CAMKIIγ suppresses an efferocytosis pathway in macrophages and promotes atherosclerotic plaque necrosis

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Atherosclerosis is the underlying etiology of cardiovascular disease, the leading cause of death worldwide. Atherosclerosis is a heterogeneous disease in which only a small fraction of lesions lead to heart attack, stroke, or sudden cardiac death. A distinct type of plaque containing large necrotic cores with thin fibrous caps often precipitates these acute events. Here, we show that Ca2+/calmodulin-dependent protein kinase γ (CaMKIIγ) in macrophages plays a major role in the development of necrotic, thin-capped plaques. Macrophages in necrotic and symptomatic atherosclerotic plaques in humans as well as advanced atherosclerotic lesions in mice demonstrated activation of CaMKIIγ. Western diet-fed LDL receptor-deficient (Ldlr−/−) mice with myeloid-specific deletion of CaMKIIγ had smaller necrotic cores with concomitantly thicker collagen caps. These lesions demonstrated evidence of enhanced efferocytosis, which was associated with increased expression of the macrophage efferocytosis receptor MerTK. Mechanistic studies revealed that CaMKIIγ-deficient macrophages and atherosclerotic lesions lacking myeloid CaMKIIγ had increased expression of the transcription factor ATF6. We determined that ATF6 induces liver X receptor-α (LXRα), an MerTK-inducing transcription factor, and that increased MerTK expression and efferocytosis in CaMKIIγ-deficient macrophages is dependent on LXRα. These findings identify a macrophage CaMKIIγ/ATF6/LXRα/MerTK pathway as a key factor in the development of necrotic atherosclerotic plaques.

Introduction

Despite major medical advances in the past 50 years, cardiovascular disease remains the major cause of morbidity and mortality worldwide (1). Ischemic cardiovascular disease is caused by atherosclerosis, a lipoprotein-driven inflammatory process in which lipid, macrophages and other immune cells, and fibrinous elements accumulate in the intima of large arteries at sites of disturbed blood flow (2, 3). While most atherosclerotic plaques are clinically silent, a subset of lesions can trigger thrombotic vascular occlusion, leading to unstable angina, myocardial infarction, stroke, or sudden cardiac death. Two morphologically distinct lesion types can lead to occlusive thrombosis: rupture-prone plaques with necrotic cores and thin fibrous caps (thin-cap fibroatheroma [TCFA]) and fibrous plaques with superficial endothelial erosion (4, 5). Given the clinical importance of TCFA, understanding the processes that contribute to the development of the necrotic core is essential.

In normal physiology, apoptotic cells are rapidly and efficiently cleared by phagocytes through efferocytosis, which is the receptor-mediated process by which macrophages and other phagocytes recognize, engulf, and degrade apoptotic cells. When efferocytosis fails, dead cells undergo secondary necrosis and generate an intense inflammatory response (6). Studies have shown that macrophage-mediated efferocytosis is defective in advanced human plaques that have not yet ruptured (7, 8). Further, experiments using genetically engineered mice, i.e., mice that lack the key macrophage efferocytosis receptor MerTK, have demonstrated a causal relationship between defective macrophage-mediated efferocytosis and plaque necrosis (9, 10). Recently, our group has demonstrated that MerTK expression is decreased in symptomatic carotid artery lesions as compared with asymptomatic lesions (9). The mechanisms that contribute to defective efferocytosis in advanced atherosclerosis, however, have not yet been fully elucidated.

Ca2+/calmodulin-dependent protein kinase II (CaMKII) is a serine-threonine kinase that is a key mediator of Ca2+-regulated processes in many different cell types (11, 12). There are 4 CaMKII isoforms, α, β, δ, and γ, each encoded by a distinct gene. Recent studies from our lab have delineated a central role for overactive CaMKIIγ in hepatocytes in the development of obesity-induced insulin resistance and diabetes (13–15). Interestingly, the same isoform is expressed by macrophages, and previous in vitro work from our lab suggested that CaMKIIγ could participate in certain types of apoptosis pathways in macrophages (16). In view of these findings, and with an eye toward identifying common upstream

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Figure 1. Expression of p-CaMKII is increased in advanced and symptomatic atherosclerotic lesions of humans and mice. (A) Human carotid specimens were obtained at time of endarterectomy from asymptomatic (Asympt) or symptomatic (Sympt) subjects with recent TIA/stroke. Fixed sections were costained for p-CaMKII (green), CD68 (red), and DAPI (nuclei; blue). Sections stained with isotype control antibodies for p-CaMKII and CD68 showed absence of signal. p-CaMKII staining was quantified as MFI within CD68+ cells. Data are presented relative to the average value obtained for the asymptomatic subjects’ specimens (n = 7 asymptomatic and 5 symptomatic subjects, 2 slides per patient). Scale bar: 500 μm. (B) Left: adjacent sections from the human carotid endarterectomy specimens in panel A were stained with H&E, and necrotic area was quantitated. Right: relationship between necrotic area and p-CaMKII fluorescence intensity in macrophages per low power field (LPF) was plotted ($r^2 = 0.6195$, $P = 0.0024$). Correlation coefficient ($r^2$) and $P$ values were calculated using Pearson product-moment correlation analysis. (C) Sections of human coronary arteries obtained from individual subjects at autopsy were classified as PIT or TCFA and stained and quantified as above. Data are presented relative to the average value obtained for the PIT specimens (n = 5 subjects in each group, 2 slides per patient). Scale bar: 250 μm. (D) Frozen aortic root sections from 8-week and 16-week WD-fed Ldlr−/− mice were costained for p-CaMKII (green), Mac-3 (red), and DAPI (blue). p-CaMKII staining was quantified as MFI within Mac-3+ cells. Data are presented relative to the average value obtained for the 8-week specimens (n = 5 mice in each group, 2 slides per mouse). Scale bar: 150 μm. *$P < 0.05$, **$P < 0.01$, Mann-Whitney U test.
pathogenic signaling pathways in different cell types that contribute to cardiometabolic disease, we sought to understand the role of macrophage CaMKII in advanced atherosclerosis.

In this report, we show that macrophage CaMKII is activated in advanced and symptomatic human carotid atherosclerotic lesions versus those that are less advanced or asymptomatic as well as in advanced versus early aortic root lesions in Western diet-fed (WD-fed) LDL receptor-deficient (Ldlr−/−) mice. Moreover, when myeloid CaMKII is deleted in WD-fed Ldlr−/− mice, the formation of necrotic lesions is suppressed. Studies with cultured macrophages suggest that the mechanism involves a pathway through which CaMKII in macrophages impairs efferocytosis by suppressing an ATF6/liver X receptor-α (LXRα)/MerTK pathway. These findings reveal an inverse link between CaMKII and LXRα/MerTK in macrophages that is relevant to necrotic plaque formation in advanced atherosclerosis and involves a new means of transcriptional regulation of LXRα by ATF6.

