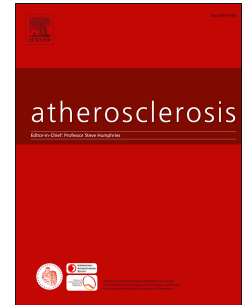# Accepted Manuscript

Cellular subtype expression and activation of CaMKII regulate the fate of atherosclerotic plaque

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CELLULAR SUBTYPE EXPRESSION AND ACTIVATION OF CAMKII REGULATE THE FATE OF ATHEROSCLEROTIC PLAQUE

Cellular subtype expression and activation of CaMKII regulate the fate of atherosclerotic plaque

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Key Words: Atherosclerosis, CaMKII, VSMCs, Macrophages, Stable plaques, Unstable plaques.

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Abstract

Background and aims: Atherosclerosis is a degenerative process of the arterial wall implicating activation of macrophages and proliferation of vascular smooth muscle cells. Calcium-calmodulin dependent kinase type II (CaMKII) in vascular smooth muscle cells (VSMCs) regulates proliferation, while in macrophages, this kinase governs diapedesis, infiltration and release of extracellular matrix enzymes. We aimed at understanding the possible role of CaMKII in atherosclerosis plaques to regulate plaque evolution towards stability or instability.

Methods: Clinically defined stable and unstable plaques obtained from patients undergoing carotid end arteriectomy were processed for evaluation of CaMKs protein expression, activity and localization.

Results: The larger content of CaMKII was found in CD14$^+$myeloid cells that were more abundant in unstable rather than stable plaques. To test the biological effect of activated CD14$^+$myeloid cells, VSMCs were exposed to the conditioned medium (CM) of macrophages extracted from carotid plaques. CM induced attenuation of CaMKs expression and activity in VSMCs, leading to the reduction of VSMCs proliferation. This appears to be due to the CaMKII dependent release of cytokines

Conclusions: These results indicate a pivotal role of CaMKs in atherosclerosis by regulating activated myeloid cells on VSMCs activity. CaMKII could represent a possible target for therapeutic strategies based on macrophages specific inhibition for the stabilization of arteriosclerotic lesions.

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Introduction

Atherosclerosis is a degenerative process of the vascular wall leading to clinical manifestations such as stroke and myocardial infarction[1]. The underlying mechanism results from the altered equilibrium in the cellular content of the vascular wall. Indeed, vessel walls harbour vascular smooth muscle cells (VSMCs), fibroblasts, endothelial cells (ECs), whereas in atherosclerotic vessels, inflammatory cells such as activated macrophages of different subtypes, neutrophils and lymphocytes, are also present[2].

Plaque stability is a concept deriving from the clinical evidence that some atherosclerotic plaques are more often associated with cardiovascular events. Histology differences between stable (SP) and unstable (UnSP) plaques show different cellular content with a more relevant inflammatory cell component in UnSPs.

Ca^{2+} is involved in atherosclerosis at different extent. It is well recognized that inflammatory macrophages co-localize with Ca^{2+}phosphate deposits in developing atherosclerotic lesions and can promote calcification. Pathological calcification is not merely a passive consequence of chronic inflammatory disease, but it may lead to a positive feedback of calcification and inflammation driving disease progression.

The equilibrium of all cellular populations present in the vascular wall is tuned by calcium (Ca^{2+}), which regulates both acute and adaptive responses. Moreover, basic Ca^{2+} phosphate deposition underlies the development of arterial calcification, a frequent component of atherosclerosis, which predicts coronary artery disease events[3]. Ca^{2+} signalling surges and promotes the generation of reactive oxygen species (ROS) in VSMCs and ECs[2], thus maintaining chronic inflammation and release of pro-inflammatory cytokines[2].

Ca^{2+} signalling plays an important role in the regulation of cell proliferation through the crosstalk of multifunctional calcium-calmodulin dependent kinases (CaMKs) and a number of other signalling

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pathways, such as extracellular regulated kinase (ERK)[4]. CaMKs have never been taken into account while looking at mechanisms of atherosclerosis, but our recent studies demonstrate that a murine model lacking calcium-calmodulin dependent kinase IV (CaMKIV) presents early onset arteriosclerosis[5], as well as increased calcium-calmodulin dependent kinase II (CaMKII) activity in selected cell types, thus suggesting a role for Ca^{2+}-CaMKII signalling in this process[4].

CaMKs regulate biological processes that are relevant to atherosclerosis, by affecting the biology of vascular wall cells and infiltrating macrophages. In particular, CaMKII plays a critical role in blood pressure regulation through the control of endothelial function[6] such as proliferation of VSMCs and ECs[7-9]. CaMKs actively regulate chemiotaxis, diapedesis, chemokine production in macrophages[10]. Indeed, CaMKII has an important role in the modulation of immune responses, such as T-cell activation[11],survival[10], maturation and antigen presentation of dendritic cells[12]. Several lines of evidence link CaMKII activation with mechanisms that sustain plaque formation. In particular, oxidized CaMKII may be responsible for the increased risk of death among patients with diabetes, following a heart attack[13]. Furthermore, CaMKII activity results in the suppression of angiotensin-converting enzyme shedding in endothelial cells, suggesting its possible correlation with blood pressure control[14]. Therefore, CaMKs are potentially involved, at several levels, in the mechanisms of the atherosclerotic lesion[15]. Considering the complex nature of atherosclerosis, our hypothesis is that CaMKII participates in the development of the disease at different levels, and we found its involvement in macrophages and VSMCs activity, through which it regulates plaque instability.

