrophage Rac1's specificity in regulating pro-inflammatory signaling.

ELISA was used to measure cytokines (IL-1 β , IL-1 α , and TNF- α) from cell culture supernatant. Note, that using the supernatant allowed for measurement of only the mature form of IL-1 β that is secreted out into the cell media to occur. BMDMs were pretreated with or without 4-hydroxytamoxifen (1 μ M) and then subjected to a inflammasome stimulation assay (primed with or without LPS (10ng/mL) for 2 hours followed by exposure to cholesterol crystals (1000 μ g/mL) or vehicle control for 12 hours at 37°C). It was expected that supernatant from *Rac1*-deleted BMDMs would demonstrate reduced IL-1 β expression but similar levels of IL-1 α and TNF- α when compared to vehicle control samples. Knockdown of endogenous Rac1 expression in BMDMs reduced the

expression of IL-1 β (**Figure 5.6A**). Comparable levels of IL-1 α and TNF- α protein were observed between tamoxifen and vehicle control treated cultures (**Figure 5.6B and 5.6C**).

Loss of Macrophage Rac1 expression does not modulated Atherosclerotic Calcification by Impairing Phagocytosis of Cholesterol Crystals

After demonstrating that macrophage Rac1 regulates IL-1 β *in vitro* and mimics the pattern observed in systemic serum expression among myeloid specific *Rac1*-deleted mice *in vivo*, the next step was to explore the mechanisms by which Rac1 modulates IL-1 β expression. Rac1 is known to play an important function in cytoskeleton remodeling and macrophage phagocytic activity depends on proper regulation of the cytoskeleton.^{309,523,524} Macrophage phagocytosis of cellular debris and crystals in the plaque environment can trigger proinflammatory signaling via NLRP3 inflammasome activation.⁴⁹ Therefore, it was important to assess whether macrophage Rac1 may be impairing macrophage phagocytosis of cholesterol crystals, leading to reduced inflammasome activation.

To test this hypothesis, *ApoE*^{-/-}*CSF1R*^{mcm}*Rac1*^{fl/fl} BMDMs were pretreated with or without 4-hydroxytamoxifen (1 μ M) and then primed with or without LPS (10ng/mL) followed by exposure to fluorescent cholesterol crystals (1000 μ g/mL) or vehicle control. The plasma membranes and nuclei were stained to visually observe the degree of crystals phagocytosis by the macrophages. Given that Rac1 is a major regulator of the cytoskeleton, macrophage *Rac1*-deletion was expected to have visibly fewer fluorescent

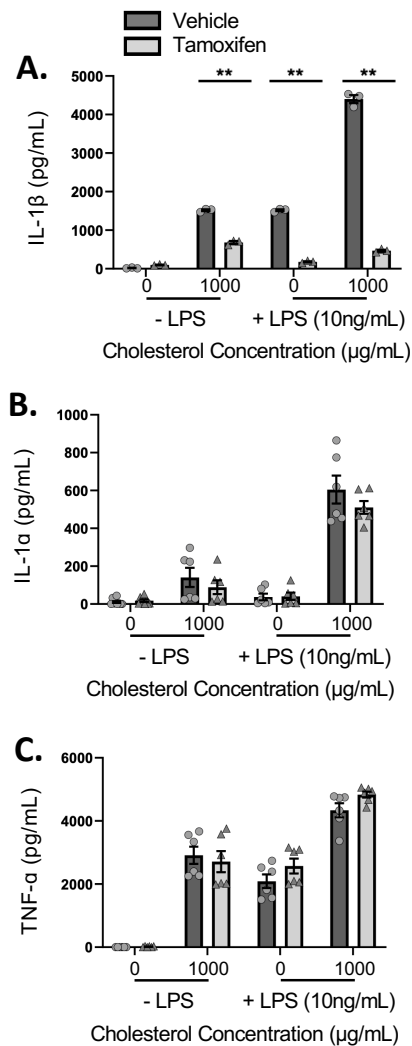


Figure 5.6 Macrophage Rac1 regulates IL-1 β expression.

A. Primary mouse BMDMs from vehicle control or tamoxifen treated *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} mice were primed with or without LPS (10 ng/ml) and exposed to cholesterol crystals (0 or 1000 μ g/mL) for 24 hours to induce inflammasome activation, followed by Enzyme-linked immunosorbent assay (ELISA) on culture supernatants for secreted, mature IL-1 β protein concentrations (Cholesterol treated; **, P<0.0001 ANOVA; n=6 biological replicates from 2 mice; 1 male and 1 female; LPS treated; **, P<0.0001 ANOVA; n=6 biological replicates from 2 mice; 1 male and 1 female; LPS and Cholesterol treated; **, P<0.0001 ANOVA; n=6 biological replicates from 2 mice; 1 male and 1 female). **B.** IL-1 α supernatant protein concentrations (not significant by ANOVA; n=12 biological replicates from 4 mice; 2 males and 2 females). **C.** TNF- α supernatant protein concentrations (not significant by ANOVA; n=12 biological replicates from 4 mice; 2 males and 2 females).

crystals overlapping with the plasma membrane staining, which would indicate reduced phagocytosis. Tamoxifen-induced knockdown of *Rac1* exhibited a qualitatively similar appearance of cholesterol crystals engulfed by macrophages as observed in vehicle control cells (**Figure 5.7A**).

To further investigate *Rac1*'s role in macrophage phagocytosis, a quantitative approach was used to more rigorously assess potential differences in cholesterol crystal phagocytosis. To do this, flow cytometry was utilized to assess the relative amount of macrophage phagocytosis by measuring the fluorescence emitted by cholesterol crystals that were internalized by individual cells. Given this more robust approach a reduction in fluorescence among the tamoxifen treated cells compared to controls was expected. However, no difference in phagocytotic activity between tamoxifen (induced *Rac1*-deletion) and vehicle treated macrophages was observed (**Figure 5.7B and 5.7C**).

Macrophage *Rac1* does not modify Reactive Oxygen Species Production under Cholesterol Crystal Inflammasome Stimulation

Another potential mechanism whereby *Rac1* may regulate IL-1 β expression is through *Rac1*-mediated of reactive oxygen species (ROS) production. *Rac1* activity can alter ROS production via NADPH oxidase. ³⁸⁰ *Rac1*-mediated ROS can also potentially trigger NF- κ B activity. ^{370,428} Both ROS (via NADPH oxidase) and NF- κ B are activators of the NACHT, LRR, and PYD domains-containing protein 3 (NALP3), which is critical for NRLP3 inflammasome formation. ⁴²⁹ The NRLP3 inflammasome complex facilitates the cleavage of inactive pro- IL-1 β to generate mature IL-1 β that can then be secreted. ⁵²⁵

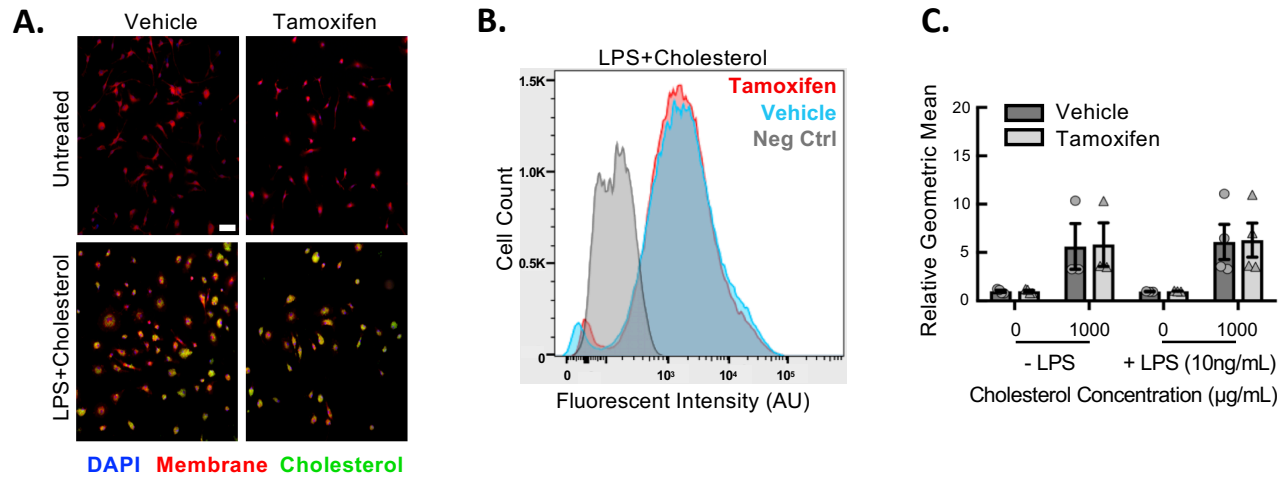


Figure 5.7 Loss of macrophage Rac1 expression does not affect phagocytosis.

A. Primary mouse BMDMs from vehicle control or tamoxifen treated *ApoE^{-/-} CSF1R^{mcm} Rac1^{fl/fl}* mice were primed with or without LPS (10 ng/ml) and exposed to fluorescent cholesterol crystals (0 or 1000 µg/mL) for 24 hours to induce inflammasome activation. Immunofluorescence micrographs of macrophages stained with DAPI and the plasma membrane stained red using CellMask™ Deep Red. The fluorescence from the cholesterol crystals appear green. **B.** Histogram generated by flow cytometry measuring the fluorescent intensity (from engulfed fluorescent cholesterol crystals) and the number of corresponding cells with various intensities from vehicle and tamoxifen treated macrophages. The negative control measured background fluorescence from vehicle treated macrophages that were exposed to standard non-fluorescent cholesterol crystals. **C.** For quantitative analysis, the geometric means were generated from the histograms of fluorescent intensity and the number of corresponding cells with various intensities. The geometric means were then normalized to the unstimulated (no LPS and no cholesterol) vehicle control treated cells (not significant by ANOVA; n=8 biological replicates from 8 mice; 4 males and 4 females).

From previous work, *ApoE*^{-/-} *Rac2*^{-/-} BMDMs have been shown to have elevated ROS production associated with increased IL-1 β expression.⁷⁸ Treatment of these macrophages with small molecule inhibition of NADPH oxidase using diphenyleneiodonium significantly reduced IL-1 β expression (mRNA and protein).⁷⁸ This suggested that Rac1 regulation of IL-1 β expression may be, in part, through ROS production. However, contrary to the findings with the *ApoE*^{-/-} *Rac2*^{-/-} BMDMs, statin-treated BMDMs demonstrated increased IL-1 β expression but reduced ROS production compared to vehicle treated cells.²⁹⁵ This finding suggested that in the setting of statin-induced Rac1 activity IL-1 β expression may not require increased ROS production.

Therefore, the dependence of ROS production on and endogenous macrophage Rac1 was evaluated. The hypothesis being tested was that ROS production regulated IL-1 β expression downstream of endogenous Rac1 expression in macrophages. To investigate this, *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} BMDMs were pretreated with or without 4-hydroxytamoxifen (~1 μ M) and then primed with or without LPS (10ng/mL) followed by exposure to cholesterol crystals (1000 μ g/mL) or vehicle control. ROS production (i.e. hydrogen peroxide) was then measured among these live cells using a luminescence assay. There are many different ROS products but we choose to measure hydrogen peroxide for two reasons: (1.) It has the longest half-life of all ROS (2.) many other ROS are eventually converted into hydrogen peroxide. Therefore, by measuring hydrogen peroxide one can measure a relatively stable metric of ROS production and it acts as a rough proxy for many other ROS chemical species. Reduced ROS production from Rac1-deleted cells (compared to vehicle controls) was expected to occur. However, Rac1

knockdown macrophages demonstrated comparable ROS levels to vehicle control cells, suggesting that Rac1 expression did not impact ROS production (**Figure 5.8**).