**Results**

CaMKII activation is increased in symptomatic and advanced human atherosclerotic disease and in advanced mouse atherosclerosis. Based on previous studies with cultured macrophages (16, 17), we hypothesized that CaMKII would be activated in macrophages in advanced atherosclerotic lesions and that this process would further promote the development of advanced atherosclerotic plaque characteristics. To begin to explore this hypothesis, we sought evidence of CaMKII activation in macrophages of human advanced atherosclerotic lesions. Because the major indicator of CaMKII activation is autophosphorylation of Thr287 (18), we compared immunoreactive phospho-Thr287-CaMKII (p-CaMKII) in sections of human plaques that differed in terms of clinical or pathologic progression. First, we compared sections of carotid plaques obtained from endarterectomy of patients who were either asymptomatic or had recently suffered a transient ischemic attack (TIA) or stroke. We found that most of the p-CaMKII signal colocalized with macrophages and that this signal was higher in plaques from symptomatic versus asymptomatic subjects (Figure 1A). Note that the mean fluorescence intensity (MFI) of p-CaMKII was determined per CD68-positive lesion area to correct for the potentially higher number of macrophages in the more advanced lesions. Importantly, the symptomatic lesions were more necrotic, and when all lesions were considered, there was a strong positive correlation between p-CaMKII and the necrotic area (Figure 1B). In contrast, there was no significant correlation between total CaMKII signal in lesional macrophages and the necrotic area (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI94735DS1). Furthermore, there was a significant relationship between necrotic area and p-CaMKII MFI per total lesion area (Supplemental Figure 1B). Given the role of CaMKII in smooth muscle cells (11, 19), we compared p-CaMKII MFI associated with smooth muscle α-actin-positive (SMαA-positive) cells in lesions and found no significant differences between the plaques of asymptomatic and symptomatic patients (Supplemental Figure 1C). Thus, although CaMKII is active in both macrophages and smooth muscle cells in atherosclerosis, it is primarily the activity in macrophages that increases in symptomatic lesions. Finally, in a separate cohort of patients, we assayed lesional p-CaMKII using sections of coronary artery plaque classified as either pathological intimal thickening (PIT), which is a relatively stable type of lesion, or TCFA, which is an advanced type of lesion. The p-CaMKII signal per CD68-positive lesion area was higher in the TCFA lesions versus PIT lesional macrophages (Figure 1C).

In anticipation of the molecular-genetic causation studies in mice that follow, we assayed p-CaMKII in atherosclerotic lesions from Ldlr−/− mice fed a WD for 8 weeks, which resulted in relatively early lesions, and in mice fed the WD for 16 weeks, which led to advanced plaques. As with human lesions, p-CaMKII colocalized mostly with lesional macrophages, and the p-CaMKII signal was more intense in the advanced versus early plaques (Figure 1D; isotype controls are shown in Supplemental Figure 1D). No difference was found in total CaMKII fluorescence in the macrophage-rich areas of 8-week versus 16-week groups, indicating a true increase in p-CaMKII/total CaMKII ratio (Supplemental Figure 1E).

Genetic targeting of myeloid CaMKII in WD-fed Ldlr−/− mice leads to smaller necrotic core area and improved efferocytosis in atherosclerotic plaques. Given the increase in p-CaMKII in advanced human and mouse lesions, and because macrophages exclusively express the γ isoform of CaMKII (16, 17), we hypothesized that the loss of CaMKIIγ in myeloid cells would suppress development of advanced atherosclerotic plaque characteristics. To test this hypothesis, we bred Camk2gΔ/Δ mice onto the Ldlr−/− background and then further crossed these mice to lysozyme M-Cre (LysM-Cre) mice in order to drive myeloid-lineage-specific deletion of the gene. The resulting Camk2gΔ/Δ Ldlr−/− LysM-Cre−/− mice (M-CaMKII–KO mice) and littermate control Camk2gΔ/Δ Ldlr−/− mice (control mice) were fed the WD for 12 weeks. There were no significant differences in body weight, insulin, glucose, plasma cholesterol, plasma lipoprotein distribution of cholesterol, or plasma triglycerides between the 2 groups of mice (Supplemental Figure 2, A–F). Additionally, we found no differences in circulating total white blood cell, monocyte, neutrophil, or platelet numbers (Supplemental Figure 2, G–J), and there was also no difference in the percentage of lesional macrophages, total CD3+ T cells, or regulatory T cells between the 2 groups (Supplemental Figure 2, K–M). As expected from the specificity of LysM-Cre (20), M-CaMKII–KO mice showed no detectable CaMKIIγ signal in macrophage-rich areas of aortic root lesions (Supplemental Figure 1E), but the signal in lesional SMαA+ cells and vWF+ endothelial cells was retained (Supplemental Figure 1F).

Our primary end points were 2 features associated with the clinical progression of human plaques: necrotic core area and thinning of the collag enous fibrous cap that covers and “stabilizes” advanced lesions (21). Inspection of aortic root plaques suggested a significant decrease in necrotic core area in the M-CaMKII–KO lesions compared with control lesions, and this was verified by quantitative image analysis (Figure 2A). Importantly, overall lesion area (Figure 2B) was similar between the 2 cohorts, indicating a true decrease in the necrotic core/lesion area ratio (Figure 2C). A decrease in this ratio is often seen when a process specific to advanced plaque progression has been targeted (21, 22). We also found a substantial increase in fibrous cap thickness in the M-CaMKII–KO lesions (Figure 2D), which is another indicator of plaque stabilization. Consistent with this interpretation, we found...
that M-CaMKII–KO lesions had less collagenase activity (Figure 2E), which might contribute to the thicker collagen caps in the lesions from these mice (23, 24). A similar decrease in necrotic core/lesion area was seen in brachiocephalic arterial lesions of M-CaMKII–KO versus control mice (Supplemental Figure 3, A–C).