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Materials and methods

Patient classification

This study included a total of 54 consecutive patients (28M/26F, 62±9 years) undergoing carotid endarterectomy for occlusive artery disease in the Surgery Unit of the "Federico II" University of Naples. Carotid atherosclerosis was assessed by carotid ultrasonography. The patients selected for surgery had either a high-grade stenosis (>70%) or an ulcerated lesion of a medium grade based on echo-Doppler analysis[16]. The carotid atherosclerotic plaques were classified as complex lesion or "unstable" (UnSP) and noncomplex or "stable" (SP). UnSP showed one or more of the following features: ulceration, irregular surface, presence of mobile thrombi on the plaque surface or intraluminal thrombus, predominant echolucency (hypoechoic plaques), and heterogeneity with substantial intraplaque-echolucent areas. SP were required to have all of the following features: smooth and regular surface and homogeneity and uniform echogeneity or dominant echogeneity with small areas of echolucency. All ultrasonographic examinations were performed by experienced ultrasonographers blinded to clinical and angiographic findings.

The investigations were approved by the Ethical Committee of "Federico II" University of Naples. Written informed consent was obtained from all participants according to the declaration of Helsinki.

Cell culture, conditioned media and reagents

CD14$^+$ myeloid cells (i.e. monocytes and macrophages) were isolated from human atherosclerotic plaques using magnetic beads bound to anti-CD14 antibody (Invitrogen, Carlsbad, CA, USA), as previously described[17]. Alternatively, monocytes were purified from buffy coats of healthy donors obtained from the Leukapheresis Unit of "Federico II" University of Naples, as explained above[18].

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Conditioned media was collected from cultured monocytes, stimulated with 10 ng/mL of lipopolysaccharide (LPS) for 24 hours[19]. The CAMKII selective peptide inhibitor tat-CN17β[20] (5 µMoL/L for 30 min) was used to block CaMKII activity, as previously described[20]

Human VSMCs were purchased from ATCC-Tech LGC Standard UK.

Western blot analysis

Human atherosclerotic plaques and VSMCs were lysed in ice-cold RIPA-SDS buffer. Equal amounts of total cellular extracts were subjected to Western blot (WB) analysis, as previously described and validated[21].

CaMKII activity assay

Total extract from atherosclerotic plaques or VSMCs was immunoprecipitated for CaMKII[21]. Purified CaMKII was then subjected to reaction with the CaMKII substrate autocamtide in the presence of $[^{32}P]$-γATP (GE-Amersham, Piscataway, NJ)[4,22,23].

Cell proliferation and $[^3H]$-thymidine incorporation

Serum starved VSMCs (50,000 cells/well) were treated with cell-free supernatants of cultured CD14^{+} myeloid cells. After 24 and 48 h, cells were detached from the plates, and counted on haemocytometer[4]. To determine DNA synthesis, cells were treated, as described previously, in presence of 0.5µCi $[^3H]$-thymidine (Amersham, Buckinghamshire)[24].

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Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

After reverse transcription reaction from SP and UnSP, real time quantitative polymerase chain reaction (RT-PCR) was performed with SYBR Green Real Time PCR master mix kit (Applied Biosystems, Foster City, CA,USA), as previously described[21].

Immunofluorescence and histology

Plaques obtained from human carotid endarteriectomy were fixed in formalin, embedded in paraffin and sectioned at 5 µm with a rotary microtome. Sections were incubated with anti-CD68, CaMKII, phospho-CaMKII, α-actin (Santa Cruz, Dallas, Texas, USA) primary antibodies at room temperature and subsequently exposed to specific fluorescent labeled secondary antibodies (Alexa Fluor, Invitrogen, Carlsbad, CA, USA). Images were taken with an Eclipse E1000 Fluorescence Microscope (Nikon, Chiyoda, Tokyo, Japan) and acquired with Sigma Scan Pro software (Jandel). Images were digitalized and the relative staining areas were quantified by NIH Image (ImageJ). Data are expressed as CD68 to α-actin positive cell ratio. Paraffin-embedded sections of stable and unstable plaques were stained with Masson trichrome, as explained above[25].

Cytokines quantification

CD14$^+$ myeloid cell conditioned media were screened for the concentration of interleukin (IL)-1β, IL-6, IL-10, IL-12 (p70), tumor necrosis factor alpha(TNF-α), vascular endothelial growth factor (VEGF), soluble CD40 ligand (sCD40L), matrix metalloproteinase 9 (MMP-9). Measurements were performed with the Bio-Plex human cytokine kit from BioRad (BioRad, Hercules, CA, USA), according to the manufacturer's protocol.

Statistical analysis

All values are presented as mean+SEM. One-way and two-way ANOVA were performed as appropriated to compare the different parameters with controls or among the different groups. A significance level of $p<0.05$ was assumed for all statistical evaluations. Statistics were computed with GraphPad Prism software (San Diego, CA).

Extended details of experimental procedures are provided in Supplementary materials.

Results

CaMKs in atherosclerotic plaques

To evaluate CaMKs expression in human atherosclerotic plaques, we performed Western blot analysis on protein extracts from 10 UnSP and 10 SP; a significant reduction of CaMKII expression levels was observed in UnSP when compared with SP (Fig. 1A upper panel and Fig. 1B). Interestingly, UnSP are characterized by significant increase of CaMKIV protein levels when compared with SP (Fig. 1A middle panel and Fig. 1C). Quantitative RT-PCR on 6 UnSP and 6SP confirmed that UnSP presented lower CaMKII and higher CaMKIV mRNA levels than SP (Fig. 1D).

To define CaMKII activity in human atherosclerotic plaques, we observed that CaMKII phosphorylation levels were enhanced in SP compared to the unstable ones (Fig.1E and F). Subsequently, *in vitro* ^{32}P kinase assay with endogenous CaMKII indicated that kinase activity was more active in human SP compared to UnSP (Fig.1G).

Cell type and CaMKII content in atherosclerotic plaques

VSMCs produce the connective tissue to maintain the structural strength of the plaque[26], and a decreased number of VSMCs favors plaque rupture and thrombus formation[27]. Therefore, the tendency of the plaques to stability or instability depends on the predominant cellular population (VSMCs or myeloid cell).