Macrophage Rac1 regulates Nuclear NF- κ B Activity under Inflammasome Cholesterol Crystal Stimulation

NF- κ B is an important transcription factor for macrophage IL-1 β expression and Rac1 activity has been reported to regulate NF- κ B activity in HeLa cells.^{526,527} It is important to note that HeLa cells are immortalized cervical cancer cells, and translating these findings into normal primary cells like macrophages can be challenging. But considering these findings, Rac1 activity may potentially regulate IL-1 β expression through downstream NF- κ B nuclear localization and activity. Additionally, this potential pathway is supported by findings that enhanced macrophage Rac1 activity was associated with increased NF- κ B activity *in vitro* in both of the prior macrophage models, the *Rac2*-deletion and statin-treatment.^{78,295}

In order to study the relationship between endogenous macrophage Rac1 expression and NF- κ B activity, *ApoE^{-/-}CSF1R^{mcm}Rac1^{fl/fl}* BMDMs were pretreated with or without 4-hydroxytamoxifen (1 μ M) and then underwent inflammasome stimulation by being primed with or without LPS (10ng/mL) followed by exposure to cholesterol crystals (1000 μ g/mL) or vehicle control. The nuclear protein of these cells was isolated by centrifugation and nuclear fractionation, and then ELISA-based colorimetric assay was used to measure the NF- κ B activity. This approach was chosen because it measures the amount of nuclear NF- κ B that is actively binding to double stranded DNA to promote

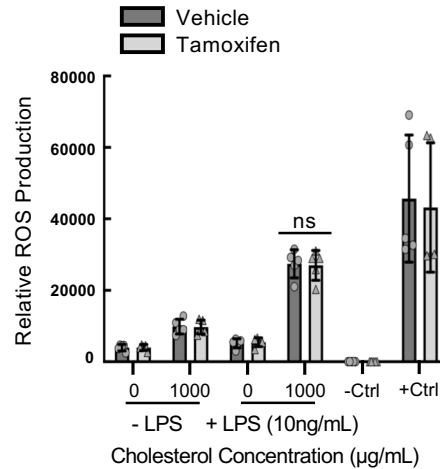


Figure 5.8 Loss of macrophage *Rac1* expression does not affect ROS production.

Relative luminescence as a measure of reactive oxygen species (ROS) production in primary mouse BMDMs from vehicle control or tamoxifen treated *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} mice were primed with or without LPS (10 ng/ml) and exposed to cholesterol crystals (0 or 1000 µg/mL) to induce inflammasome activation. The negative control measured a cell-free culture to detect background noise and menadione (50µM) treated cells were used as a positive control (not significant by ANOVA; n=10 biological replicates from 8 mice; 4 males and 4 females) .

transcription. This excludes the potential risk of any inactive cytosolic NF- κ B from being measured and also ensures that only nuclear NF- κ B that has potential to bind to DNA is being measured to more closely gauge the level of transcriptional activity. Moreover, this is a highly sensitive assay which is ideal when trying to measure low levels of an activated nuclear protein. Based on prior findings, tamoxifen-treated macrophages were expected to exhibit reduced NF- κ B activity.^{78,295} Inflammasome stimulated *Rac1*-deleted BMDMs exhibited significantly reduced NF- κ B activity (~40% reduction) compared to vehicle control cells with endogenous *Rac1* expression (that were also stimulated with LPS and cholesterol crystals) (**Figure 5.9**).

Summary

Prior investigation using *Rac2* knockout animals or animals on a statin supplemented HFD have demonstrated that enhanced macrophage *Rac1* activity contributes to increased IL-1 β expression and calcification. In this chapter, the loss of endogenous macrophage *Rac1* expression (via gene deletion) and consequently the loss of basal *Rac1* activity levels has been shown to be sufficient to modulate IL-1 β expression and atherosclerotic plaque calcification. Moreover, the global *Rac2* deficient mice and *ApoE* deficient mice fed a statin diet, both exert systemic effects on various cell types. These systemic effects may be required for a *Rac1*-IL-1 β pathway to effectively modulate atherosclerotic calcification. This chapter has clarified that macrophage *Rac1* signaling can directly contribute to plaque development independently of the confounding factors that were relevant in earlier reports.

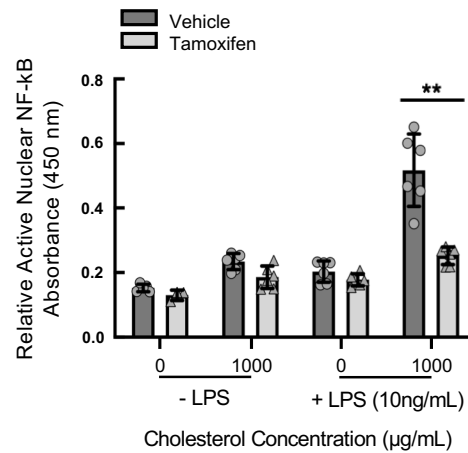


Figure 5.9 Macrophage Rac1 expression regulates nuclear NF-κB activity .

Relative nuclear NF-κB activity measured by absorbance in primary mouse BMDMs from vehicle control or tamoxifen treated *ApoE*^{-/-} *CSF1R*^{mcm} *Rac1*^{fl/fl} mice were primed with or without LPS (10 ng/ml) and exposed to cholesterol crystals (0 or 1000 µg/mL) to induce inflammasome activation (**, P<0.001 ANOVA; n=12 biological replicates from 4 mice; 2 male and 2 female).

The mechanism by which macrophage Rac1 regulates IL-1 β expression appears to be facilitated through nuclear NF- κ B activity, a known transcription factor for IL-1 β . Findings from the studies of global Rac2 deleted and statin treated animals also complement this proposed mechanism. These studies observed an increase in NF- κ B activity associated with increased macrophage Rac1 activity.

The observation that loss of endogenous macrophage Rac1 expression led to a female-specific reduction in serum LDL (and consequently serum total cholesterol) is unexpected and has not been previously reported. This finding presents new opportunities for future studies of a macrophage Rac1 specific pathway among females that regulates cholesterol and could potentially lead to novel therapies to lower LDL levels among female patients. Despite this reduction in serum LDL among female animals, it was insufficient to alter plaque formation and development. Therefore, it did not act as a confounding factor in this study.

Collectively, the findings reported in this chapter demonstrate that endogenous macrophage Rac1 expression is sufficient to contribute to IL-1 β expression (both *in vitro* and *in vivo*) and atherosclerotic plaque calcification. Moreover, these findings complement prior work in suggesting that macrophage Rac1 regulates IL-1 β expression through nuclear NF- κ B activity.

Chapter 6:

Endogenous Macrophage IL-1 β expression contributes to Atherosclerotic Calcification

Joshua M. Berus, Cadence Lee, Willie Dong, Chris Mantsounga, and Alan R. Morrison

Author contributions: A.R.M conceived the study. J.M.B., C.L. performed the *in vitro* and animal experiments. J.M.B., C.L., W.D., C.M., and A.R.M. analyzed the data. All data presented in Figures 6.1, 6.2, 6.3, and 6.4 are a result of experiments conducted solely by J.M.B., whereas Figure 6.5 is from a collaboration between J.M.B. and C.L.

Findings and Reasoning leading to the Study of the Role of Macrophage IL-1 β Expression in Atherosclerotic Calcification

The Morrison laboratory's previous study of *Rac2*-deletion and statin treatment, along with the recent findings of the previous chapter regarding myeloid *Rac1*-deleted mice identified a positive association between and IL-1 β expression and calcification in both *in vitro* and *in vivo* experimental models. In BMDMs, endogenous *Rac1* expression has been shown to regulate nuclear NF- κ B activity, and NF- κ B is a known transcription factor for IL-1 β . We hypothesized that IL-1 β acts downstream of macrophage *Rac1*-NF- κ B signaling to promote calcification and that myeloid IL-1 β is sufficient to regulate calcification *in vivo*. In support of this hypothesis, the Morrison laboratory previously found that the systemic inhibition of IL-1 receptor signaling with IL-1 receptor antagonist treatment can inhibit the progression of aortic calcification in *ApoE*^{-/-} *Rac2*^{-/-} mice on a high-fat diet.⁷⁸ However, this finding is not necessarily specific to IL-1 β , as IL-1 α can be

affected, nor is this systemic treatment necessarily specific to macrophage IL-1 β expression. Thus, a mechanistic relationship has never been demonstrated between macrophage IL-1 β expression and atherosclerotic calcification of the aorta *in vivo*. Therefore, the effects of endogenous myeloid IL-1 β expression in an experimental animal model of atherosclerosis were evaluated to test if it was sufficient to modulate aortic calcification.

ApoE^{-/-} *CSF1R*^{mcm} IL-1 β ^{fl/fl} mice were bred and placed on HFD *ad libitum* for 18 weeks. *CSF1R*^{mcm} IL-1 β ^{fl/fl} allows for tamoxifen-inducible *IL-1 β* - gene deletion in a myeloid-specific manner. Tamoxifen treatment of BMDMs from these mice demonstrated 75% reduction in both IL-1 β mRNA and protein expression when subjected to strong inflammasome stimulation with LPS priming and cholesterol crystal exposure.⁴⁵¹ With these animals, one can downregulate IL-1 β when the mice are young adults at 10-12 weeks of age with a pulse treatment of 10 days tamoxifen without affecting vascular development. These newly generated animals allow both age- and cell-specific changes to IL-1 β expression *in vivo* for the first time. All prior *in vivo* studies of IL-1 β have been conducted using IL-1 β global deletion animals or treating animals with systemically administered anti-IL-1 β antibodies and have focused on measuring plaque burden and not atherosclerotic calcification.^{528 192} These previous strategies present challenges to understanding the cell-specific roles of IL-1 β expression, and global deletion models can alter animals' early development, which can serve as a confounding factor in result interpretation.

Myeloid IL-1 β expression does not affect Serum Lipid Levels

Elevated cholesterol level is a risk factor for atherosclerosis. As presented early, female-specific myeloid Rac1 expression was found to contribute to systemic LDL levels. In the female Rac1 knockdown mice, serum IL-1 β was also observed to be decreased. Cytokines have been previously reported to be capable of participating in cholesterol metabolism.^{529,530} Therefore, IL-1 β expression could be potentially altering well-established lipid-based mechanisms involved in atherosclerotic disease progression, which could potentially modify the development of calcification. Moreover, it was important to assess if the female-specific effect of myeloid Rac1 expression on serum LDL levels was specific to Rac1 or acting downstream of Rac1 through IL-1 β . Thus, the lipid profile of serum from both male and female mice were studied separately.

To investigate the potential role of myeloid IL-1 β regulating systemic lipid levels, including total cholesterol, LDL, HDL, and triglycerides, the Yale Center for Clinical Investigation analyzed serum samples collected at week 18 on HFD from *ApoE*^{-/-} *CSF1R*^{mcm} IL-1 β ^{fl/fl}. No change in circulating lipid content was expected as it was hypothesized that myeloid IL-1 β was primarily modulating atherosclerotic plaque development by promoting an osteogenic phenotype among VSMCs in the intima. The Morrison laboratory previously reported that recombinant IL-1 β can trigger VSMC-mediated calcification *in vitro*.⁷⁸ Myeloid IL-1 β knockdown animals in this study demonstrated similar serum levels of total cholesterol, HDL, LDL, and triglycerides compared to vehicle controls (**Figure 6.1A**). No sex specific differences were observed (**Figure 6.1B**).

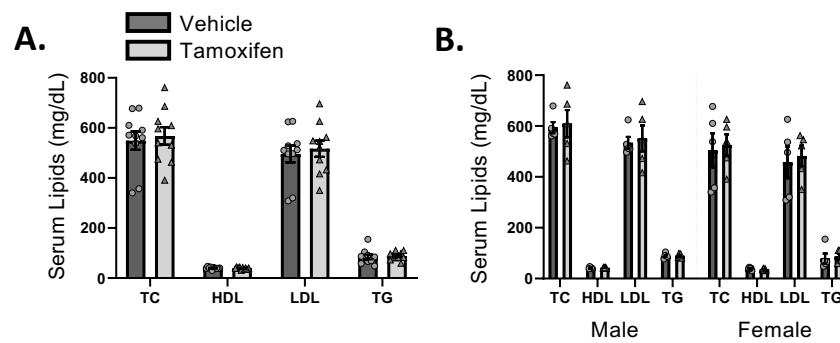


Figure 6.1 Myeloid IL-1 β does not modulates serum cholesterol.