We then sought to further characterize important features of advanced atherosclerotic disease in these mice. Elevated ER stress, particularly increased expression of mediators of the unfolded protein response (UPR), is another feature of advanced human and mouse plaques (22, 25, 26). Using laser capture microdissection (LCM) of aortic root sections followed by quantitative real-time quantitative PCR (RT-qPCR), we found significantly less expression of mRNAs encoding 3 genes that are induced by the UPR: Ddit3 (encoding CHOP), spliced Xbp1, and Dnajb9 (encoding ERdj4) (Supplemental Figure 4, A–C). Because advanced lesions are characterized by the presence of proinflammatory cytokines, we also determined the levels of Il1b, Il6, and Tnfa mRNA in LCM samples of aortic root lesions and found significantly lower levels of all 3 cytokine mRNAs in the lesions of M-CaMKII–KO mice (Supplemental Figure 4, D–F). To gain insight into whether these differences are cell intrinsic due to the absence of CaMKIIγ in macrophages, we examined the response of control and CaMKII-KO bone marrow–derived macrophages to low-dose LPS. There was a marked reduction in the expression of Il6 in CaMKII-KO macrophages and a smaller, though significant, reduction in Il1b, but Tnfa was not reduced (Supplemental Figure 4, G–I). Thus, the decrease in inflammation in M-CaMKII–KO lesions may be caused by the direct effect of CaMKIIγ deficiency on inflammatory signaling in macrophages as well as by secondary causes, such as plaque necrosis. Taken together, these findings demonstrate that genetic targeting of myeloid CaMKIIγ protects WD-fed Ldlr–/– mice from developing several key features of a type of advanced plaque that can precipitate acute vascular events in humans.
A major cause of necrotic core formation in advanced atherosclerosis is defective phagocytic clearance of apoptotic cells (efferocytosis) within lesions, leading to secondary necrosis of the uncleared cells and necrotic cell–induced inflammation (24). We first assayed apoptotic cells in lesions by measuring the percentage of lesional cells that showed either TUNEL positivity or caspase-3 activation. By either measurement, the lesions of M-CaMKII–KO mice had fewer apoptotic cells compared with control mice (Figure 3, A and B). To determine whether this finding was associated with defective efferocytosis, we used a standard assay in which lesions costained for TUNEL and macrophages (Mac-3) are quantified for TUNEL+ cells that are either associated with a macrophage, indicative of efferocytosis, or not associated with macrophages (free) (10). Data are presented as the ratio of associated/free TUNEL+ cells, with higher values representing higher levels of efferocytosis. We found a marked increase in this measure of efferocytosis in the M-CaMKII–KO lesions compared with lesions from the control group (Figure 3C). These data suggest that at least one mechanism for the decreased number of apoptotic cells in the lesions of M-CaMKII–KO mice is improved efferocytosis. Most importantly, improved efferocytosis could also explain the decrease in plaque necrosis in M-CaMKII–KO mice, i.e., by preventing secondary necrosis of lesional apoptotic cells.

CaMKII-KO macrophages and lesions express higher levels of the efferocytosis receptor MerTK. To further elucidate the role of macrophage CaMKII in efferocytosis, we assayed efferocytosis in vitro using bone marrow–derived macrophages from control and CaMKII-KO mice. Consistent with the lesional data, CaMKII-KO macrophages displayed increased efferocytosis compared with control macrophages (Figure 4A). Because efferocytosis is a 2-step process in which macrophages first bind apoptotic cells via specialized efferocytosis receptors and then internalize the apoptotic cells, we repeated this assay at 4°C, which allows for binding of apoptotic cells, but not their ATP-dependent internalization. Under these conditions, CaMKII-KO macrophages showed enhanced apoptotic cell binding (Figure 4B), suggesting improved apoptotic cell recognition. We then compared CaMKII-KO and control macrophages for a number of efferocytosis receptors proposed to have a role in atherosclerosis. We found no differences in the expression of β5 integrin, CD36, CD61, CD206, CD300F, or Tim4. CaMKII-KO macrophages did, however, have a marked enhancement in cell-surface expression of MerTK (Figure 4C), which plays an important role in efferocytosis in advanced atherosclerotic lesions (9, 10, 27). CaMKII-KO macrophages also had an increase in MerTK mRNA (Figure 4D). To confirm our findings, we used the CaMKII inhibitor KN93 (16) and showed that this compound, but not the inactive homolog KN92, induced an increase in MerTK mRNA, cell-surface MerTK, and efferocytosis (Supplemental Figure 5, A–C). Moreover, KN93 was unable to induce further increases in these parameters in CaMKII-KO macrophages, indicating an on-target effect of the inhibitor. Finally, we examined control and M-CaMKII-KO atherosclerotic lesions for MerTK expression by immunofluorescence microscopy and found that M-CaMKII-KO lesions expressed significantly more of the protein than control lesions (Figure 4E). Thus, the increase in efferocytosis in CaMKII-KO macrophages in vitro and in atherosclerosis is associated with an increase in MerTK, which is a known mediator of macrophage efferocytosis in atherosclerosis.

CaMKII deficiency in macrophages increases the expression of LXRs, which induces MerTK. To explain the link between CaMKII deficiency and increased MerTK-mediated efferocytosis, we reasoned that CaMKII deficiency in macrophages might lead to an increase in a MerTK inducer. In this context, we assayed an MerTK inducer that is relevant to atherosclerosis, LXR (28), and found that Nr1h3 mRNA (encoding LXRα), LXRα protein, and one of LXR’s gene targets, Abca1, were indeed increased in CaMKII-KO macrophages (Figure 5, A and B). Interestingly, Nr1h2 mRNA (encoding LXRβ) was not increased (Figure 5A). Confirming these findings, KN93, but not KN92, induced an increase in Nr1h3 and Abca1 mRNA without any change in Nr1h2 levels (Supplemental Figure 5, D–F). To test causation with regard to MerTK induction, we treated...
macrophages from control and CaMKII-KO mice with siRNA targeting LXRα. As above, CaMKII-KO macrophages expressed higher levels of Mertk mRNA than control macrophages, and in response to siLXRα, this increment was abrogated (Figure 5C). Similar results were found for Abca1 mRNA, cell-surface MerTK, and efferocytosis (Figure 5, C–E). We next examined the potential in vivo relevance of our findings by using LCM to assess relevant mRNAs in the atherosclerotic lesions of control and M-CaMKII–KO mice. Consistent with our in vitro findings, lesions from M-CaMKII–KO mice contained significantly higher levels of Nr1h3 (LXRα) and Abca1 mRNAs than did lesions from control mice, while there was no significant difference in the level of Nr1h2 (LXRβ) mRNA (Figure 5F). These data provide a plausible mechanism linking macrophage CaMKIIγ deficiency to increased MerTK-mediated efferocytosis, namely, through an LXRα/MerTK induction pathway.