Immunofluorescence studies performed on human SP (Fig. 2A) and UnSP (Fig. 2B) indicate that CaMKII was expressed mainly in F4/80 positive cells, while this kinase showed a very low expression in VSMCs (α-actin positive cells). Moreover, UnSPs were characterized by lower prevalence of VSMC compared to the CD68 positive cells population in contrast to what observed in SP (Fig. 2C). Finally, Masson trichrome staining, performed in SP and UnSP to identify

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cellularity (red) and the fibrous content (blue), showed that cellular infiltrates in UnSP (Fig. 2D, left panel) were irregularly distributed within the collagen matrix, which was less abundant. On the other hand, SP (Fig.2D, right panel) were characterized by an elongated shape and regular distribution alternated to collagen distribution.

Monocytes and macrophages reduce VSMCs proliferation

The inverse proportion of CD68 positive cells and VSMCs in SP and UnSP suggests that myeloid cells might interfere with VSMCs biology. To assess this, we tested medium conditioned by CD14$^+$ myeloid cells (prevalently monocytes and macrophages) extracted from 10 UnSPs (CM), to identify the soluble factors released by human plaque macrophages (Fig.3A). As expected, IL6 was the most abundant factor released by CD14$^+$ myeloid cells from these plaques (Fig. 4A). Next, we assessed the effect of CM on VSMCs proliferation and [^{3}H] thymidine uptake. We found that treatment with CM caused a significant reduction of cellular proliferation at both 24 and 48 hours compared to control medium (Ctr) (Fig.3B). Furthermore, CM also decreased [^{3}H] thymidine incorporation by VSMCs (Fig.3C). These results suggest that soluble factors released by CD14$^+$ myeloid cells reduce VSMCs proliferation.

The activation of CD14$^+$ myeloid cellsreduces CaMKs activity in VSMCs

To assess whether CM is able to impair CaMKII expression and activation in VSMCs, we treated these cells with CM of CD14$^+$ myeloid cells extracted from UnSP, for 24 h. We found that CM did not induce a difference of CaMKII expression levels (Fig.4A upper panel, and Fig.4B) but caused an important reduction of CaMKII phosphorylation (Fig.4A middle panel, and Fig.4C) and activity (Fig.4D). Interestingly, CM induced a significant increase of CaMKIV expression levels (Fig.4A lower panel, and Fig.4E). These data demonstrate also *in vitro* that soluble factors released by

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CD14$^+$ myeloid cells are able to impair CaMKII activity and induce CaMKIV expression in humans VSMCs.

CaMKII inhibition prevents CD14$^+$ myeloid cells activation

CD14$^+$ myeloid cells appear to negatively regulate VSMCs proliferation by inducing CaMKII inhibition. Since we showed that CaMKII is abundantly expressed also in plaques CD68 positive cells, we tested the effect of CaMKII inhibition on activated macrophages to release soluble factors in the extracellular media. Human CD14$^+$myeloid cells purified from buffy coats of healthy donors were treated with LPS in the presence or absence of the CaMKII selective peptide inhibitor tat-CN17β[20]. We observed that CaMKII inhibitor significantly reduced the release of IL6, TNFβ and IFNγ[28] in the cultured media by LPS stimulated macrophages (Fig.5A). Then we tested the effect of CaMKII inhibition in macrophages on VSMCs growth. CM of CD14$^+$ myeloid cells activated with LPS induced the inhibition of VSMC proliferation, as tested by cell count (Fig.5B) and [^{3}H] thymidine incorporation (Fig.5C), which was prevented by pretreatment with the CaMKII selective inhibitor tat-CN17β (Fig.5C and D). These results demonstrate that CaMKII activity is an essential component in the control of the release of cytokines regulating VSMCs proliferative responses by macrophages.

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Discussion

The main finding of our report is that CaMKII is an important checkpoint in the fate of human atherosclerotic plaques. First, we showed that CaMKII is highly expressed in arteriosclerotic plaques, in particular, we found that CaMKII is more expressed in SP than UnSP. Second, we observed that UnSP are characterized by increased expression of CaMKIV. Nevertheless, this difference was not enough to explain the complexity of the pathophysiology of two different lesions.

Third, we observed that SP and UnSP express different ratio of colonizing macrophages over resident VSMCs since a prevalence of this latter is observed in unstable plaques. This observation must be taken into consideration when comparing total expression and activity of CaMKII between the two lesion types.

The prevalence of one cell type over the other regulates the fate of the arteriosclerotic plaque towards stability or unstability. The accumulation of macrophages in the vascular wall produces two events that facilitate plaque instability and rupture, reduced VSMCs proliferation and matrix degradation[29,30]. Both events are depending upon the release of cytokines[29]. Plaques with a poor VSMC content and elevated infiltration of macrophages are more prone to rupture than plaques rich in proliferating VSMCs, which will be more stable.

In both VSMCs and plaque infiltrating macrophages, the pathophysiological role of CaMKII is extremely relevant. Indeed, we describe that CaMKII is increased in macrophages within the unstable plaque. This finding is in agreement with previous data showing the pivotal role of CaMKII in activating macrophages[14]. On the contrary, in SP, poor in infiltrating macrophages, VSMCs remain the most relevant reservoir of the kinase. In these cells, CaMKII activation leads to cellular proliferation. Our report places in perspective the opposite role of CaMKII in macrophages and VSMCs. We show that in CD14^{+} cells, CaMKII activation produces cytokines that inhibit CaMKII activity and cell proliferation in VSMCs. This finding is paralleled by increased CaMKIV

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expression. Taken together, these findings confirm that CaMKII and CaMKIV are counter-regulated in proliferative cells[31]. The evidence that CM of activated macrophages inhibit VSMCs proliferation supports the central role of macrophage derived cytokines. Our results in human macrophages are well in agreement with this vision. In this model, we recapitulated the inhibitory effects of macro-CM on VSMCs' CaMKII activation and cell proliferation. Moreover, we proved that macrophages' release of cytokines is CaMKII dependent since it can be prevented by the concomitant exposure of these cells to the CaMKII selective inhibitor tat-CN17β. Our data allow depicting a pathophysiological scenario of atherosclerosis, in which the abundance of CaMKII in infiltrating CD14^{+} cells leads to the humoral inhibition of VSMCs proliferation and plaque instability. On the contrary, in stable plaque, VSMCs overexpress CaMKII, which induces VSMCs proliferation, favoring plaque stability (Fig.5D).