Lipid panel analysis of serum from *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1 β* ^{fl/fl} vehicle control or tamoxifen treated mice fed a high-fat diet (HFD) for 18 weeks. TC = Total Cholesterol, HDL = High-Density Lipoprotein, LDL = Low-Density Lipoprotein, TG = Triglycerides. **A.** Analysis by condition (not significant by ANOVA; n=20 mice; 10 males and 10 females) **B.** Analysis by sex (not significant by ANOVA; n=10 mice; 5 vehicle and 5 tamoxifen).

Myeloid IL-1 β does not affect Aortic Plaque Lipid Burden

Both *Rac2*-deleted and statin fed mice with enhanced *Rac1* activity and consequent increased serum IL-1 β demonstrated comparable global aortic plaque lipid burden compared to vehicle controls with basal serum IL-1 β levels.^{78,295} However, both of these studies have confounding factors that may have negated myeloid IL-1 β from modulating plaque lipid burden. The *Rac2*-deletion study altered the expression of all cells, not just macrophages, and the statin study presented potential pleotropic effects that may confound the study's interpretation. From these other studies it appears that elevated serum IL-1 β does not affect the prevalence of plaque. However, it is unclear if basal IL-1 β levels contribute to plaque formation. Therefore, to evaluate if myeloid IL-1 β affects the prevalence of plaques en face Oil Red O staining of aortas from *ApoE*^{-/-} *CSF1R*^{mcm} IL-1 β ^{fl/fl} after 18 weeks on HFD was used. No significant difference in plaque burden between vehicle control and myeloid IL-1 β knockdown animals was expected. Comparable levels of Oil Red O staining were observed between vehicle and tamoxifen treated animals (**Figure 6.2A and 6.2B**).

Myeloid IL-1 β contributes to Plaque Calcification but not Plaque Area or Fibrosis

To assess myeloid IL-1 β 's effect on the phenotypic features of atherosclerotic plaque traditional histological sectioning and staining at the level of the aortic sinus was used. Brightfield microscopy was used to assess plaque area (hematoxylin & eosin), fibrosis by collagen staining (Masson's trichrome), and calcification (Alizarin Red). Sinus plaque area and sinus plaque collagen area were comparable between vehicle and

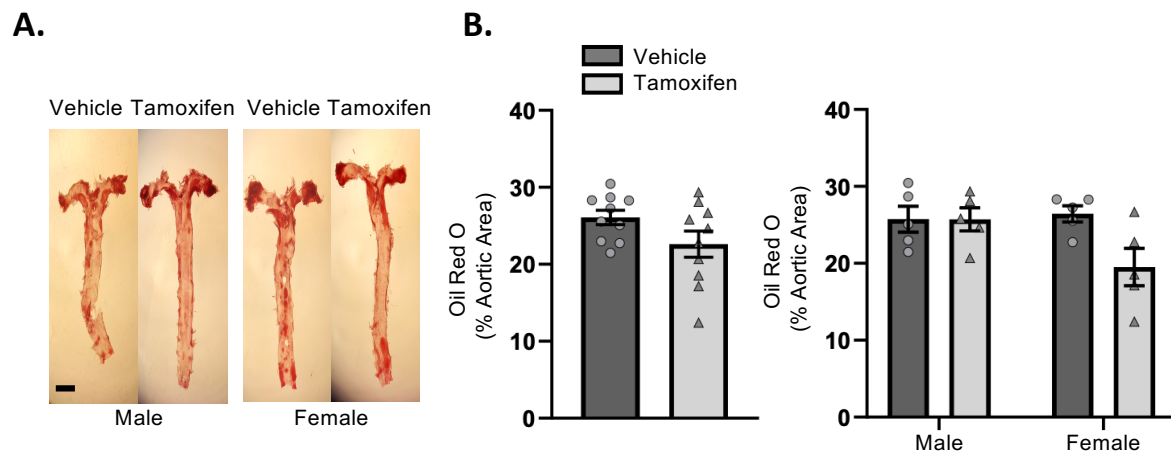


Figure 6.2 Myeloid IL-1 β does not affect plaque lipid burden.

A. En face Oil Red O lipid staining of aortic whole mounts from *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1 β* ^{fl/fl} vehicle control or tamoxifen treated mice fed a high-fat diet (HFD) for 18 weeks of HFD, (**B**) along with quantification of percent aortic area stained positive for Oil Red O by condition and sex. Bar=0.5 cm. (Not significant by ANOVA; n=20 mice; 10 males and 10 females.)

tamoxifen treated (induced myeloid *IL-1 β* -deletion) animals (**Figure 6.3A and 6.3B**). However, sinus plaque calcification area by Alizarin Red staining was decreased in plaques from tamoxifen treated animals consistent with calcification being a myeloid-specific *IL-1 β* -dependent process. (**Figure 6.3A and 6.3B**).

Myeloid IL-1 β is critical for Aortic Atherosclerotic Calcification

In order to measure the global degree of atherosclerotic calcification of the aorta, a calcium-chelating bisphosphonate-conjugated near-infrared agent that has a high affinity for hydroxyapatite was used. Treating the mice with this agent prior to tissue harvesting allows for *ex vivo* molecular imaging and quantification of global calcification in aortic tissue. ^{453,454,457,461} Tamoxifen-induced myeloid-specific *IL-1 β* gene-deletion in *ApoE*^{-/-} *CSF1R*^{*mcm*} *IL-1 β* ^{*fl/fl*} on HFD was expected to result in decreased atherosclerotic plaque calcification compared to vehicle control treated mice. Myeloid *IL-1 β* gene-deletion resulted in significantly less in atherosclerotic calcification compared to vehicle control mice with endogenous myeloid *IL-1 β* expression (**Figure 6.4A and 6.4B**).

Myeloid IL-1 β is a Major Source of Systemic IL-1 β in Serum

Myeloid *Rac1*-deletion mice, which had reduced calcification, exhibited decreased systemic *IL-1 β* levels in serum. This suggests that macrophage *Rac1* activity may potentially be modulating vascular calcification at least in-part through downstream macrophage-derived systemic *IL-1 β* expression. In support of this hypothesis, serum *IL-1 β* was previously observed to be associated with cardiovascular death in patients with high coronary calcium burden and *IL-1 β* has been shown to be sufficient to promote

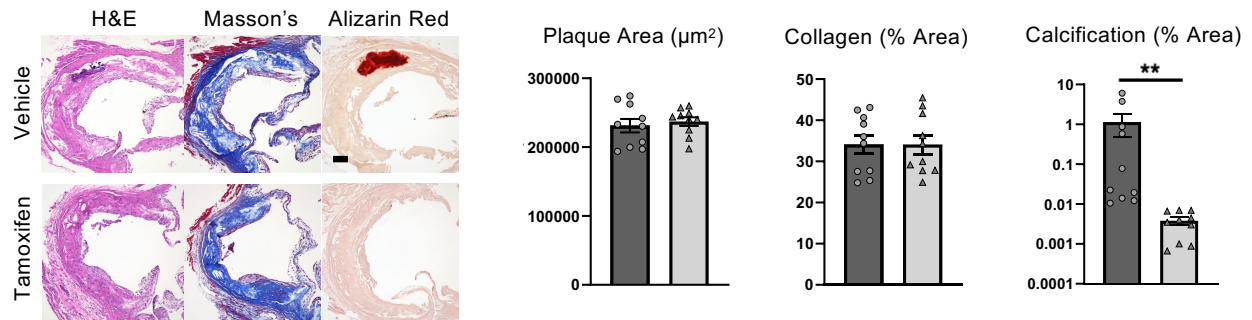


Figure 6.3 Calcified plaque is associated with myeloid IL-1 β expression.

A. Hematoxylin and eosin (H&E) staining, Masson's trichrome staining, and Alizarin Red staining of adjacent aortic sinus sections at the level of the aortic valve from *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1 β* ^{fl/fl} vehicle control or tamoxifen mice fed a high-fat diet (HFD) for 18 weeks. Bar, 100 μm . **B.** Quantification of aortic plaque area (not significant by ANOVA; n=20 mice; 10 male and 10 female), the percentage of collagen within the plaque area (as indicated by positive Masson's trichrome staining) (not significant by ANOVA; n=20 mice; 10 male and 10 female), and the percentage of calcification within the plaque area (as indicated by positive Alizarin Red staining) (**, P<0.05 Mann-Whitney test; n=20 mice; 10 male and 10 female).

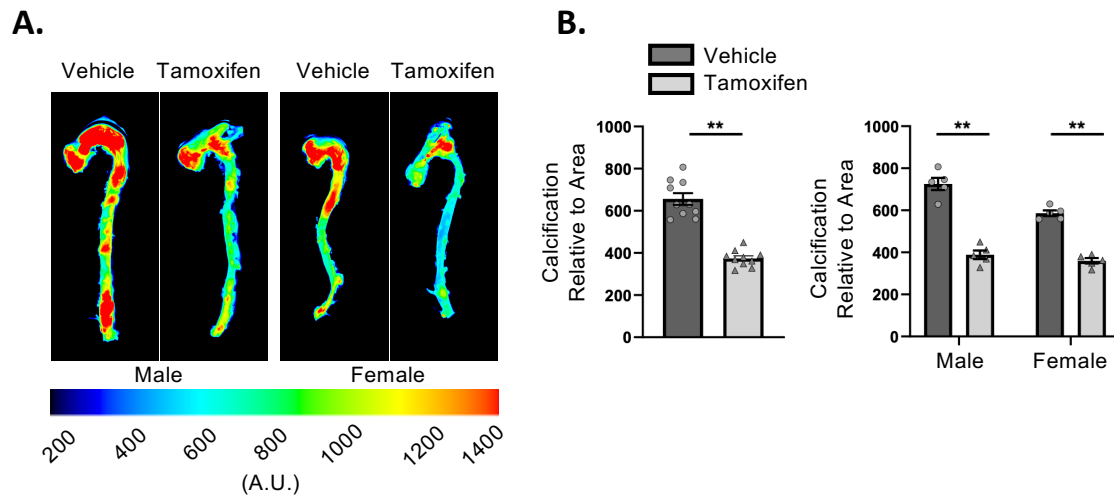


Figure 6.4 Global aortic calcification is associated with myeloid IL-1 β expression.

A. *Ex vivo* near-infrared calcium imaging from *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1 β* ^{fl/fl} vehicle control or tamoxifen treated mice fed a high-fat diet (HFD) for 18 weeks along with **(B)** quantification of calcification signal relative to the area of the aorta by condition (**, $P < 0.0001$ ANOVA; $n = 20$; 10 males and 10 females) and by sex (Males; **, $P < 0.0001$ ANOVA; $n = 10$, 5 vehicle and 5 tamoxifen; Females; **, $P < 0.0001$ ANOVA; $n = 10$, 5 vehicle and 5 tamoxifen).

VSMC calcification *in vitro*.⁷⁸ In order to test if myeloid IL-1 β is a major source of circulating IL-1 β expression in serum, ELISA was used to measure IL-1 β protein from serum samples from *ApoE*^{-/-} *CSF1R*^{mcm} IL-1 β ^{fl/fl} on a HFD after 18 weeks. Week 18 on HFD was shown earlier to be the period of peak systemic IL-1 β expression this study's animal model for atherosclerosis (*ApoE*^{-/-}). Systemic levels of IL-1 β were expected to be significantly reduced among myeloid-specific *IL-1 β* -deletion mice compared to vehicle controls. Among these myeloid IL-1 β knockdown mice, peak serum IL-1 β concentrations at week 18 of HFD were reduced (**Figure 6.5A**).

In order to test the specificity of myeloid IL-1 β 's influence on atherosclerosis, it was important to assess if knocking down IL-1 β modulated other systemic inflammatory cytokines, such as IL-1 α and TNF- α . Therefore, ELISA was used to also measure IL-1 α and TNF- α among serum samples from the *ApoE*^{-/-} *CSF1R*^{mcm} IL-1 β ^{fl/fl} on a HFD after 18 weeks. Deletion of myeloid *IL-1 β* was not expected to affect systemic IL-1 α nor TNF- α expression. This prediction was based on previous findings that reduced systemic myeloid IL-1 β levels in *Rac1* knockdown animals was associated with no changes in IL-1 α nor TNF- α levels compared to vehicle controls. Also, findings from the statin study demonstrated that changes in serum IL-1 β were not associated with any changes in systemic IL-1 α and TNF- α expression.²⁹⁵ Reduced myeloid IL-1 β expression had no effect serum levels of the pro-inflammatory cytokines, IL-1 α and TNF- α , between vehicle control and tamoxifen treated mice (**Figure 6.5B and 6.5C**).