CaMKII deficiency in macrophages disrupts an HDAC4/DACH1/ATF6 pathway that regulates LXR and MerTK. Previous studies from our lab have elucidated a pathway in hepatocytes in which activated CaMKII phosphorylates HDAC4, causing its nuclear exclusion (14). Cytoplasmic HDAC4 is unable to SUMOylate and target the nuclear corepressor DACH1 for degradation. The resulting increase in nuclear DACH1 represses expression of the transcription factor ATF6, leading to decreased expression of ATF6 gene targets (14).

We therefore sought to determine whether this pathway might also be present in macrophages and possibly relevant to the increase in LXRα in CaMKIIγ-deficient macrophages. As was the case with hepatocytes, pHDAC4 and DACH1 were lower and ATF6 was higher in CaMKII-KO versus control macrophages (Figure 6A). Note that the increase in ATF6 was apparent at both the protein level (Figure 6A, showing cleaved/nuclear form) and the mRNA level (Figure 6B). Next, we wondered whether Nr1h3...
(LXRα) might be a transcriptional target of ATF6. Indeed, we found that the Nr1h3 (LXRα) gene, but not the Nr1h2 (LXRβ) gene, has a consensus ATF6-binding site in intron 1 that is conserved across species, and ChIP analysis showed interaction of ATF6 with this site (Figure 6C). Moreover, according to the ENCODE ChIP-seq database, this site is surrounded by strong signals for POLR2A and H3K4m3, suggesting that it is an active transcriptional regulatory region (29–31). To determine whether ATF6 might regulate LXR expression, we silenced ATF6 using siRNA and found significant decreases in Nr1h3 (LXRα) mRNA and its target Abca1 (Figure 6D).

Figure 5. CaMKII deficiency in macrophages increases the expression of LXRα, which induces MerTK. (A) mRNA was harvested from control and CaMKII-KO macrophages and quantified by RT-qPCR analysis for Nrth3 (LXRα), Nrth2 (LXRβ), and Abca1 and normalized to Rplp0 (36B4). Data are shown as relative to the value for control macrophages. Results are shown as mean ± SEM. *P < 0.001, unpaired t test. (B) Lysates of control and CaMKII-KO macrophages were immunoblotted for LXRα, ABCA1, and β-actin. *P < 0.001, 2-way ANOVA with post hoc Tukey’s analysis. (C) Macrophages were transfected with scrambled RNA (scr) or siRNA targeting LXRα (siLXRα) and, after 48 hours, assayed for Nrth3, Nrth2, and Abca1 mRNA. Data are shown as relative to the value for control macrophages. Results are shown as mean ± SEM. *P < 0.001, 2-way ANOVA with post hoc Tukey’s analysis. (D) Macrophages were labeled with MerTK antibody at 4°C and subjected to flow cytometric analysis. MFI data are shown as relative to the value for control macrophages. Results are shown as mean ± SEM. *P < 0.001, 2-way ANOVA with post hoc Tukey’s analysis. (E) Macrophages were incubated for 45 minutes with a 3:1 ratio of PKH67–green linker–labeled apoptotic Jurkat cells to macrophages and assayed for efferocytosis by image analysis of fluorescent microscopy images. Results are shown as mean ± SEM. *P < 0.001, 2-way ANOVA with post hoc Tukey’s analysis. (F) RNA was obtained from aortic root sections by LCM. Nr1h3, Nrth2, and Abca1 mRNA was quantified by RT-qPCR with normalization to Gapdh. Data are shown as relative to the value for lesional mRNA from the control cohort. n = 5 in each group. **P < 0.05, Mann-Whitney U test.
Figure 6. CaMKII deficiency in macrophages disrupts an HDAC4/DACH1/ATF6 pathway that regulates LXRxu and Mertk. (A) Whole cell or nuclear extracts from control and CaMKII-KO macrophages were immunoblotted using antibodies against p-HDAC4 (pHDAC4), HDAC4, DACH1, β-actin, ATF6, and nucleophosmin (NPM). Left: representative immunoblot. Right: densitometry. *P < 0.05; **P < 0.01, unpaired t test. (B) Macrophage mRNA was quantified by RT-qPCR for Atf6. Data are presented as the relative value to control macrophages. ***P < 0.001, unpaired t test. (C) Left: conserved ATF6 consensus sequence in intron 1 of the Nrlh3 (LXRδ) gene. Right: macrophages from control and CaMKII-KO mice were subjected to ChIP analysis using anti-ATF6 or IgG control. The intronic region containing the ATF6-binding site and a nonconsensus sequence were amplified by RT-qPCR and normalized to Input DNA. n = 4 biological replicates. *P < 0.05, unpaired t test. (D) Macrophages were transfected with siRNA targeting ATF6 (siATF6) and analyzed for the indicated mRNAs as in Figure 5C. **P < 0.01; ***P < 0.001, 2-way ANOVA with post hoc Tukey’s analysis. (E–G) Macrophages were transfected with siATF6 as above and analyzed for Mertk mRNA, cell-surface MerTK by flow cytometry, and efferocytosis. **P < 0.001, 2-way ANOVA with post hoc Tukey’s analysis. (H) Human bone marrow–derived macrophages with a viral vector expressing of macrophage CaMKII as discussed below, could have contributed to the plaque phenotype in these mice.

Because CaMKIγ is activated as atherosclerosis progresses, we tested whether overexpression of an active form of CaMKII in cultured, nonstimulated macrophages was sufficient to suppress ATF6, LXRxu, and efferocytosis. For this purpose, we transduced bone marrow–derived macrophages with a viral vector encoding a constitutively active form of CaMKII (CaMKIIγ, which has a T287D mutation) (15, 32) or a control virus expressing LacZ. In macrophages transduced with Ca-CaMKII, there was significantly less Atf6, Nrlh3 (LXRxu), and Mertk mRNA (Figure 7A–C) and decreased levels of LXRxu and MerTK protein (Figure 7D) compared with macrophages transduced with LacZ. Further, macrophages transduced with Ca-CaMKII showed a decrease in efferocytosis (Figure 7E). These data, together with both the lesional data presented earlier and the previously published studies on the role of MerTK in advanced plaques (9, 10, 27), support the concept that activation of CaMKIIγ in lesional macrophages, by suppressing an ATF6-LXRxu-MerTK module, contributes to defective efferocytosis and plaque necrosis in advanced atherosclerotic lesions.