Our investigation leaves the nature of CD14^{+} cells, extracted from the plaques, unexplored. Indeed, different macrophages subtypes as well as neutrophils and lymphocytes have been described in the plaques[32]. Nevertheless, macrophages remain the most abundant cell type expressed in the plaques and, most likely, our cultured CD14^{+} cells containing mostly macrophages[33]. The relative role of these different immune cell types in atherosclerotic plaques remains still to be elucidate and steps beyond the aim of the present investigation.

In summary, our data demonstrate that in atherosclerotic lesions, the crosstalk between macrophages and VSMCs is regulated by CaMKII and, above all, indicate that cell-type specific inhibition through small molecule can potentially modify the fate of cellular components of the plaque and its morphological features and evolution to rupture. Our data pose the bases of new therapeutic strategies based on cell type specific inhibition of CaMKII, allowing stabilization of atherosclerotic lesions. This strategy has the potential to modify the clinical outcome of vascular events of those conditions that promote macrophages infiltration in the vascular wall, such as diabetes or obesity.

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Cardiovascular diseases represent a leading cause of morbidity and mortality, with an ever increasing burden on health care systems. Acute events recognize atherosclerosis as the common pathogenesis. There are many pieces of evidence in the literature about the mechanisms and processes involved in atherosclerosis, the leading cause of death in the industrialized world. It has recently been demonstrated that CaMKs regulate biological processes relevant to the process of arteriosclerosis, by affecting the biology of vascular wall cells and infiltration[2], and this is the first time that CaMKs are taken into account. The characterization of the role of the CaMKs in the pathogenesis of arteriosclerosis, especially in the inflammatory and proliferative events determining its evolution, contributes to the understanding of the molecular mechanism underpinning atherosclerotic plaque progression, and brings the potential to innovative therapeutic strategies.

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Figure Legends

Fig. 1. CaMKs in atherosclerotic plaques. (A) To evaluate the expression of CaMKs, total cell lysates from human SPs and UnSPs were analyzed, with specific antibodies, by Western blotting for total CaMKII, CaMKIV and phosphothreonine 286 CaMKII (PCaMKII) . Immunoblot for actin is included to show total protein load. Images are representative of three separate experiments.

Densitometric analysis from immunoblot quantified (B) CaMKII and (C) CaMKIV levels corrected for actin densitometry. *$p<0.05$ *vs.* SP. (D) Total RNA was isolated from human atherosclerotic plaques using TRIzol reagent, and cDNA was synthesized by means of a Thermo-Script RT-PCR System, following the manufacturer's instruction. Gene expression for CaMKII and CaMKIV was evaluated by RT-PCR. The graphs indicate the relative amounts of transcripts for CaMKs in SP and UnSP. Cycle threshold (Ct) values from 3 independent experiments (n=6/group for A, n=10/group for B) were normalized to the internal β-actin control. The ratio of fold change was calculated using the Pfaffl method. *$p<0.05$ SP *vs.* UnSP. (E) Densitometric analysis from immunoblot quantified phosphothreonine 286 CaMKII (P-CaMKII) levels corrected for actin densitometry. * $p<0.05$ *vs.* SP. (F) CamKII activity assay was performed using endogenous kinase, obtained from SPs and UnSPs, which was immunoprecipitated using anti-CaMKII antibody and incubated with its substrate autocamtide in a reaction mixture containing [γ^{32}P]-ATP. Quantification of incorporated radioactivity was performed by liquid scintillation. (G) The results are presented as total incorporation (CPM, count per min.). Each data point in all graphs represents the mean ±SEM of 3 independent experiments.

Fig.2. Cellular localization of CaMKII in atherosclerotic plaques. Section of SP and UnSP were double-stained with anti-CaMKII, anti-CD68 (to identify macrophages) and anti-α-actin (to recognize VSMCs) antibodies. Specific fluorescent labeled secondary antibodies were used and images were taken as described in Materials and methods. (A and B) Representative
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immunofluorescence images from (A) Sp and (B) UnSP show CaMKII (red), macrophages (green), actin (blue). Immunofluorescence images from SP and UnSP were digitalized and the relative staining areas were quantified by NIH Image (ImageJ). Quantification of the relative cellular component was performed by counting the number of CD68 or α-actin positive cells in 5 observation fields at 100X amplification. Data are expressed as CD68 to α-actin positive cell ratio. (D) Paraffin-blocked tissues from SPs and UnSPs, sectioned and stained with Masson's trichrome, showed a decrease of interstitial fibrosis and reduced cellularity (red cells) in UnSP. Images are representative of 3 independent experiments (magnification 60X; black bar = 100 μm).

Fig.3. Macrophages reduce VSMCs proliferation. (A) CD14$^+$ myeloid cells (i.e. monocytes and macrophages) isolated from UnSPs were cultured to obtain conditioned medium (CM). To quantify the concentration of factors released in the supernatant of cultured CD14$^+$ myeloid cells, Bio-Rad Bio-Plex Pro™ Human Cytokine Magnetic Bead Panels were used, according to manufacturer's instruction. (B) 5x10^4 VSMC were plated in 6-well plates, serum starved overnight, and cultured with CM. After 24 and 48 h, cells were harvested and counted by hemocytometer. *$p<0.05$ SP *vs.* UnSP. (C) Serum starved 5x10^4 VSMC, plated in 6-well plates, were cultured in CM in the presence of 0.5 μCi/mL [^{3}H]-thymidine. After 24 h, the rate of [^{3}H] thymidine incorporation was determined by β-counter. Values of [^{3}H] thymidine are presented as total incorporation (CPM, counts per min.). * $p<0.05$ SP *vs.* UnSP. Each data point in all graphs represents the mean±SEM of 3 independent experiments.