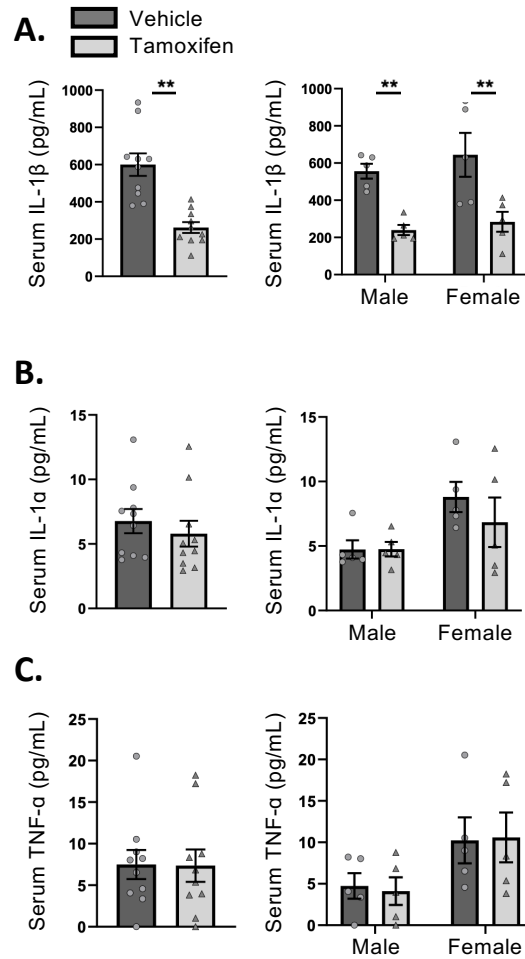


Figure 6.5 Systemic serum IL-1 β is significantly derived from myeloid cells.

A. Serum IL-1 β protein concentrations by Enzyme-linked immunosorbent assay (ELISA) from *ApoE*^{-/-} *CSF1R*^{mcm} *IL-1 β* ^{fl/fl} vehicle control or tamoxifen treated mice fed a high-fat diet (HFD) for 18 weeks. Measurements were analyzed by condition (**, $P < 0.0002$ ANOVA; $n = 20$; 10 males and 10 females) and by sex (Males; **, $P < 0.02$ ANOVA; $n = 10$, 5 vehicle, 5 tamoxifen; Females; **, $P < 0.01$ ANOVA; $n = 10$, 5 vehicle, 5 tamoxifen). **B.** Serum IL-1 α protein concentrations at week 18 analyzed by condition and sex (not significant by ANOVA; $n = 20$ mice; 10 males and 10 females). **C.** Serum TNF α protein concentrations at week 18 analyzed by condition and sex (not significant by ANOVA; $n = 20$ mice; 10 males and 10 females).

Summary

All previous findings reported in this body of work has demonstrated a repeated association between IL-1 β and atherosclerotic plaque calcification. ^{78,295} However, it remained unclear if IL-1 β signaling played a causal role in calcification. The data presented in this chapter has shown that IL-1 β expression directly contributes to the development of plaque calcification. Moreover, this work has shown that endogenous macrophage specific IL-1 β expression alone is sufficient to modulate calcification. From the studies of *Rac2*-deleted animals and statin treatment, elevated levels of serum IL-1 β expression were associated with calcification. ^{78,295} Given that the loss of endogenous macrophage IL-1 β expression is sufficient to reduce calcification, it stands to reason that elevated serum IL-1 β levels may also further contribute to greater plaque calcification. This possibility is supported by findings of the statin study. When macrophage *Rac1* was deleted among mice on a statin diet, the statin enhancing effect on calcification was abolished. ²⁹⁵ Likewise, among these same mice a statin enhance serum IL-1 β was also abolished when macrophage *Rac1* was deleted. ²⁹⁵ Therefore, these collective findings suggest that both endogenous expression and enhanced expression of macrophage IL-1 β can contribute to atherosclerotic calcification.

It is also important to note that this is the first *in vivo* study of a macrophage specific *IL-1 β* -deleted model. Previous studies have used either global *IL-1 β* knockout animals or used anti-IL-1 β antibodies, which also suppresses global IL-1 β signaling from all cell types. ^{531,532} This study further points to importance of inflammation in atherosclerotic calcification and highlights the critical role that macrophages play.

Chapter 7:

Discussion

Statins Disrupt Macrophage Rac1 Regulation Leading to Increased Atherosclerotic Plaque Calcification

Ischemic heart disease caused by atherosclerosis is the leading cause of morbidity and mortality in the world.⁵³³ The current standard of care depends on 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also known as statins.^{333,485-488} Statins are very effective at reducing cardiovascular events.^{334,335,489,490} The therapeutic benefit of statins has long been attributed to their lipid-lowering ability (*e.g.* cholesterol). However, multiple studies have demonstrated that statins reduce the risk of myocardial infarction out of proportion to their lipid-lowering effects.^{334,335,489,490} These pleiotropic effects of statins have been attributed to their effects on the posttranslational isoprenylation of proteins, such as Racs. Statins disrupt this modification by inhibiting the formation of isoprenoids within the cholesterol synthesis pathway downstream of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity.^{336,534} The relationship between statin therapy and atherosclerotic calcification has been controversial. Initial studies suggested that statins inhibit calcification, but later studies reported that statin therapy promoted calcification.⁴⁹²⁻⁴⁹⁵ Moreover, recent studies have continued to support a pro-calcifying effect from statin therapy as demonstrated by increased plaque calcium composition

relative to lipid composition in statin treated patients. Despite observing this relationship, no mechanism has been identified to explain how statins alter calcification. 493,496,497

The Morrison laboratory previously identified Rac2 as a major regulatory determinant of the degree of macrophage IL-1 β expression by its regulation of Rac1 activity upstream of NF- κ B and reactive oxygen species production.⁷⁸ Consequently, the *Rac2* gene-deletion model led to progressive inflammatory atherosclerotic calcification that was dependent on IL-1 β signaling, and *Rac2*^{-/-} macrophages demonstrated increases in Rac1 activation and Rac1-dependent IL-1 β expression.⁷⁸

In brief summary, (1) statins treatment is associated with vascular calcification by an unknown mechanism (2) statins are reported to modify Rac protein and their activity (3) *Rac2* deficient mice exhibit enhanced calcification in a macrophage Rac1-IL-1 β dependent manner. Collectively, these findings led to the investigation presented here that investigated if statins could be regulating vascular calcification by disrupting a macrophage-specific Rac1-IL-1 β signaling axis.

Statin treatment is Independently associated with Coronary Artery

Calcification among Patients

From this investigation, among patients statin therapy was found to be independently associated with increased coronary calcification when adjusted for other cardiovascular risks.²⁹⁵ Limitations of this clinical study include it being conducted at a single site and a retrospective analysis of primarily male veterans, increasing risk of a

selection bias. However, these findings are consistent with more recent clinical studies that demonstrated increased plaque calcification with statin use.⁴⁹²⁻⁴⁹⁷ It remains unclear if the effects of statin-enhanced calcification are protective or harmful. Further research is needed to evaluate the clinical impact of statin-induced calcification. Moreover, a deeper investigation into the structure of plaque calcification among patients on statin and its ultimate effects on plaque stability need to be conducted.

To answer these questions in future studies, a prospective clinical trial could be conducted. Patients can be recruited and filtered through for confounding factors such as smoking, diabetes, hypertension, etc. The trial participants would receive annual computed tomography scans (or “CT” scans) to assess the degree and structure of aortic plaque calcification. Over the course of this study, records of cardiovascular events and patient mortality would be kept. Patients data would be organized for analysis in the following groups: not receiving statin, low-intensity statin therapy, high-intensity statin therapy. Using this information, one would be able to assess the effects of statin-induced calcification on patient outcomes. Moreover, one could assess if statins modulated the structure of plaque calcification among patients and if calcification’s structure also impacts health outcomes. This future study would not only establish an understanding of the clinical impact of statin-induced calcification but it would also assess how the structure of calcification may directly influence health outcomes.

After observing that statin treatment was associated with increased vascular calcification among patients, it was important to then investigate the mechanisms

involved, which have remained largely unknown. Isoprenylation of Racs have been shown to be important in facilitating binding to its inhibitor RhoGDI at its hydrophobic pocket.^{535,536} Therefore, it was hypothesized that statins prevent macrophage Rac isoprenylation and disrupt Rac binding to RhoGDI leading to enhanced Rac1 activity. Moreover, it was postulated that if this were the case then statin-enhanced Rac1 activity may promote calcification as previously observed with *Rac2*-deficient mice, which developed Rac1-dependent enhanced calcification.⁷⁸

Statin treatment is associated with Enhanced Monocyte Rac1 Activity in Patients

In primary human monocytes (macrophage precursors) from patients on or off statin medical therapy, it was found that increased Rac1 activity was associated with statin treatment. Moreover, this pattern remained consistent using bone-marrow derived macrophages (BMDMs) from *ApoE* deficient animals for the study of atherosclerotic calcification. Statin treatment led to increased Rac1 activity in a dose-dependent manner in BMDMs. These findings are consistent with another study that found statin-enhanced Rac1 activity among immortalized monocytes (THP-1) and microglial (BV-2) cells.⁵³⁴ One notable limitation of this study, is that this assessment of statin's effects on monocyte Rac1 activity does not convey the degree by which statin treatment at various dosages alters Rac1 activity in humans. Samples were collected from patients on a variety of dosages. Therefore, while this study does demonstrate that statin treatment can alter myeloid Rac1 activity in patients, it does not clarify the exact relationship between dosage and Rac1 activity. Moreover, this study did not assess the degree of medication

compliance among individuals who donated samples for analysis. The large distribution of Rac1 activity that was measured among patients may be explained by both variance in statin dosage and patient's medication compliance.

Future prospective clinical studies can clarify the exact relationship between statin dosage and its pleiotropic effects on monocyte Rac1 activity as follows: Patient data on statin dosage can be collected and recorded in association with collected blood samples. This way, one could repeat the assay described above to assess monocyte Rac1 activity and then analyze the relationship between statin dosage and Rac1 activity among all the samples. Moreover, to assess the degree of medication compliance pill counting can be used.⁵³⁷ Patients enrolled in the potential study could agree to arrive at a pharmacy after a specified time in which they should no longer have any medication left and will provide the pharmacist with their recent pill bottle. The pharmacist can then count any remaining pills that were not taken and record which individuals in the study missed any medication. With this data, samples from non-compliant patients can be excluded to avoid confounding the study's analysis.

Statin disrupts Macrophage Rac1 Isoprenylation leading to enhance Rac1 activity, NF- κ B activity, and IL-1 β signaling *in vitro*

After identifying that statins can enhance macrophage Rac1 activity, more mechanistic studies of macrophage Rac1 activity were conducted. Moreover, it was important to further evaluate if statin altered Rac1 activity modulated IL-1 β in the atherosclerotic mouse model as observed in the Morrison laboratory's previous study of

Rac2 deficient mice. Statin-induced inhibition of macrophage Rac1 isoprenylation was identified to be a critical mediator of its complex formation with RhoGDI, resulting in increased Rac1 activation, increased activation of the Rac1 effector NF- κ B, and consequently, increased IL-1 β mRNA and mature protein production upon LPS and cholesterol crystal stimulation. The data are supported by the ability of FPP to partially rescue and of GGPP to fully rescue the statin-mediated effects on Rac1 activity and IL-1 β expression, whereas squalene, which lies downstream of FPP in the cholesterol synthesis pathway, was unable to mitigate the statin-mediated effects. Although statins are known to reduce the isoprenylation state of multiple Rac family members, conditional *Rac1* knockdown, using an inducible myeloid specific promoter for Cre recombinase expression crossed with loxP flanked *Rac1*, confirmed the statin-mediated effect on IL-1 β expression to be specific to macrophage Rac1 isoprenylation.