No significant change in Nrlh2 (LXRδ) was observed. Consistent with a link between LXRxu and MerTK, silencing of ATF6 lowered Mertk mRNA, cell-surface expression of MerTK, and efferocytosis in both control and, most notably, in CaMKII-KO macrophages (Figure 6, E–G). Furthermore, we found significantly higher levels of Atf6 mRNA in the lesions of M-CaMKII-KO mice versus control mice (Figure 6H). Finally, the key findings were reproduced in human monocyte-derived macrophages: approximately 50% silencing of CAMK2G mRNA using siRNA led to significant decreases in ATF6, Nrlh3 (LXRxu), and MERTK mRNA expression (Figure 6I). These combined data show that CaMKIIγ deficiency in macrophages enhances MerTK-mediated efferocytosis through a new pathway in which ATF6 induces LXRxu.

Discussion

The development of large necrotic cores with thin overlying collagen caps is a hallmark of a particular type of human plaque that can trigger acute thrombo-occlusive vascular events (5, 21). Two processes that can lead to plaque necrosis are defective efferocytosis of apoptotic cells and primary cellular necroptosis (7, 33–37). Defective efferocytosis can result if apoptotic cells resist uptake by phagocytes, as described recently for atherosclerosis (38), or if the phagocytes themselves have an impairment in apoptotic cell binding or internalization. The work here defines a process that may help explain defective apoptotic cell recognition by advanced lesional macrophages. As described, activated CaMKII in these macrophages leads to decreased expression of a key atherosclerosis-relevant efferocytosis receptor, MerTK. Interestingly, another process in advanced lesions can lead to the ADAM17-mediated proteolytic degradation of MerTK and prevention of this process using genetic engineering to render MerTK resistant to proteolysis also suppresses plaque progression (39).

While we have accumulated substantial evidence to suggest that the ATF6/LXRxu/MerTK/efferocytosis pathway is a major mechanism contributing to the improvement in necrotic core area in the M-CaMKII-KO mouse, it is possible that other mechanisms contributed to the overall lesional phenotype. Our laboratory previously reported that CaMKIIγ activation in macrophages can trigger apoptosis (16), and we did see a decrease in apoptotic cells in M-CaMKII-KO lesions (Figure 3A). However, based on the in situ efferocytosis data and our mechanistic work in the current study, together with the previous literature on lesional efferocytosis and MerTK (9, 10, 27), we believe that the dominant mechanism accounting for the decrease in apoptotic cells in M-CaMKII-KO lesions is the enhanced clearance of these cells by lesional macrophages. Nonetheless, it is possible that decreased lesional apoptosis, perhaps due to a separate effect of macrophage CaMKII as discussed below, could have contributed to the plaque phenotype in these mice.

The increase in fibrous cap thickness and lower collagenase activity in M-CaMKII-KO lesions raises the question of whether overactive macrophage CaMKII impairs inflammation resolution-induced repair processes in advanced lesions. Inflammation resolution, a process through which specific molecules restore tissue homeostasis after an inflammatory response, is impaired in advanced human and mouse plaques (40–42). Moreover, therapeutically restoring resolution mediators can block plaque progression in mice (41–44). In this context, it is possible that the loss of macrophage CaMKIIγ improves resolution in plaques. If so, one mechanism could be related to our recent finding that CaMKIIγ activation in macrophages preferentially channels the common precursor lipid mediator arachidonic acid into proinflammatory leukotriene B4, rather than proresolving lipoxin A4 (17). If the resolution process is improved in M-CaMKII-KO lesions, as we predict, this might explain the improvement in protective fibrous cap formation (45). However, it is also possible that M-CaMKII-KO directly inhibits expression or activity of matrix metalloproteinases (see Figure 2E) (46, 47), which have been implicated in advanced plaque progression (48, 49). Another possible link between inflammation resolution and the findings reported herein is that efferocytosis in general, and MerTK signaling in par-
is related to apoptosis induction, as CHOP deficiency does not improve efferocytosis (22), but other links between ER stress and impaired efferocytosis are possible (57). Second, suppression of the UPR in M-CaMKII–KO mice may lower lesional inflammation. For example, when the UPR is activated by cholesterol loading of macrophages, elevated CHOP drives Erk1/2-dependent production of IL-6 (58). Moreover, hematopoietic deficiency of CHOP in a mouse model of colitis led to a less severe inflammatory response, at least in part due to a reduction in IL-6 (59). In the context of these two reports, it is interesting to note that the cytokine mRNA showing the greatest reduction in M-CaMKII–KO lesions and macrophages was Il6 (Supplemental Figure 4, D–I). Finally, the ER stress that occurs in advanced lesions may actually promote CaMKII activation, which would create a pathologic amplification loop. This idea is based on the finding that induction of ER stress in cultured macrophages activates CaMKII (16). While we have shown that the PERK and IRE1 branches of the UPR are less active in the M-CaMKII–KO lesions, the expression of ATF6 is concomitantly increased. Although this may seem counterintuitive, there is a precedent for this pattern of UPR activation, both in our work and...
that of others (13–15, 60–63). In this context, ATF6 may serve to regulate the PERK and IRE1 branches of the UPR in order to modulate the response to chronic stress (64).

We have identified ATF6 as a transcriptional regulator of LXRα, and our mechanistic and in vivo data are consistent with a model in which LXRα mediates the increase in MerTK-mediated atherosclerotic lesional efferocytosis and the suppression of plaque necrosis when CaMKII is deleted in macrophages. A previous study demonstrating that LXR can induce MerTK provided us with a clue as to how CaMKII deficiency and ATF6 might be linked to MerTK/efferocytosis (28). This study showed that macrophages from LXRα/β double-knockout mice had reduced expression of MerTK and impaired efferocytosis (28). In addition, efferocytosis itself activated LXR, perhaps through changes in cellular lipids (28), and another study reported that MerTK signaling can directly activate LXR (65). Thus, there may exist a positive feedback loop between efferocytosis and LXR. Finally, mice treated with LXR agonists have been shown to have both decreased lesion area and smaller necrotic cores (66, 67), which is consistent with these previous studies and the new findings herein.

Another possible link between CaMKII and impaired LXR action was suggested by a prior study showing that phosphorylation of LXRβ by CaMKII abrogated LXR-mediated transrepression of inflammatory genes in several cell lines (68). In addition, CaMKII can phosphorylate components of the nuclear corepressor (NCoR) complex directly, leading to derepression of inflammatory genes (68). In this model, treatment of macrophages with the CaMKII inhibitor KN93 led to increased LXR-mediated transrepression and suppression of inflammation, suggesting that in the absence of active CaMKII, LXR acts unopposed to interact with the NCoR complex. While we have not examined this pathway in lesional macrophages, it may represent a complementary mechanism whereby CaMKII antagonizes LXR activity and thereby promotes lesional inflammation.