Fig.4. The activation of CD14$^+$ myeloid cells reduces CaMKs activity in VSMCs. VSMCs were cultured for 24 h in conditioned medium (CM) obtained by cultured CD14$^+$ myeloid cells isolated by UnSPs. (A) Whole extracts underwent Western blotting to visualize phosphothreonine 286

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CaMKII (PCaMKII), total CaMKII and CaMKIV. Immunoblot for actin is included to show total protein load. Densitometric analysis from immunoblot quantified (B) PCaMKII, (C) CaMKII and (D) CaMKIV levels corrected for actin densitometry. * $p<0.05$ *vs.* SP. (E) Total cell lysates were immunoprecipitated using anti-CamKII antibody and samples were subjected to CaMKII kinase assay as indicated in the Materials and methods. The results are presented as total incorporation (CPM, counts per min.). *$p<0.05$ VSMC- Ctr *vs.* VSMC-CM. Images are representative of three separate experiments. Each data point in all graphs represents the mean±SEM of 3 independent experiments. * $p<0.05$ SP *vs*. UnSP.

Fig.5. CaMKII inhibition prevents CD14^{+} myeloid cells activation. Human CD14^{+} myeloid cells (monocytes and macrophages) purified from buffy coats of healthy donors were pre-treated with CaMKII selective inhibitor tat-CN17β (5 μMoL/L for 30 min.) and stimulated with LPS (10 nMoL/L) for 24 h to obtain the conditioned medium. (A) Bio-Rad Bio-Plex Pro™ Human Cytokine Magnetic Bead Panels were used to quantify the cytokines concentration released in the medium by human CD14^{+} myeloid cells treated with LPS, in the presence or absence of tat-CN17β. * $p<0.05$ *vs.* VSMC-Ctr. (B) 5×10^4 VSMC were plated in 6-well plates, serum starved overnight, and cultured with supernatant of human CD14^{+} myeloid cells treated with LPS, in the presence or absence of tat-CN17β. After 24 and 48 h, cells were harvested and counted by hemocytometer. *$p<0.05$ *vs*. VSMC-Ctr. (C) Serum starved 5×10^4 VSMC, plated in 6-well plates, were cultured in CM in the presence of 0.5 μCi/mL [³H]-thymidine. After 24 h, the rate of [³H]-thymidine incorporation was determined by β-counter. Values of [³H] thymidine are presented as total incorporation (CPM, counts per min.). * $p<0.05$ *vs*. VSMC-Ctr. Each data point in all graphs represents the mean±SEM of 3 independent experiments. (D)A new model of CaMKII function in atherosclerotic plaques is proposed. In UnsP, CaMKII activation of infiltrating CD14^{+} myeloid cells induces the release of cytokines that inhibit CaMKII activation in VSMCs, resulting in CaMKIV