**Statin enhanced Atherosclerotic Calcification in *ApoE* deficient animals is
Dependent upon Myeloid Rac1 expression**

Moreover, *in vivo* studies using animals with inducible myeloid *Rac1* deletion and HFD supplemented with atorvastatin further demonstrated statin treatment to be a determinant for the degree of atherosclerotic plaque calcification in a macrophage Rac1-dependent manner. The dosage of statin in the chow that was chosen appeared to impact the immune response but did not impact chronic lipid metabolism as cholesterol and plaque lipid burden were relatively similar between treatment groups. The estimated daily mouse ad libitum dosing of atorvastatin at 6mg/kg was near the recommended high-intensity statin dosing for humans (1.07mg/kg) and consistent with other studies that

suggest a higher dose is required to have a more significant impact on cholesterol and atherosclerosis burden in mice fed this type of HFD for over 20 weeks. ⁴⁵² Importantly, this statin dose was associated with increased circulating IL-1 β expression and increased plaque ALP and RUNX2 expression. This observation is consistent with prior work by the Morrison laboratory, which demonstrated that increased IL-1 β expression appears to trigger an osteoblast-like differentiation among vascular smooth muscle cells leading to calcifying plaque. ⁷⁸ Prior literature supports that a close association between inflammatory macrophages and calcifying vascular cells appears required to activate vascular smooth muscle cell to osteoblast-like phenotypic conversion, and a number of those studies involve macrophage-secreted factors like TNF- α or IL-1 β . ^{75,78,163,538-541} The Morrison laboratory has observed increased vascular smooth muscle cell osteogenic transcription factor expression, including RUNX2, SOX9 (SRY-box transcription factor 9), OSX (osterix), and MSX2 (Msh homeobox 2), in response to IL-1 β , which raised the question of a direct mechanism of IL-1R (interleukin-1 receptor) signaling in the promotion of an osteogenic program in mesenchymal cells. ⁷⁸ Further studies are required to address the molecular mechanisms that determine IL-1 β responsiveness along with consequent changes in gene programming in vascular smooth muscle cells *in vivo*. In this discussion, the focus is primarily oriented towards the mechanisms upstream of IL-1 β that appear to be impacted by statin therapy, specifically, Rac1-dependent IL-1 β expression.

Endogenous Macrophage Rac1 expression is contributes to Atherosclerotic Plaque Calcification and Systemic IL-1 β

Both the prior study of *Rac2* deficient mice and the more recent findings on statin enhanced atherosclerotic calcification have been discussed. Both reports implicated vascular calcification as being dependent on Rac1 activity. However, neither of these studies clearly demonstrated if endogenous macrophage Rac1 is independently and sufficiently capable of modulating atherosclerotic plaque calcification due to the limitations of the other studies' experimental designs. The global *Rac2* deficient mice and *ApoE* deficient mice fed a statin diet, both exert systemic effects on various cell types. These systemic effects may be required for a Rac1-IL-1 β pathway to effectively modulate atherosclerotic calcification. Also, both *Rac2* deletion and statin treatment give rise to enhanced Rac1 activity (above their basal level). Thus, in a context where Rac2 signaling is not disrupted and the pleiotropic effects of statin are absent, it was important to ask if this Rac1-IL-1 β pathway is still relevant to the natural progression atherosclerotic calcification.

In order to answer this question, *ApoE* deficient mice that could have myeloid *Rac1* expression conditionally knocked-down with tamoxifen treatment were generated and placed on a HFD. This *in vivo* study demonstrates that the degree of plaque calcification is dependent on endogenous myeloid Rac1 expression. Consistent with the Morrison laboratory's working model of a macrophage Rac1-IL-1 β pathway, circulating IL-1 β expression was observed to be myeloid Rac1-dependent. Serial testing of serum IL-1 β levels from animals with endogenous myeloid Rac1 expression indicated that systemic

inflammation is elevated by week 12 and peaks around week 18 on HFD before declining at week 24. By week 24, serum IL-1 β levels were comparable to measurements taken at week 0 (before the HFD began). This pattern of systemic IL-1 β signaling is similar to the pattern of macrophage infiltration observed by Aikawa and colleagues *in vivo* using *ApoE* deficient mice. After peak of infiltration of macrophages, a significant decline followed weeks later (which was then associated with a peak in osteogenic activity). ⁷⁵

Potential Mechanisms for Female-Specific Macrophage

Rac1 Regulation of Serum LDL Levels

During the course of this study, one notably unexpected finding was observed. Myeloid *Rac1* deletion resulted in a modest but statistically significant decrease in serum LDL (and consequently total cholesterol levels), exclusively in females. The reduction in LDL levels alone was somewhat surprising as sex-specific, Rac-related effects on cholesterol metabolism not observed in prior studies.

However, in prior animal studies, Rac1 activity was increased above basal level while in this more recent study, Rac1 expression (and presumably its total activity level) was significantly reduced below its endogenous state. Note that neither statin nor *Rac2*-deletion affected basal Rac1 expression but only its activity level. Together these findings suggest that some basal level of Rac1 activity in females may be required in the upregulation of serum LDL levels by myeloid cell lineages and increasing Rac1 activity any further has no additional effect. Also, a reduction in basal Rac1 expression may also be of consequence as that may ultimately reduce Rac1 activity levels below baseline.

Despite that modest but statistically significant reduction of serum LDL in female mice with reduced myeloid Rac1, this effect appears insufficient to modulate the progression of atherosclerotic calcification and total plaque burden (indicated by Oil Red O staining). This inability to alter the course of the disease is not entirely surprising given that the reduced LDL levels are still higher than healthy C57BL/7J mouse on normal diet. ⁵⁴²

Rac1's localization, activity, and function in cell migration can be modified by concentrations of intracellular or plasma membrane-bound free cholesterol in a variety of cell types, including macrophages. ^{543,544} Increased Rac1 activity has been associated with macrophage cholesterol efflux, the release of excess cholesterol to the extracellular environment. Minimal investigation of Rac1 and cholesterol efflux has been conducted but c-Jun N-terminal kinase (JNK) signaling has been identified as a downstream effector of Rac1 in its regulation of cholesterol efflux. ^{545,546} JNK signaling in macrophages is known to primarily play a role in inflammatory signaling. ⁵⁴⁷ Given this study's recent findings that loss of myeloid Rac1 modestly reduces systemic LDL levels and others' research, one could postulate that the reduced LDL levels are a result of reduced macrophage Rac1-induced cholesterol efflux.

While this argument presents a case for why no effects on systemic cholesterol levels were previously observed in studies with enhanced Rac1 activity, it does not explain how this effect on cholesterol is sex-specific. Other studies have identified links between (sex-specific) estrogen signaling, Rac1, and macrophage-specific cholesterol metabolism. ^{548,549} Estrogen participates in lipid metabolism and acts as a down-regulator

of serum LDL levels. ⁵⁵⁰⁻⁵⁵² Before menopause, serum LDL levels are lower in women compared to men of the same age but after menopause women's LDL levels rise and are typically greater than that of men at the same age. ⁵⁵³ Moreover, postmenopausal women receiving an oral supplementation of estrogen experience reduced LDL levels. ^{550,553}

Estrogen can also inhibit Rac1 activity in VSMCs and macrophages, *in vitro*, and can modify macrophage cell function in a Rac1 dependent manner. ^{554 555,556 557} In support of these observations, women with elevated estrogen levels have been reported to have blood-derived monocytes with reduced Rac1 activity. ⁵⁵⁵ However, the exact mechanism linking estrogen to Rac1 activity is unclear. However, estrogen can reduce macrophage Rac1 mRNA and protein expression, which may explain the observed reduction in Rac1 activity to some degree. ^{554,555} Given that reduced myeloid Rac1 expression reduces LDL levels exclusively in female mice, macrophage Rac1 may be participating in cholesterol metabolism in an estrogen-regulated pathway. Estrogen receptor signaling can mediate LDL oxidation in both primary and immortalized macrophage cell cultures. ⁵⁵⁸⁻⁵⁶⁰ (Testosterone treatment exhibits no such effects.) In breast cancer cell culture, small molecular inhibition (via EHT 1864) of Rac activity leads to reduced estrogen receptor expression. ⁵⁶¹ Such a relationship in macrophages could link Rac1 to estrogen signaling but no studies of macrophages have investigated this potential link. These studies and results reported here collectively suggest that macrophage Rac1 is involved in cholesterol metabolism (potentially through cholesterol efflux) and regulated in a sex-dependent manner through estrogen signaling. Further research into the relationship between Rac1, LDL, and estrogen is warranted. One of the first steps for future studies to explore the

relationship between macrophage Rac1 and estrogen signaling could begin by culturing BMDMs exposed to estrogen and then treated the cells with either Rac1 inhibitor, EHT 1864, or vehicle control. These cells could then be lysed and mRNA could be isolated to measure estrogen receptor transcription. This would elucidate if macrophage Rac1 is involved in estrogen signaling through the regulation of estrogen receptor expression.

Macrophage Rac1 Regulation of IL-1 β

Rac1 is involved in many cell functions and diseases.^{309,318} It is ubiquitously expressed in both humans and mice.³⁰⁷⁻³⁰⁹ In macrophages, this study has found that Rac1 activity is associated with vascular calcification and systemic IL-1 β expression. Moreover, using BMDMs IL-1 β expression was found to be dependent on Rac1 expression. There is no known mechanism reported to link Rac1 to IL-1 β expression. However, Rac1 has been reported be important in phagocytosis, ROS production, and NF- κ B activity, which all present plausible links to IL-1 β . Therefore, these three potential mechanisms that could mediate Rac1's regulation of IL-1 β were investigated.

Loss of Rac1 expression does not affect

Macrophage Phagocytosis of Cholesterol Crystals

In the context of atherosclerosis, macrophage phagocytosis is critical to the early stages of plaque development. Macrophages develop into foam cells through the phagocytosis of cholesterol crystals that form in the intima.^{49,562,563} These cholesterol crystals activate the NLRP3 inflammasome, which leads to the release of mature IL-1 β .^{47,49} Eventually these foam cells undergo apoptosis, which further triggers

inflammation and contributes to the plaques necrotic core.^{564,565} Phagocytosis is dependent on tight regulation of the cytoskeleton.^{566,567} Rac1 is a key regulator of cytoskeletal structure through localized polymerization of actin within the cell.^{523,568} Rac1's regulation of the cytoskeleton effects macrophage morphology, migration, cell cycling, and phagocytosis.^{352,353,358-360,363-365} It was hypothesized that knocking-down macrophage *Rac1* may reduce IL-1 β by impairing phagocytosis of cholesterol crystals and preventing NLRP3 inflammasome activation and subsequent IL-1 β maturation. Using both qualitative and quantitative approaches, knocking-down *Rac1* expression in BMDMs was found to not affect phagocytosis of cholesterol crystals. This suggests that while Rac1 is involved in regulating the cytoskeleton, it is not required for macrophage phagocytosis of cholesterol crystals.

Other studies have reported that Fc receptor-mediated phagocytosis requires Rac1 activity in myeloid cells.^{358,569-571} However, complement receptor 3 (CR3)-mediated phagocytosis in myeloid cells does not require Rac1 but instead depends upon another GTPase, RhoG (Ras homology growth-related).^{572,573} Therefore, the findings presented here and other reports suggest that the pathway for macrophage phagocytosis as it pertains to cholesterol crystals is either independent of Rac1 or redundancy through other small GTPases allows for preservation of phagocytosis in the absence of Rac1. Note that like Rac1, RhoG is a member of the Rho family and shares 72% of Rac1's amino acid sequence.³¹²

A recent report suggested that the primary mechanism for the internalization of cholesterol crystals and NLRP3 activation in primary human monocytes is through Rac1-independent CR3-phagocytosis.⁵⁷⁴ The process by which macrophages recognize and then phagocytosis crystals structures, such as cholesterol crystals, is still not fully understood and may depend on the particular lipid composition of the crystal structure.⁵⁷⁵ Further investigation is needed to identify the exact mechanisms behind macrophage Rac1-independent cholesterol crystal phagocytosis.