In summary, the present study elucidates a role for macrophage CaMKII in the development of advanced atherosclerotic plaque features that are clinically important in humans. We have shown that increased activation of CaMKII in lesional macrophages is associated with advanced and symptomatic atherosclerotic disease in both humans and mice and that deletion of myeloid CaMKIIγ in atheroprone mice suppresses development of these advanced plaque characteristics. When considered together with the possible roles of CaMKII in lesional smooth muscle cells (69, 70), overactive hepatocyte CaMKII in obesity-associated insulin resistance and type 2 diabetes (13–15), and the role of overactive CaMKII in cardiomyocytes in heart failure (12), our findings suggest the potential of targeting this common upstream pathway as an integrated therapeutic approach to cardiometabolic disease.

Methods

Mice. Ldlr<sup>−/−</sup> mice on the C57BL/6j background (stock 002207) and Lysozyme M-Cre (LysM-Cre) mice also on the C57BL/6j background (stock 004781) were purchased from The Jackson Laboratory. Mer<sup>−/−</sup> mice have been described previously (39). Camk2g<sup>−/−</sup>β mice were generated as previously described (39). Camk2g<sup>−/−</sup>β mice were bred to Ldlr<sup>−/−</sup> mice to obtain homozygotes for both genes of interest and then subsequently crossed to LysM-Cre<sup>−/−</sup> mice to obtain Camk2g<sup>−/−</sup>β Ldlr<sup>−/−</sup> LysM-Cre<sup>−/−</sup> mice and littermate control mice (Camk2g<sup>−/−</sup>β Ldlr<sup>−/−</sup>). Throughout the manuscript, these mice are referred to as M-CaMKII-KO mice, where M refers to myeloid, and control mice, respectively. Mice were backcrossed for more than 10 generations to the C57BL/6j background.

Measurement of lipids, insulin, and glucose and complete blood counts. Fasting blood glucose was measured using a glucometer (One Touch Ultra, Lifescan) in mice that were fasted for 4 hours with free access to water. Standard kits were used to measure plasma insulin (Crystal Chem, catalog 90080), plasma triglycerides (Wako, Triglyceride M Kit), and cholesterol (Wako, Cholesterol E kit) per the manufacturer’s instructions. Complete blood cell count including leukocyte differential was obtained using a FORCYTE Hematology Analyzer (Oxford Science).

Human plaque histology. The specimens used in the PIT versus TCFA coronary plaque study (65% men, age 71.7 ± 12.4 years) were obtained at autopsy. Specimens were formalin fixed, paraffin embedded, and sectioned prior to analysis. Lesions were classified according to Virmani et al. (71). The carotid artery specimens were obtained from patients undergoing carotid endarterectomy for high-grade stenosis of the internal carotid artery at the Division of Vascular Surgery, University Medical Center, Johannes-Gutenberg University. According to preoperative assessment by a consulting neurologist, carotid stenoses were classified as either asymptomatic or symptomatic, defined as TIA or stroke within the past 6 months. After retrieval in the operating room, the excised plaques were rinsed in physiologic saline, immediately snap-frozen in liquid nitrogen, and then transferred to −80°C for storage until processing. Samples were fixed in 4% formalin overnight, dehydrated in EDTA for 4 days, and then embedded in paraffin blocks. Serial sections were obtained at 5-μm intervals. Prior to immunofluorescence staining, all sections from both cohorts were deparaffinized in xylene and then rehydrated in a graded series of ethanol concentrations. Sections were then boiled for 20 minutes in an antigen retrieval buffer (10 mM Tris, 0.5 M EDTA, 0.05% Tween-20). After rinsing in PBS and blocking in serum-free protein blocking buffer (DAKO, catalog X0909) for 1 hour, sections were incubated overnight at 4°C with primary antibodies directed toward p-CaMKII (Novus Biologicals, catalog NB300-184, 1:100), CD68 (DAKO, catalog M0814, 1:100), or SMα (Sigma-Aldrich, catalog C6198, 1:100). After rinsing in PBS, slides were incubated with secondary antibodies for 2 hours at room temperature. Parallel slides were used for staining with isotype controls. Slides were mounted with DAPI-containing mounting solution, and images were obtained using a Leica microscope and analyzed using FIJI/ImageJ software (NIH). Two slides, each with 2 sections, were assessed for each patient. In parallel, adjacent sections from asymptomatic and symptomatic carotid artery specimens were stained with Harris’ H&E. Total lesion and necrotic core areas were defined as previously described (43).

Mouse atherosclerotic lesion analysis. Eight to ten-week-old male control or M-CaMKII-KO mice were placed on a WD (Envigo, TD 88137) for 8, 12, or 16 weeks. Three temporally separate cohorts were structured to include 10 to 14 mice per group with birthdates within 1 to 2 weeks of each other. At the time of harvest, mice were euthanized using isoflurane. Blood was removed by left ventricular puncture, and the vasculature was then perfused with cold PBS. Aortic roots were placed in OCT and immediately frozen or fixed in paraformaldehyde and paraffin embedded. Serial 6-μm (paraffin) or 8-μm (frozen) sections were obtained for analysis. For morphometric analysis, 6 paraf-
fin sections 60 μm apart were stained with Harris’ H&E. Total lesion and necrotic core areas were defined as previously described (43). Collagen staining was performed using picrosirius red (Polysciences, catalog 24901A) per the manufacturer’s instructions. Collagen cap thickness was quantified from 3 distinct regions of the plaque as previously described (43). TUNEL staining was performed using a kit per the manufacturer’s instructions (Roche, catalog 12156792910). For immunofluorescence staining, frozen sections were fixed in cold acetone for 10 minutes. After blocking for 1 hour in serum-free protein blocking buffer (DAKO, catalog X0909), sections were incubated overnight at 4°C with the following primary antibodies: Mac3 (BD Biosciences — Pharamingen, catalog 550292, 1:100), SMAα (Sigma-Aldrich, catalog C6198, 1:100), CD3 (Abcam, catalog ab16669, 1:200), Foxp3 (eBioscience, 14-5773-82, 1:200), vWF (Abcam, catalog ab1713, 1:200), p-CaMKII (Novus Biologicals, catalog NB300-184, 1:100), CaMKIIγ (Novus Biologicals, catalog NB2P-15685, 1:100), or cleaved caspase-3 (Cell Signaling Technologies, catalog 9661, 1:100). Two slides, each with 2 sections, were assessed for each mouse. Parallel slides were used for staining with isotype controls. After rinsing in PBS, slides were incubated with secondary antibodies for 2 hours at room temperature. Slides were counterstained with DAPI, and data were quantified as the percentage of cells positive for markers of interest out of total DAPI nuclei. All images were captured using a Zeiss fluorescence microscope and analyzed using ImageJ by an observer blinded to the group assignment of each sample.