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up-regulation and inhibition of proliferation. In SP, VSMCs represent the major source of active CaMKII, and this kinase favors the proliferative events of this cell type.
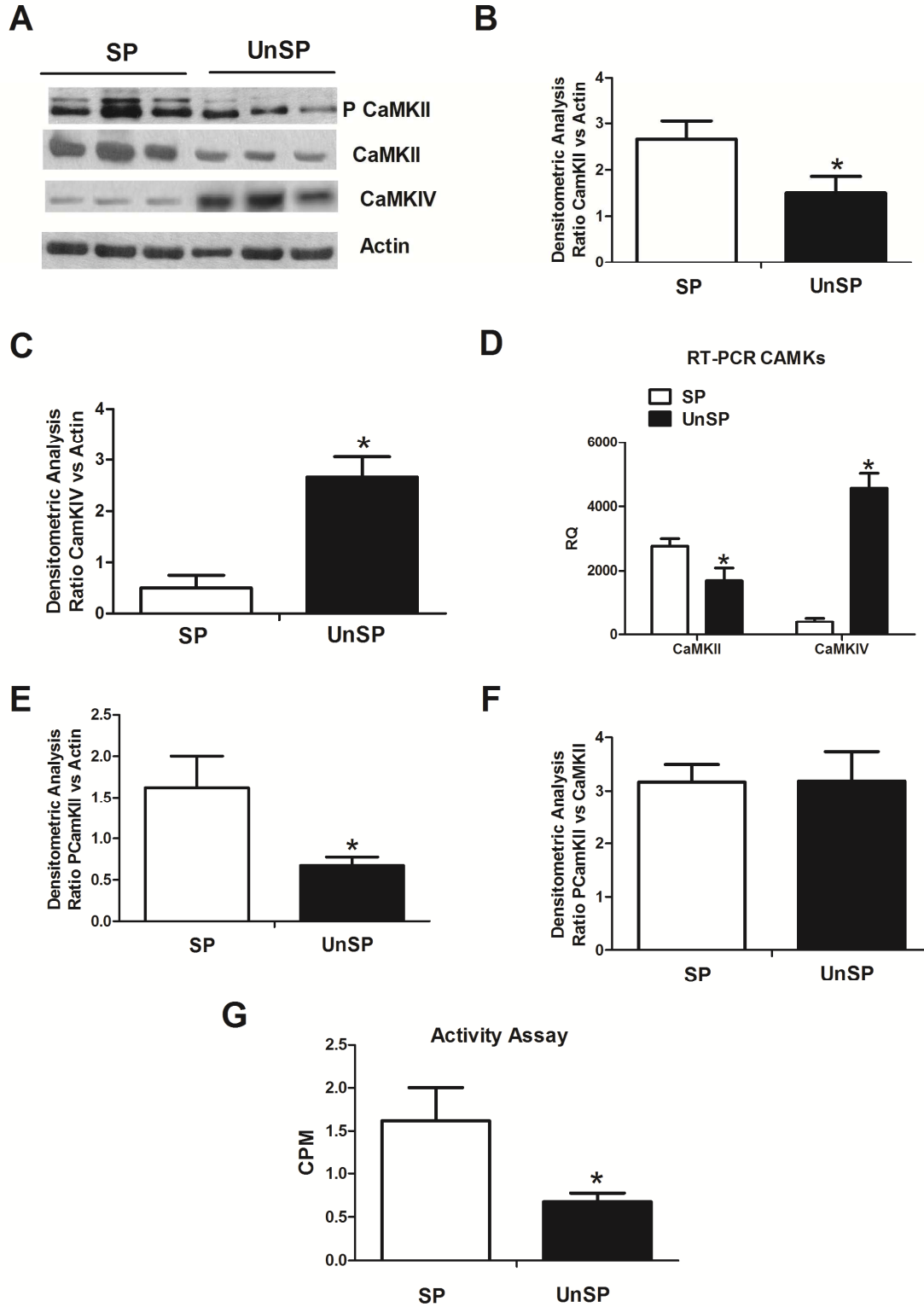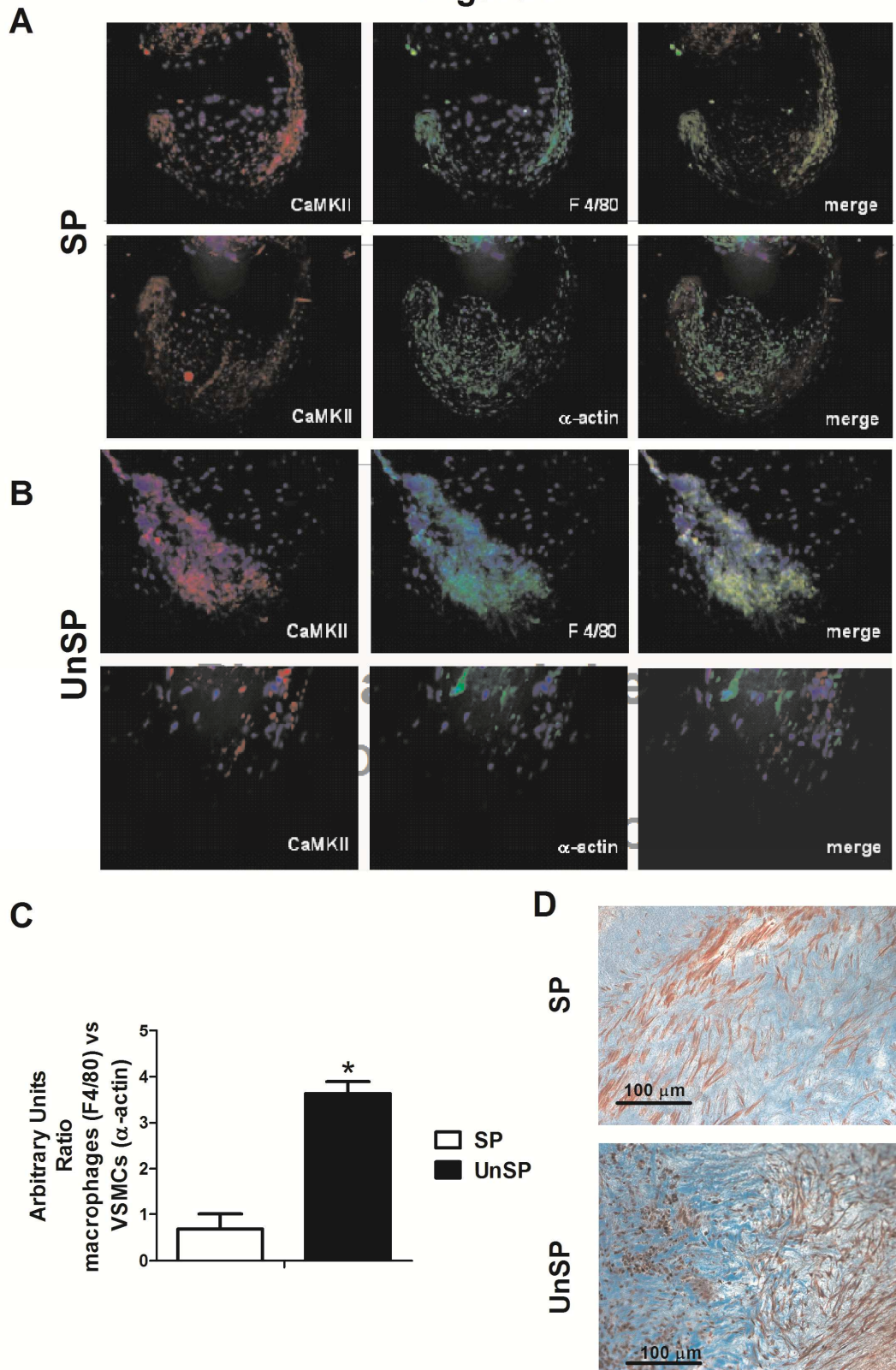
Figure 1