Finding that macrophage cholesterol crystal phagocytosis is not impacted by *Rac1*-deletion holds significant implications for one's understanding of Rac1's role in atherosclerotic calcification and the its role (or lack thereof) in phagocytosis. Prior work by the Morrison laboratory and the findings reported here demonstrated that through genetic or pharmaceutical interventions that enhanced macrophage Rac1 activity can promote vascular calcification by increasing IL-1 β . Additional investigation followed by these findings then observed that endogenous Rac1 expression was sufficient to contribute to calcification and was associated with decreased IL-1 β . The Morrison laboratory's hypothesis has been that macrophage Rac1 regulates IL-1 β signaling to modify calcification. However, given Rac1's role in phagocytosis it was possible that the observed reduction of calcification in *Rac1* knockdown animals may be attributed partially or fully to the inability to phagocytose NLRP3-activating cholesterol crystals. The finding that *Rac1* deletion does not affect phagocytosis supports that endogenous Rac1-IL-1 β signaling directly contributes to vascular calcification.

A key limitation of the study presented here regarding Rac1 and phagocytosis is that it measured the uptake of cholesterol crystals only after 12 hours of exposure. It is possible that the kinetics of phagocytosis was affected by the loss of Rac1. The rate of crystal internalization could have been impaired through *Rac1* deletion but enough time passed for the macrophages to reach maximum crystal intake. Thus, this would result in no measurable differences in the internalization of the crystals, despite that Rac1 impaired the rate of macrophage phagocytosis. To clarify if this is the case, future studies could repeat this study by measuring crystal uptake at several earlier time points (such as 1, 4, and 8 hours), post-crystal exposure. This would allow for a more robust evaluation of Rac1's role in macrophage phagocytosis of cholesterol crystals by assessing its relationship to the kinetics of phagocytic activity.

Endogenous Macrophage Rac1 expression does not regulate IL-1 β expression by modulating ROS Production

ROS production can trigger NLRP3 inflammasome formation for the maturation and release of IL-1 β . The exact mechanism by which ROS production activates the inflammasome is unclear. It is thought that ROS may activate the inflammasome by either directly generating a disulfide bond between highly conserved residues (Cys-8 and Cys-108) or indirectly by altering the conformation of unknown redox-sensitive proteins that contribute to inflammasome formation.^{576,577} Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a plasma membrane-bound enzyme complex that converts oxygen (O₂) into superoxide anions (O₂⁻), which are spontaneously converted to hydrogen peroxide (via a dismutation reaction with positively charged hydrogen ions).^{578,579} Hydrogen peroxide can then undergo further reactions to generate other ROS

products, which can be used in the innate immune response as cytotoxins against microbial pathogens.^{580,581} In the context of atherosclerosis, ROS production can cause oxidative stress in the plaque environment, participates in the accumulation of oxidized LDL, and can promote cell proliferation.^{435,582,583} In phagocytes, like macrophages, NADPH oxidase remains in its unassembled inactive state until the immune response is triggered, which promotes the translocation of NADPH's cytosolic components to form a membrane-bound complex.⁵⁸⁰ This complex consists of p47_{phox}, p67_{phox}, a NOX isoform (primarily NOX2 in phagocytes), and depending on the cell type, Rac1 or Rac2.^{373,377,378} Rac1 is associated with macrophages while Rac2 is associated with neutrophils in NADPH oxidase formation.^{373,377,378} Activated Rac interacts with p67_{phox} (which is bound to p47_{phox}) and is important in translocating it from the cytosol to the membrane for NADPH complex formation.^{373,584}

Rac1 activity has been reported to alter ROS production via NADPH oxidase.³⁸⁰ ROS production is known to stimulate NLRP3 inflammasome formation, which cleaves and releases mature IL-1 β .^{429,525} The Morrison laboratory previously observed that *Rac2* deleted macrophages have elevated ROS production associated with increased Rac1 activity and IL-1 β expression.⁷⁸ Inhibition of Rac1 activity among *Rac2* deleted macrophages using small molecule, EHT 1864, resulted in reduced IL-1 β expression.⁷⁸ These results suggested that Rac1 may be modulating IL-1 β through downstream ROS signaling. However, in the study of statin induced calcification, increased Rac1 activity and IL-1 β expression was associated with decreased ROS production.²⁹⁵ Both the *Rac2*-gene deletion and the statin studies present somewhat contradictory data regarding the

exact role of ROS in proposed Rac1-IL-1 β pathway. Using BMDMs primed with LPS (10ng/mL) and exposed to cholesterol crystals (1000 μ g/mL), deletion of endogenous *Rac1* had no effect on ROS production as measure by quantified H₂O₂. Collectively, these findings suggest two plausible explanations for the relationship between ROS and this Rac1-IL-1 β pathway, as follows:

Scenario 1: “Sufficient Rac1 Activity”, artificially enhanced Rac1 activity promotes ROS but endogenous levels of Rac1 activity are insufficient. This would explain why one observes both *Rac1* knock-down not affecting ROS production and *Rac2* deletion with enhanced Rac1 activity increasing ROS. In regards to statin, the reduced ROS (despite elevated Rac1 activity) may be explained by impaired Rac binding to the membrane (via isoprenylation). Thus, statin would prevent Rac1 from participating in NADPH oxidase assembly at the plasma membrane (regardless of its activation state).

Scenario 2: “Redundancy”, endogenous Rac1 activity contributes to ROS production and when Rac1 is lost, Rac2 compensates for the loss and maintains endogenous ROS production. Thus, one would observe no change in ROS production among *Rac1* knockdown cells but would still observe increased ROS when Rac1 activity is enhanced (by *Rac2* deletion). This still doesn’t explain how statins result in reduced macrophage ROS production despite increased Rac1 activity. To address this point, one could hypothesize that statin-impaired Rac binding to membranes (via isoprenylation) would prevent it from participating in NADPH oxidase assembly (despite enhanced Rac1 activity).

In order to further test if either one of these scenarios is plausible, future studies could analyze the following:

1. Test the degree of Rac1 activity required for ROS production to be modified by treating BMDMs with decreasing dosages of Rac1-specific inhibitor (NSC23766) and increasing doses of non-hydrolyzable GTP.

2. Test if Rac2 compensates for loss of endogenous Rac1 activity in the context of ROS production. This can be tested using BMDMs with both *Rac1* and *Rac2* deletion or using *Rac1* deleted BMDMs and treating them with inhibitor EHT1864 to suppress Rac2 activity.

3. To test if isoprenylation is important for Rac mediated NADPH oxidase activity, BMDMs could be treated with increasing dosages of GGTI-2133 and measure ROS production. GGTI-2133 is an inhibitor of geranylgeranyl transferase I, which is responsible for the isoprenylation of Racs, which facilitates membrane binding.

Note that these tests and the data recently discussed are still bound by some limits. These cell culture experiments will never perfectly replicate the *in vivo* environment and do not account for the effects of aging, which can influence many components of atherosclerosis including macrophage cell function. ⁵⁸⁵⁻⁵⁸⁷ However, future *in vitro* studies could better account for the effects of aging by generating cultures from aged animals, which has been shown to influence the functions of BMDMs. ^{588,589}

Rac1 regulates Macrophage NF- κ B Activity

The NLRP3 inflammasome is a protein complex, which is primarily expressed in macrophages, and it is important for the maturation of IL-1 β .^{432,590,591} After the complex forms, caspase-1 becomes active and then cleaves pro-IL-1 β (the immature form) to generate mature IL-1 β that is then released into the extracellular environment.⁵⁹² Macrophage infiltration of the intima and subsequent inflammation plays an important role in the development of atherosclerotic plaque.⁷⁵ NLRP3 inflammasome activation can be triggered by cholesterol crystals, oxidized LDL, and hydroxyapatite crystals.^{593,594} All of these stimuli can be observed in plaque. The NLRP3 inflammasome has also been implicated in other pathologies such as autoimmune and neurological disorders.^{595,596}

Macrophage NLRP3 inflammasome formation requires nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B) activity. NF- κ B primes macrophages for inflammasome activation by upregulating the transcription of both the NLRP3 protein and pro-IL-1 β .^{591,597} However, NF- κ B activity alone is insufficient for inflammasome activation.^{527,591} Only after NF- κ B-priming, which generates a sufficient amount of NLRP3 for the inflammasome assembly, can stimuli such as cholesterol crystals trigger NLRP3 inflammasome formation to occur.⁵²⁷ Pathogen-associated molecular patterns (PAMPs, like LPS) and damage-associated molecular patterns (DAMPs, like cholesterol crystals) can act as stimuli for inflammasome formation.^{525,598,599}

NF- κ B is a transcription factor that is localized in the cytosol in its inactive state and upon stimulation enters the nucleus and promotes transcription. ^{600,601} The Morrison laboratory previously observed increased NF- κ B activity in *Rac2* deleted BMDMs, which had elevated Rac1 activity and IL-1 β secretion. Similarly, as reported here statin-enhanced Rac1 activity is associated with increased NF- κ B activity. Endogenous macrophage Rac1 expression has also been observed to be important for NF- κ B activity and IL-1 β expression in inflammasome stimulated BMDMs. Given these findings and the known relationships among NF- κ B, the NLRP3 inflammasome, and IL-1 β , it appears that Rac1 is regulating IL-1 β through NF- κ B activity.

Findings reported here regarding NF- κ B activity are complementary to other studies that have observed Rac1-dependent NF- κ B activity in other cell types.^{366,385,602,603} Note though that Rac1 has been observed to act as both an up-regulator and down-regulator of NF- κ B depending on the cell type. ³⁶⁸ In intestinal epithelial cells Rac1 appears to down-regulate nucleotide-binding oligomerization domain-containing protein 2 (Nod2)-dependent NF- κ B activity by sequestering Nod2 to the plasma membrane. ⁶⁰⁴ Rac1 up-regulation in colorectal endothelial cells appears to be mediated by a Rac1-dependent transcription of the NF- κ B subunit, p105. ⁶⁰⁵ In support of this model for Rac1 modifying IL-1 β through NF- κ B activity, another study using macrophages observed that peptidoglycan stimulation resulted in Rac1-GTP dependent up-regulation of NF- κ B activation. ⁶⁰⁶

In conclusion, the findings reported in this dissertation support that macrophage Rac1 activity promotes NF- κ B activity and suggests that Rac1-dependent IL-1 β expression is facilitated through the regulation of its transcription factor, NF- κ B. These findings also open new opportunities to therapeutically disrupt this Rac1-IL-1 β pathway to inhibit the development of atherosclerotic calcification. However, a notable limitation of the findings reported here is that while NF- κ B activity was demonstrated to be associated with Rac1 expression, this study did not show if reduced NF- κ B activity was sufficient to reduce IL-1 β expression as is presented in the proposed Rac1-IL-1 β pathway here. In the study of Rac2-deleted macrophages, IL-1 β protein expression was inversely correlated with increasing dosages of an NF- κ B inhibitor, which suggests IL-1 β is being regulated by NF- κ B activity in macrophages under inflammasome stimulation.⁷⁸ Despite that Rac1 appears to be associated with NF- κ B activity, loss of Rac1 could be downregulating IL-1 β through a NF- κ B-independent mechanism. Therefore, to better establish the relationship among Rac1, NF- κ B, and IL-1 β future investigations should be conducted. In order to better elucidate this pathway, one could treat inflammasome stimulated BMDMs with increasing dosages of an NF- κ B inhibitor and measure the mature IL-1 β protein levels secreted into culture supernatant using ELISA.