In situ zymography for collagenase activity. Frozen sections were warmed to 37°C and incubated with 20 μg/ml solution of DQ collagen IV (Invitrogen, catalog D12052) or DQ collagen I (Invitrogen, catalog D12060) at 37°C overnight. Slides were washed in PBS and then mounted with DAPI-containing solution. Images were captured using a Zeiss fluorescence microscope and analyzed using FIJI/ImageJ by an observer blinded to the group assignment of each sample.

In situ effrocytosis. Experimentation was carried out as previously described by our laboratory (10, 72). In brief, aortic root sections were stained with TUNEL (Roche) followed by Mac-3 (BD Biosciences — Pharamingen) to label lesional macrophages. Apoptotic cells were then determined to be either macrophage associated (colocalizing or juxtaposed with macrophages) or free (not associated with macrophages). Data were plotted as a ratio of associated to free cells to represent effrocytosis efficiency. Images were captured using a Zeiss fluorescence microscope and analyzed using FIJI/ImageJ by an observer blinded to the group assignment of each sample.

LCM. Aortic root lesional material from 5 mice was captured (twelve 5-μm sections per mouse) using a PALM LCM microscope as previously described (72). RNA was isolated using the RNeasy Mini Kit (QIAGEN), and cDNA was synthesized from RNA (ranging from 500 ng to 1 μg per reaction) using a cDNA synthesis kit (Applied Biosystems, catalog 4374966). Real-time PCR was performed using a 7500 Real-Time PCR System and SYBR Green reagents (Applied Biosystems). Specific primer sets used were as follows: Abca1 forward CCAATGAAGAGATATGGG; Abca1 reverse CACTGGCTAGTGCGGT; Dnajb9 forward GATGGGGTTGATGAACTCCACC; Dnajb9 reverse CTGCACCTGCTGCGGAG. Commercially available human Abca1 reagents (Active Motif, catalog AM1751). Purity of RNA was estimated by measuring absorbance at 260 and 280 nm using a NanoDrop (ThermoScientific).

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on 4%-20% gradient SDS-polyacrylamide gels and transferred to 0.45-μm nitrocellulose membranes. Blots were blocked in Tris-buffered saline with 0.1% Tween-20 containing either 5% BSA or nonfat milk at room temperature for 1 hour. Membranes were then incubated overnight at 4°C with primary antibodies, which included those for ABCA1 (Novus Biologicals, catalog NB400-105, 1:1000), ATF6 (Novus Biologicals, catalog NB1-40256, 1:500), DACH1 (Proteintech, catalog 10914-1-AP, 1:1000), HDAC4 (Cell Signaling Technologies, catalog 2072, 1:1000), phospho-HDAC4 (Cell Signaling Technologies, catalog 3424S, 1:1000), LXRα (Abcam, catalog ab41902, 1:2000), nuleophosmin (Cell Signaling Technologies, catalog 3542, 1:1000), or β-actin (HRP conjugate, Cell Signaling Technologies, catalog 5125, 1:5000).

Transfection of siRNAs. Bone marrow-derived macrophages were plated at 0.5 × 10⁶ cells/well in a 24-well plate and allowed to adhere overnight. Commercially available siRNAs for CamKIIγ (QIAGEN), ATF6 (QIAGEN), and LXRα (QIAGEN) were used. siRNAs were prepared for transfection using Lipofectamine RNAiMax (Invitrogen, catalog 13778-150) per the manufacturer’s instructions. Cells were harvested or used for further experiments 36 to 72 hours after transfection as noted in figure legends.

LPS stimulation of bone marrow-derived macrophages. Control and CamKII-KO bone marrow-derived macrophages were plated at 0.2 × 10⁶ cells per well of a 24-well plate and allowed to adhere overnight. The following day, cells were exposed to medium containing 1 ng/ml LPS (Sigma-Aldrich, catalog L4391) or vehicle control. After incubating for 4 hours, cells were harvested for mRNA analysis.

In vitro effrocytosis. Bone marrow–derived macrophages were plated at 1 × 10⁶ cells/well in a 12-well plate and allowed to adhere overnight. In some experiments, cells were preincubated with KN92 (Calbiochem, catalog 422709) or KN93 (Calbiochem, catalog 422708). Jurkat cells were labeled with PKH67-GL (2 μM; Sigma-Aldrich) per the manufacturer’s instructions, resuspended in full medium at a density of 3 × 10⁶ cells/ml, and exposed to UV light (254 nm, 300 μW/cm²) for 5 minutes to induce apoptosis. After UV exposure, cells were incubated in a 37°C incubator with 5% CO₂ for 1 hour. We routinely obtained approximately 80% early ACs (annexin V+ PI−) using this procedure. Macrophage medium was replaced with medium containing Jurkat cells to achieve a cell ratio of 3:1 Jurkat cells/macrophages. After incubation for 45 minutes at 37°C, the medium was aspirated and the macrophages were washed twice with cold PBS. Images were obtained using a Nikon fluorescent microscope and analyzed using FIJI/ImageJ software.

Flow cytometry. Cells were suspended in FACS staining buffer (PBS containing 2% FBS) at an approximate density of 1 × 10⁶ cells/100 μl and preincubated with Fc block for 20 minutes on ice. Cells were then labeled with FITC- or PE-conjugated antibodies (eBioscience, catalog 11-4801-82) and biotinylated anti-MerTK (R&D Systems, catalog BA5951) or isotypic controls for 60 minutes on ice. Cells were washed and then incubated with secondary antibody directed at streptavidin for 45 minutes on ice. Cells were washed in FACS buffer and then resuspended for analysis on a BD FACS Canto II. Data were analyzed using FlowJo software.

Confocal microscopy for MerTK staining. Frozen sections were prepared as above. Sections were fixed in cold acetone for 5 minutes, washed in PBS, and then incubated in biotin-blocking buffer (Thermo Scientific, catalog E21390) for 1 hour. Sections were then incubated with biotinylated anti-MerTK (R&D Systems, catalog BA591) at 4°C overnight, followed by incubation with Alexa Fluor 488–conjugated streptavidin for 1 hour. Slides were mounted with DAPI-containing solution, viewed on a Nikon A1 confocal microscope, and analyzed using FIJI/ImageJ software.