Figure 2

Figure 2

A

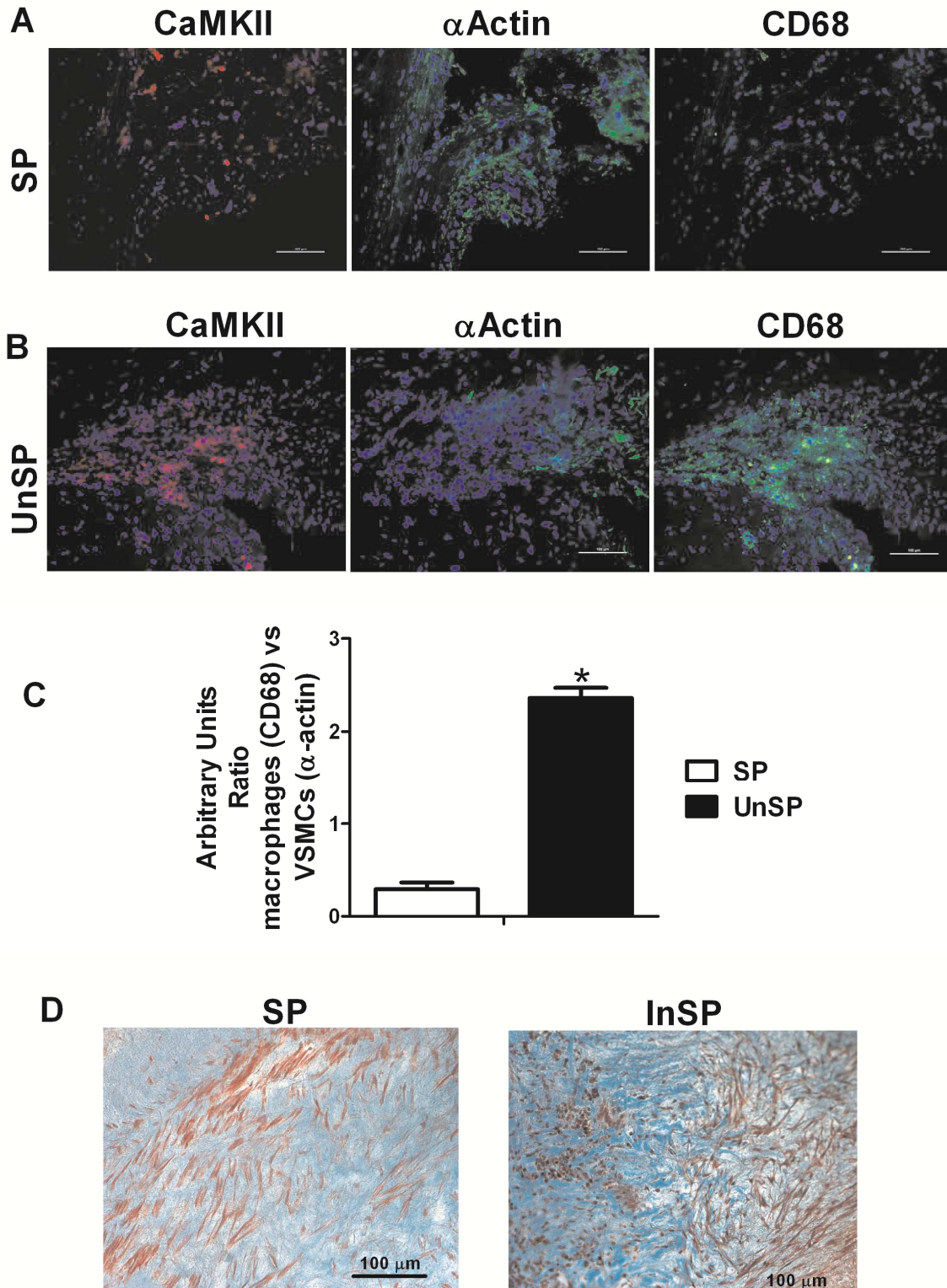CaMKII αActin CD68

SP

B

CaMKII αActin CD68

UnSP

C

□ SP
■ UnSP

D

SP InSP

Figure 3

A

B

C
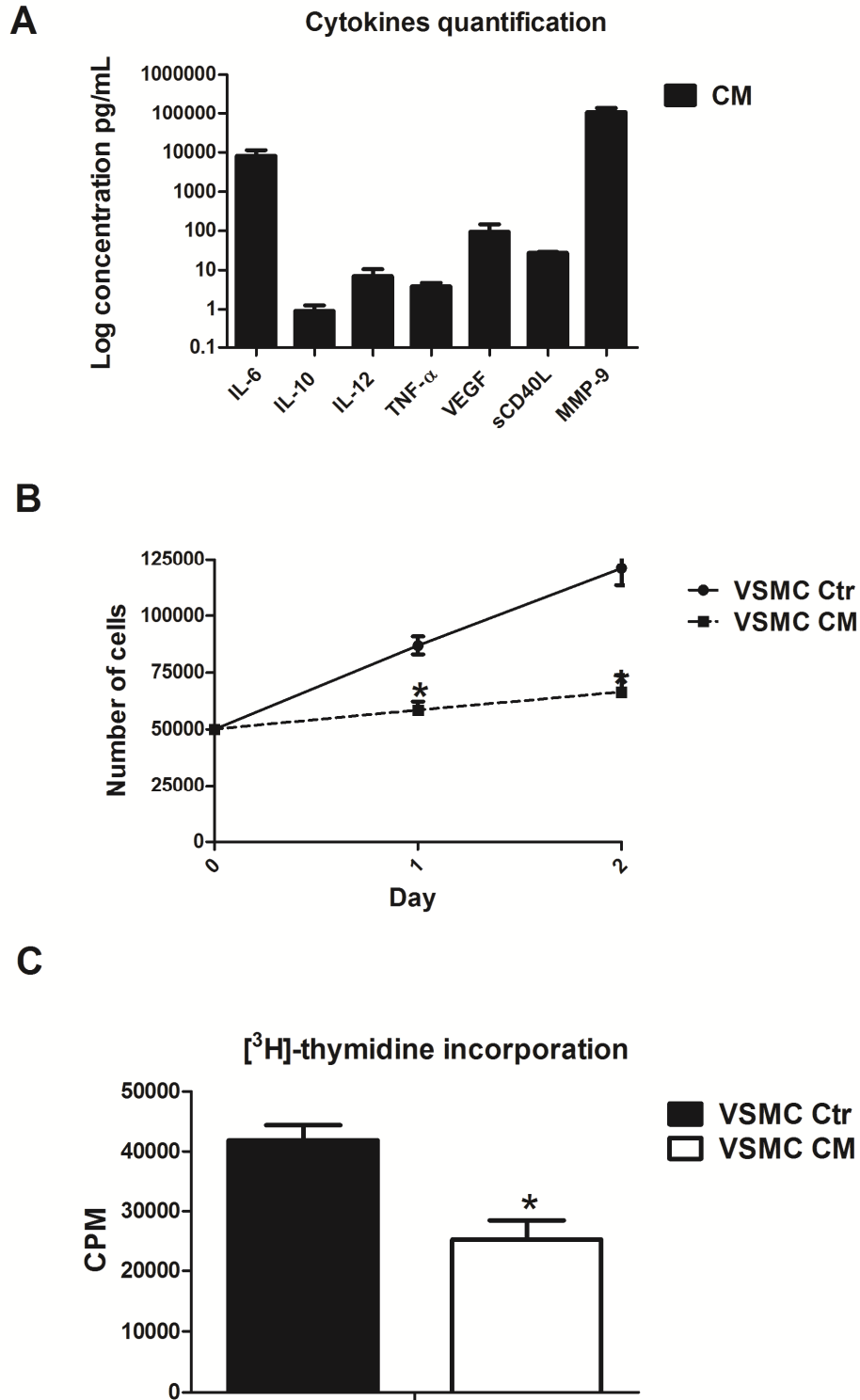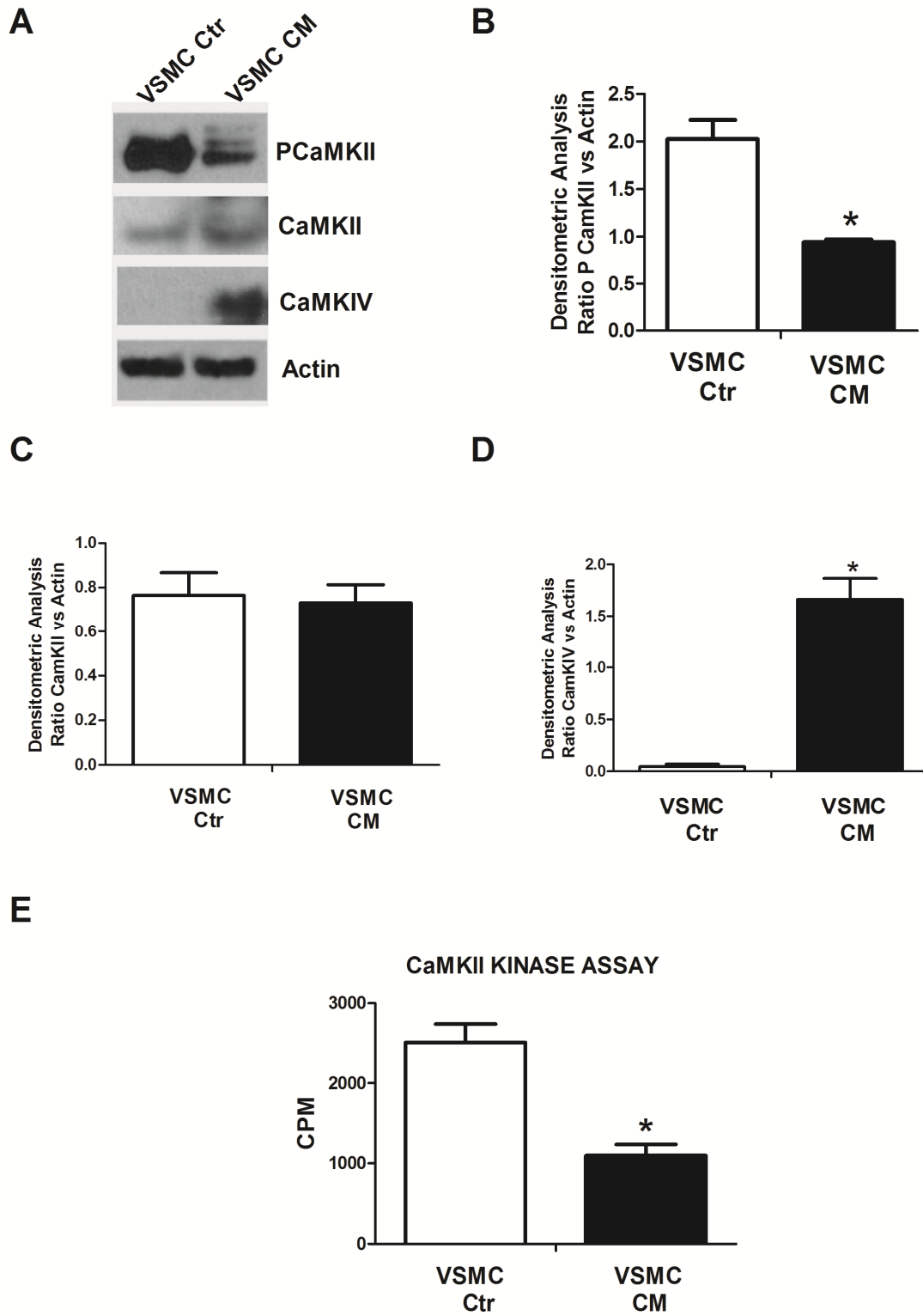
Figure 4

Figure 5

CELLULAR SUBTYPE EXPRESSION AND ACTIVATION OF CAMKII REGULATE THE FATE OF ATHEROSCLEROTIC PLAQUE

Highlights

1. Our report shows CaMKII as an important checkpoint in the fate of human atherosclerotic plaques
2. The crosstalk between macrophages and VSMCs is regulated by CaMKII in atherosclerotic lesions.
3. CaMKII levels are increased in stable plaque (SP), while unstable plaque (UnSP) have

   increased expression of CaMKIV.

4. This study contributes to the understanding of the molecular mechanism underpinning atherosclerotic plaque progression,
5. Our data pose the bases of new therapeutic strategies based on cell type specific inhibition of CaMKII, allowing stabilization of atherosclerotic lesions.