Moreover, to better understand the mechanism by which Rac1 regulates NF- κ B future studies could examine the localization of Rac1. By further investigation Rac1's mechanistic role one could elucidate if Rac1 (or specifically Rac1-GTP) nuclear localization plays a functional role or if it is irrelevant in the up-regulation of nuclear NF- κ B activity and IL-1 β expression under pro-inflammatory stimuli. To study this, wild-type

Rac1 or constitutively active Rac1 plasmid constructs with and without mutations in the polybasic nuclear localization sequence have been acquired.^{347,423} This region is required for Rac1 localization to the nucleus.³⁴⁷ Future studies can culture *Rac1*-deleted BMDMs, and then transfect the cells with these plasmids to study the effects of Rac1 or Rac1-GTP translocation to the nucleus on NF- κ B activity and IL-1 β expression. If indeed Rac1 localization to the nucleus is necessary (fully or partially) to regulate NF- κ B and IL-1 β (as proposed in the Rac1-IL-1 β signaling axis presented here), then a chromatin immunoprecipitation (ChIP) assay can be utilized to test if Rac1 is interacting with DNA sequences important in the regulation of IL-1 β transcription or NF- κ B activity. These future studies hold the potential to clarify the role of Rac1 nuclear localization and identify novel functions for Rac1 that could open new opportunities for further study. These potential studies may also reveal new therapeutic strategies for the regulation of the proposed Rac1-IL-1 β signaling pathway to inhibit the development of atherosclerotic calcification.

Endogenous Macrophage IL-1 β expression contributes to Atherosclerotic Plaque Calcification

Interleukin-1 beta (IL-1 β) is a cytokine regulating inflammation and serves as an agonist to the interleukin-1 receptor.⁶⁰⁷⁻⁶¹⁰ It serves as a major regulator of the innate immune response.⁶¹¹ IL-1 β maturation requires inflammasome activation to induce caspase-1 cleavage of its inactive form (pro-IL-1 β) and generate bioactive IL-1 β that can then be released into the extracellular environment.^{612,613} IL-1 β 's effects (via the IL-1 receptor) can act via paracrine, autocrine, and endocrine signaling.⁶¹⁴⁻⁶¹⁸ A variety of

cells can express IL-1 β . However, given its role in inflammation, IL-1 β expression predominately occurs among macrophages (and monocytes), neutrophils, and natural killer cells. ⁶¹⁹

In the context of atherosclerosis, macrophage inflammation is closely associated with vascular calcification and the development of an osteogenic phenotype among VSMCs. ^{75,76} Previous studies have pointed to macrophage TNF- α as one mechanism for the induction of osteogenic expression in VSMCs. ^{163,540,541} The Morrison laboratory recently observed IL-1 β to be highly associated with vascular calcification *in vivo* and is capable of inducing VSMC osteogenic expression and calcification *in vitro*. ⁷⁸ Moreover, the inhibition of IL-1 β signaling in *ApoE* deficient mice through the systematic administration of IL-1receptor antagonist (IL-1ra) mitigated the progression of vascular calcification. ⁷⁸ Due to the systemic effects of IL-1ra the exact cell types involved in IL-1 β signaling were unclear. Moreover, the influence of other IL-1receptor binding cytokines were inhibited, confounding the potential specificity of IL-1 β 's role in atherosclerotic calcification. ⁷⁸ In this study, a positive correlation between IL-1 β expression and both aortic calcification and macrophage Rac1 activity through both *in vivo* and *in vitro* experimentation continued to be observed. After generating the first mouse model of conditionally deleted myeloid *IL-1 β* , macrophage-derived IL-1 β was demonstrated *in vivo* to significantly contribute to the development of atherosclerotic calcification. This further supports the current paradigm that atherosclerosis is an inflammatory disease. ^{620,621} In the context of hyperlipidemic atherosclerosis macrophages were also shown to act as the primary source of circulating IL-1 β . Lastly, myeloid *IL-1 β* knockdown did not modulate

serum LDL (nor total cholesterol levels) as observed in myeloid *Rac1* knockdown female mice. This suggests that *Rac1*'s effects on cholesterol are independent of IL-1 β .

Identifying macrophage IL-1 β *in vivo* to play a causal role in atherosclerotic calcification supports this study's proposed *Rac1*-IL-1 β pathway. However, these findings alone do not establish if *Rac1* may be modulating calcification partially through an IL-1 β -independent mechanism. And if *Rac1* does influence calcification only partially through IL-1 β then to what degree is *Rac1*'s effect on calcification attributed to IL-1 β and what other mechanism could be acting downstream of *Rac1*? The answers to these questions hold significant implications to understanding both *Rac1*'s and IL-1 β 's contribution to vascular calcification. Because this limitation in studying *Rac1* and IL-1 β separately, the Morrison laboratory is now actively breeding new mice to answer these questions in future research. By generating mice with both myeloid-specific, inducible constitutively active *Rac1* allele expression and *IL-1 β* knockdown, one can better assess the linear causal relationship between *Rac1* and IL-1 β in regards to vascular calcification *in vivo*. If the animals with both macrophage constitutively active *Rac1* expression and *IL-1 β* knockdown exhibit more calcification than vehicle controls then this would indicate that *Rac1* activity can partially contribute to calcification independently of its effects on IL-1 β signaling. Alternatively, if the animals with both macrophage constitutively active *Rac1* expression and *IL-1 β* knockdown demonstrate comparable degrees of plaque calcification to vehicle controls, then this finding would suggest that *Rac1*'s effect on calcification is completely dependent on IL-1 β signaling.

IL-1 β has also been reported to have an inverse relationship to the calcification inhibitor, fetuin-A, in diabetic patients and *in vitro*.^{290,294} Reports of IL-1 β 's pro-osteogenic effects on VSMCs may potentially be mediated by changes in fetuin-A, which is capable of influencing osteogenic differentiation.^{622,623} Future studies could use ELISA to measure changes in circulating levels of fetuin-A and their association with IL-1 β expression and vascular calcification. Additionally, cryo-sectioning of aortas from wild-type and myeloid *IL-1 β* knockdown mice using immunofluorescent imaging of osteogenic markers (e.g. Runx2) could further assess if loss of macrophage IL-1 β expression reduces markers of osteogenic-like cells in plaque.

CANTOS Trial Implications

The canakinumab anti-inflammatory thrombosis outcome study (CANTOS) trial studied patients (10,061) from 40 countries around the world that had a history of myocardial infarction but still at-risk and showed signs of inflammation as indicated by circulating high-sensitivity C-reactive protein (hs-CRP) levels.⁶²⁴⁻⁶²⁷ hs-CRP levels are independent measures of cardiovascular event risk, regardless of other risks such as diabetes and hypertension.^{628,629} The study set out to test if neutralizing the pro-inflammatory cytokine, IL-1 β , using canakinumab, a human anti-IL-1 β monoclonal antibody, could mitigate the course of cardiovascular disease. This study found that canakinumab treatment (particularly at higher dosages) was effective in reducing the rate of recurrent cardiovascular events (i.e. myocardial infarction and ischemia).^{624,626} These findings were limited to individuals with elevated systemic inflammation (hs-CRP $\geq$ 2mg/L)

and a history of myocardial infraction. Thus, the effects of canakinumab therapy among patients with different profiles is unknown.

Further analysis from this study also found that canakinumab-mediated inhibition of IL-1 β signaling reduces the incidence and mortality of small cell lung cancer.⁶³⁰ This is in line with prior studies of tumor-associated macrophage inflammation, which plays an important role in cancer growth and metastasis.^{631,632} Although it is worth noting that other components of the innate immune response, like natural killer cells, can actively suppress the progression of cancer.⁶³³ Canakinumab was also reported to decreased gout, osteoarthritis, and arthritis, which are all diseases that are strongly associated with inflammation.^{624,634-636} It is important to note that systemic suppression of inflammation via canakinumab treatment lead to an small but statistically significant increase in fatal infections.⁶²⁴

Another major study called the Cardiovascular Inflammation Reduction Trial (CIRT), studied 4,786 patients on low-dose methotrexate. Methotrexate is an affordable FDA approved broad spectrum anti-inflammatory drug that is commonly used in the treatment of rheumatoid arthritis and psoriasis.⁶³⁷⁻⁶⁴⁰ Compared to placebo control treated patients, no difference was observed in IL-1 β , IL-6, hs-CRP levels, nor event rates.^{638,641} Unlike the CANTOS trial, CIRT analyzed patients with a broad range of systemic inflammation. The median patient's hs-CRP score being 1.6 in CIRT while the CANTOS trial exclusively studied patients with hs-CRP ≥ 2 . Therefore, these studies strongly suggest that the progression of atherosclerosis in high-risk patients in the setting

of ongoing chronic inflammation is closely tied to IL-1 β signaling and that targeting this pathway may be less effective in patients that do have chronically elevated systemic inflammation (as indicated by hs-CRT levels) or a prior history of myocardial infarction.

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The findings reported in this dissertation further support the importance of IL-1 β in atherosclerosis and more specifically points to macrophages and plaque calcification. Atherosclerotic plaque calcification is a known marker for total plaque burden.⁶⁴² The new findings by the CANTOS trial demonstrated that IL-1 β inhibition can reduce event mortality among patients with atherosclerosis with chronically elevated systemic inflammation. This is in-line with the Morrison laboratory's previous findings that IL-1 β is an independent predictor of cardiovascular death among patients with high coronary calcium burden.⁷⁸ A recent investigation into the physiological effects of canakinumab that can pin-point the effects responsible for reduced event rates have begun and have recently found no effects on blood pressure, a known risk factor for cardiovascular events.^{643,644} Atherosclerotic plaque calcification is a known risk factor of cardiovascular and all-cause mortality, and the effects of canakinumab on calcification have not yet been studied.¹⁸ As reported here, the animal studies of macrophage IL-1 β combined with findings from human data, not only suggests that IL-1 β is associated with calcification but plays a causal role. In agreement with this working hypothesis, one study of *LDL-receptor* deficient mice found that the systemic administration of canakinumab lead to reduced plaque calcification.⁵³¹ In prior findings among *ApoE* deficient mice by the Morrison laboratory, the inhibition of IL-1receptor signaling (using IL-1receptor antagonist) has

been observed to mitigate the progression of both vascular calcification and plaque area.⁷⁸ When considering the findings in this report and the findings of other studies, they collectively suggest that the therapeutic effects of blocking IL-1 β signaling on event rates may be attributed to reduce plaque calcification to some degree. Therefore, further investigation is warranted to understand the effects of IL-1 β inhibition—especially with canakinumab—on vascular calcification. Moreover, if canakinumab were to inhibit vascular calcification in patients, it could potentially be the first therapy to be developed for vascular calcification and has significant implications for other diseases such as calcific aortic valve stenosis and chronic kidney disease associated calcification.

Interestingly, one report by the Owens laboratory studying *ApoE* deficient mice on a HFD diet found that negating systemic IL-1 β with a monoclonal antibody after advanced atherosclerosis developed led to changes in plaque that are associated with instability. ⁶⁴⁵ It was observed that anti-IL-1 β antibody treatment (along with HFD) for 3 weeks or 8 weeks after already being on a HFD for 18 or 26 weeks, respectively, reduced the presence of plaque smooth muscle cells, reduced collagen content, and increased the presence of plaque macrophages. ⁶⁴⁵ Plaques with reduced smooth muscle cells and collagen content are often associated with increased vulnerability to rupture, which can lead to a cardiovascular event. ⁶⁴⁶ Also, no changes in calcification were observed. Now, these results are unexpected because the findings from the CANTOS trial suggest that anti-IL-1 β antibody treatment may be stabilizing plaques and thus resulting in the observed reduction of cardiovascular events. However, there are key distinctions

between these two studies to consider when reviewing these seemingly conflicting findings.

The prior animal study using anti-IL-1 β antibody treatment has several differences which may explain the different findings. Firstly, the animal study used a mouse model, which possessed intrinsic differences compared to human biology. The model also did not mimic the effects of aging relevant to CANTOS patients (with an average age 61 years) and did not reflect the potential effects of therapies (such as lipid-lowering statin treatment) that over 90% of CANTOS participants were receiving.^{645,647,648} CANTOS participants' median serum LDL concentration was managed at a healthy level (82.4mg/dL) while the animal model was hypercholesterolemic (>500g/dL).^{645,647} The management of serum cholesterol may be vital for anti-IL-1 β antibody treatment to be effective at mitigating the risks of plaque rupture and ultimately reducing cardiovascular events.