ChIP. Confluent 10-cm dishes of bone marrow–derived macrophages (1 dish per experimental condition) from each genotype were prepared for use on days 7 to 10 of differentiation. Cells were washed with cold PBS containing protease inhibitors and then incubated with 2 mM disuccinimidyl glutarate in PBS for 30 minutes at room temperature. Parafomaldehyde (PFA) was added to a final concentration of 1% before incubating for an additional 15 minutes at room temperature. Crosslinking was terminated by incubating cells in 200 mM Tris-HCl pH 8 followed by 100 mM Tris-HCl pH 8, each for 15 minutes. Cells were then harvested in 1 ml of cold PBS containing protease inhibitors and pelleted by centrifugation at 2000 g for 5 minutes at 4°C. Pellets were resuspended in 1 ml of nuclei isolation buffer (50 mM Tris-HCl pH 8, 60 mM KCl, 0.5% NP-40, 1x protease inhibitors) and incubated on ice for 10 minutes. Samples were again centrifuged at 2000 g for 5 minutes at 4°C, after which pellets were resuspended in 130 μl lysis buffer (50 mM Tris-HCl pH 8, 0.5% SDS, 10 mM EDTA pH 8, 0.5 mM EGTA pH 8) and transferred to Covaris microtubes (catalog 520045). DNA was sheared to an average size of 400-500 bps using a Covaris S2 focused ultrasonicator set at duty 5%, intensity 4, cycles 200, 15 seconds for 12 total repetitions. After sonication, samples were centrifuged at maximum speed for 10 minutes at 4°C and supernatants were harvested to fresh tubes.

Fragmented chromatin (25 μl) was removed for use as input. These samples were treated with elution buffer (0.1 M NaHCO₃, 1% SDS, 0.2 M NaCl, and 0.25 μg/μl RNase A) and incubated for 30 minutes at room temperature to digest RNA, followed by incubation at 65°C for 4 hours to reverse protein crosslinks. Samples were then treated with proteinase K in 100 μl EDTA/6.5 mM Tris-HCl pH 8 for 1 hour at 45°C. The resulting products were purified using a QIAquick PCR Purification Kit (QIAGEN, catalog 28106) and quantitated using a NanoDrop spectrophotometer. For each sample, 200 ng was run on an agarose gel to ensure that even and adequate sonication of chromatin was obtained. The remaining 105 μl of the sheared chromatin sample was diluted to 200 μl in lysis buffer and then further diluted to a final volume of 1 ml in dilution buffer (1% Triton X-100, 2 mM EDTA pH 8, 150 mM NaCl, 20 mM Tris-HCl pH 8, 1x protease inhibitors). These samples were then precleared by incubating with 0.25 μg of normal mouse IgG per 1 μg of chromatin for 1 hour with end-over-end rotation at 4°C, followed by the addition of 40 μl of prewashed magnetic beads (Dynabeads, Invitrogen, catalog 10004D) for an additional hour. Using a magnetic rack to pellet the beads, the supernatant from each sample was removed and divided into two 500-μl aliquots. Each aliquot was brought to a final volume of 1 ml with dilution buffer, and then 1 μg of either normal mouse IgG or mouse anti-ATF6 antibody (Novus Biologicals, catalog NB1-40256) was added to each 5 μl of chromatin. Samples were rotated end-over-end overnight at 4°C, then centrifuged at maximum speed for 10 minutes at 4°C. Supernatants were transferred to fresh tubes, 40 μl of beads was added, and samples were then incubated for 2 hours with rotation at 4°C. After immunoprecipitation, beads were washed once for 5 minutes with wash buffer no. 1 (20 mM Tris-HCl pH 7, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8), then once with wash buffer no. 2 (10 mM Tris-HCl pH 7, 250 mM LiCl, 1% NP-40, 0.7% sodium deoxycholate, 1 mM EDTA pH 8), and then finally with Tris-EDTA buffer pH 8. After the final wash,
samples were treated with elution buffer containing RNase A and then proteinase K, and the samples were purified for PCR as described above. The presence of a Nrfl3 (LXRα) intronic region in the anti-ATF6 precipitated samples was quantified by qPCR and expressed relative to the input genomic DNA (Nrfl3’s specific primers: forward ATCTGGGGCGG- GGTAGAGTT, reverse GGCGAACACAAAGACTGGA). Primers to a region of the Nrfl3 gene that does not contain the consensus ATF6 site were used to confirm that no nonspecific binding of the ATF6 antibody was obtained (nonspecific Nrfl3 primers: forward GGCTAGGGG- GAATGAGATGC, reverse TGGCTCCAATAGGAGCACA).

Viral transduction. Bone marrow–derived macrophages were plated at 0.2 × 10^6 cells/well in a 24-well plate and allowed to adhere overnight. High-titer (5 × 10^7 pfu/ml) adenovirus containing either CA-CaMKII or LacZ was produced by Viraguest Inc. based on previously described vectors (15, 32) that were a gift of Harold Singer (Albany Medical Center). Macrophages were transduced with 200 MOI of LacZ or CA-CaMKII virus and harvested for mRNA or protein 60 hours later.

Statistics. All results are represented as mean ± SEM. Normality was determined using D’Agostino-Pearson and/or Shapiro-Wilk normality testing. P values for normally distributed data were calculated using either Student’s t test or 2-way ANOVA with post hoc Tukey’s analysis. P values for nonnormally distributed data were calculated using the Mann-Whitney U test. For the data in Figure 1B as well as Supplemental Figures 1, A and B, correlation coefficients (r) and P values were calculated using Pearson product-moment correlation analysis.

Study approval. For human studies, carotid endarterectomy samples were obtained from coauthor BD. Coronary artery samples were obtained from coauthor JCS. The specimens used in the PIT versus TCFA coronary plaque study were obtained at autopsy, with prior consent and in agreement with the code for proper secondary use of human tissue in the Netherlands (http://www.fmwv.nl). All samples were obtained with consent from patients. Use of all material conformed to the Declaration of Helsinki and was approved by the appropriate university ethics review boards. For animal studies, all mice were cared for according to NIH and IACUC guidelines in a barrier facility at Columbia University Medical Center, and procedures involving mice were performed with the approval of the Columbia University Medical Center IACUC.

Author contributions
ACD, LO, GF, and IT conceived and designed the research. BD and JCS donated patient samples and were instrumental in the interpretation of the human data. ACD, LO, BC, ZZ, CCR, GJ, and GF conducted the experiments. ACD, LO, BC, ZZ, GF, JH, ART, and IT analyzed the data. ACD and IT wrote the paper.

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