Regarding the observation that anti-IL-1 β antibody treatment did not affect calcification among *ApoE* deficient mice, this is counter-intuitive to the findings reported here that myeloid IL-1 β knockdown reduces calcification in this same animal model. However, it is important to consider that in the findings reported here only myeloid specific IL-1 β signaling was reduced, and IL-1 β expression was reduced at 10 weeks of age before being placed on a HFD (before plaque formation occurs). The *ApoE* deficient mice had IL-1 β signaling systemically suppressed and anti-IL-1 β antibody treatment began late in plaque development after the animals had already been on a HFD for 18 weeks.⁶⁴⁵

Therefore, these differing conditions do not permit a direct comparison, and the findings of both studies do not inherently contradict each other. On the contrary, these observations collectively suggest that IL-1 β signaling in early atherosclerosis mitigates the development of calcification while in highly advanced plaques IL-1 β signaling is no longer capable of modulating calcification.

A notable risk to the clinical strategy of systemically neutralizing IL-1 β (via canakinumab) is compromising the patient's capacity to prevent and survive infectious diseases.⁶²⁴ Impaired inflammation may also pose plausible risks to patients regarding wound healing.^{649,650} Findings reported here using the *ApoE*^{-/-} *CSF1R*^{mcm} IL-1 β ^{fl/fl} animal model suggest that targeting of IL-1 β in macrophages alone is sufficient to modulate the progression of atherosclerotic calcification. Future studies focusing on precision targeting of IL-1 β expression exclusively among macrophages or the plaque's neointimal environment may hold the potential to limit impairment of the innate immune response while effectively treating vascular calcification. Future studies potentially achieve this by packaging anti-IL-1 β antibodies into liposomes composed of LDL, which preferentially accumulate in plaque and are internalized by macrophages. Furthermore, targeting upstream regulators of macrophage IL-1 β through Rac1, NF- κ B, or critical Rac1 guanine exchange factors (GEFs) may also act as therapeutic targets that are efficacious and potentially pose advantages over canakinumab in regards to patient adherence, commercial viability, and affordable/access to the global population.⁶⁵¹⁻⁶⁵⁶ Of course, further studies of how well conserved this Rac1-IL-1 β pathway is in humans will need to be conducted. The cloned animal models that were studied does not account for the

effects of human heterogeneity, the effects of aging associated with cardiovascular disease, nor the effects of comorbidities and therapeutic treatments that at-risk patients receive. 657-659

Summary & Highlights

This collective work has demonstrated that atherosclerotic calcification significantly depends on macrophage Rac1-dependent IL-1 β signaling. Macrophage derived IL-1 β expression in itself is sufficient to modulate vascular calcification, and is downstream of Rac1. Macrophage Rac1 regulation of IL-1 β appears to be facilitated through NF- κ B activity. Moreover, this Rac1-IL-1 β signaling axis has been identified as a mechanism for statin-enhanced atherosclerotic calcification with supporting data from both animal models and patient data/samples. It is now understood that statins disrupt Rac1 isoprenylation, which mitigates binding by its inhibitor RhoGDI, and results in enhanced Rac1 activity, elevated systemic IL-1 β expression, and a macrophage Rac1-dependent increase in vascular calcification. 295 A summary of this macrophage Rac1- IL-1 β signaling axis is illustrated in **Figure 7.1**.

These findings demonstrate that macrophage-mediated inflammation plays a critical role in the development of plaque calcification and further supports others' reports that atherosclerosis is an inflammatory disease. 75,295,642,660 Moreover, this work and the CANTOS trial demonstrate that IL-1 β is a major component of atherosclerotic inflammation. 295,647 This study further reveals that in macrophages both endogenous Rac1 and statin-enhanced Rac1 activity are sufficient to regulate IL-1 β and subsequently

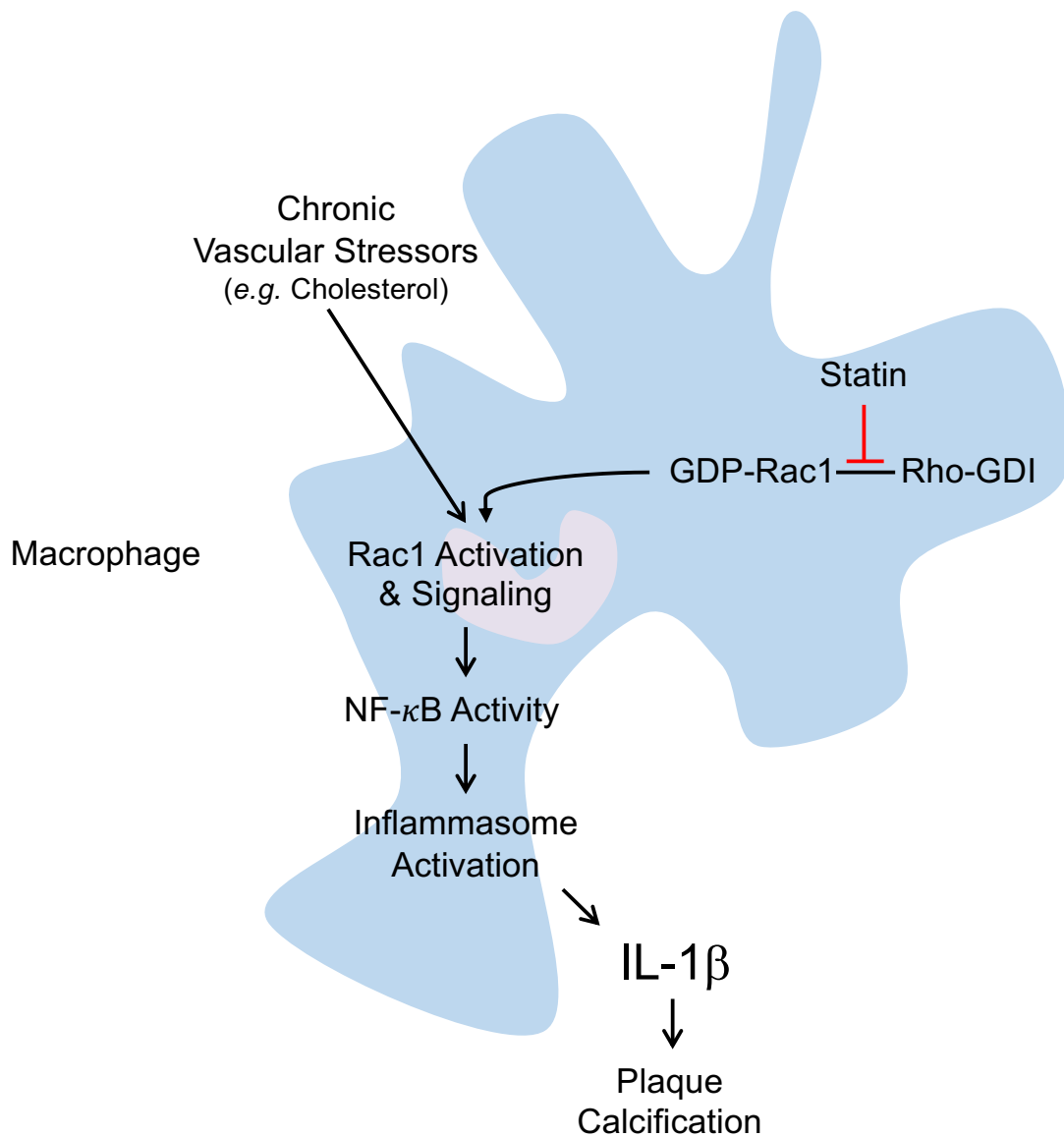


Figure 7.1 Overview of proposed macrophage Rac1-IL-1 β signaling axis for atherosclerotic plaque calcification.

Depicted above statins disrupt Rho-GDI inhibition of Rac1 (by inhibiting Rac1 isoprenylation), which leads to increased macrophage Rac1 activity. Both elevated macrophage Rac1 activity and basal levels are sufficient to modulate NF- κ B activity and IL-1 β expression. Rac1 appears to regulate IL-1 β expression through nuclear NF- κ B activity. Macrophage Rac1-dependent IL-1 β signaling is sufficient to directly contribute to plaque calcification.

impact the degree of plaque calcification without affecting other aspects of plaque development.²⁹⁵ From this and past studies, one can collectively conclude that Rac1 modulates IL-1 β through NF- κ B and not by NADPH oxidase mediated ROS production.^{78,295} As revealed here, new insights into statin's relationship to Rac1-IL-1 β signaling has led to the first demonstrated molecular mechanisms by which statin treatment promotes vascular calcification.

An overarching limitation of this collective study is the confinement of this proposed macrophage Rac1-IL-1 β pathway to atherosclerotic calcification and only in the context of hyperlipidemic non-aged mice. Clinical findings reported here, and the CANTOS trial suggest that this macrophage Rac1-IL-1 β pathway is relevant to human disease.^{78,295,647} The animal model findings reported here also do not account for other factors that may influence calcification and do not recapitulate the disease state of many patients. Some of these factors include comorbidities like diabetes and hypertension.^{644,661} Moreover, the effects of aging, which are common among patients with cardiovascular disease, is not accounted for in this investigation.^{14,16,662} However, since the tamoxifen treatments started in young adulthood, it suggests that early and persistent suppression of Rac1-IL-1 β signaling may have therapeutic potential in younger patients with early developing subclinical atherosclerosis.^{663,664}

To be clear the findings of this report are also limited to the role of Rac1-IL-1 β in atherosclerotic calcification and no other vascular calcification-associated diseases such as calcific aortic valve stenosis and chronic kidney disease. Differences observed among

these calcified diseases suggest potential differences in the mechanisms that contribute to calcification. For instance, unlike atherosclerotic calcification found in the intima, medial calcification occurs more commonly in patients with chronic kidney disease (although intimal calcification also occurs).⁶⁶⁵ Medial calcification in chronic kidney disease occurs in the absence of inflammatory macrophage cells, and its progression is not associated with the degree of atherosclerosis.⁶⁶⁶ This suggests that there are different mechanisms contributing to calcification that occurs in different tissues and disease settings among patients. However, some similarities to plaque calcification exists among these other calcific related diseases. Increased expression of the osteogenic factor, RUNX2, is associated with calcification among aortic valve disease, chronic kidney disease, and atherosclerosis.^{132,144,667-669} Moreover, the Morrison laboratory has previously discovered that blocking IL-1receptor signaling is sufficient to inhibit the progression of aortic valve thickness in the same *ApoE* deficient animal model used in this body of work.⁷⁸ Therefore, this Rac1-IL-1 β pathway in atherosclerotic calcification may have a potential role in other calcification-associated diseases, but further research is required to elucidate if that is the case. Note that all future studies suggested here are focused on mammalian animal models. Atherosclerosis is a disease that develops in arterial tissue and is largely driven by inflammation. There are no known non-mammalian vertebrates models, which share enough similarities to both human vasculature and the immune system, to develop atherosclerotic plaque calcification. Any model for this disease must account for these two factors, which is why the entirety of research conducted on plaque calcification utilizes mammalian animal models. Only zebrafish, a non-mammalian vertebrate, appears to have enough similarities to mammalian arterial tissue to study some aspects of the

vascular system, such as angiogenesis.^{670,671} Angiogenesis has been reported to participate in plaque development.^{672,673} However, this animal model does not exhibit atherosclerosis and has significant differences in the innate immunity (including differences in the proteins that form that NLRP3 inflammasome) compared to mammals.^{674,675}

Some major opportunities for future investigation stemming from findings generated in this report includes: the functional role of macrophage Rac1 nuclear localization in NF- κ B activation and IL-1 β expression under inflammasome stimulation, establishing if Rac1's effects on calcification are solely or partially dependent on IL-1 β , the potential role of Rac1-IL-1 β signaling in other calcific diseases (such as calcific aortic valve stenosis), and the relationship between Rac1-IL-1 β signaling and aging on the development of plaque calcification. Additionally, various other opportunities for future study, such as Rac1's regulation of female-specific LDL, have previously been discussed in detailed. All of these future directions possess the potential to expand the scientific community's understanding of Rac1's functions and the mechanisms of pathogenic calcification, which may one day empower humankind to develop the first therapeutic treatment for vascular calcification.

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