Human Alternative Macrophages Populate Calcified Areas of Atherosclerotic Lesions and Display Impaired RANKL-Induced Osteoclastic Bone Resorption Activity

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Running title: Alternative Macrophages in Vascular Calcification

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ABSTRACT

Rationale: Vascular calcification is a process similar to bone formation leading to an inappropriate deposition of calcium phosphate minerals in advanced atherosclerotic plaques. Monocyte-derived macrophages, located in atherosclerotic lesions and presenting heterogeneous phenotypes, from classical pro-inflammatory M1 to alternative anti-inflammatory M2 macrophages, could potentially display osteoclast-like functions.

Objective: To characterize the phenotype of macrophages located in areas surrounding the calcium deposits in human atherosclerotic plaques.

Methods and Results: Macrophages near calcium deposits display an alternative phenotype being both CD68 and mannose receptor (MR) positive (CD68+MR+), expressing carbonic anhydrase type II (CA2), but relatively low levels of cathepsin K (CTSK). In vitro IL-4-polarization of human primary monocytes into macrophages results in lower expression and activity of CTSK compared to resting unpolarized macrophages. Moreover, IL4-polarization lowers expression levels of the osteoclast transcriptional activator Nuclear Factor of Activated T cells type c-1 (NFATc-1), associated with increased gene promoter levels of the transcriptional repression mark H3K27me3. Despite higher expression of the RANK receptor, RANKL/MCSF induction of NFATc-1 and CTSK expression is defective in these macrophages due to reduced Erk/c-fos-mediated downstream signaling resulting in impaired bone resorption capacity.

Conclusions: These results indicate that macrophages surrounding calcium deposits in human atherosclerotic plaques are phenotypically defective being unable to resorb calcification.

Keywords:
Macrophage phenotype, vascular calcification, atherosclerosis.

Nonstandard Abbreviations and Acronyms:

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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LCM</td>
<td>laser capture microdissection</td>
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<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>ChIP-seq</td>
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<td>M1</td>
<td>classically activated macrophages</td>
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<td>M2</td>
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<td>MR</td>
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INTRODUCTION

Vascular calcification (VC) is an inappropriate deposition of calcium phosphate mineral, which can occur in nearly all arterial beds and in both the intimal and medial layers. Intimal calcification is associated with inflammation and the development of plaques and occlusive lesions, while adjacent regions of the vessel wall may remain remarkably normal. This intimal form of calcification is an indicator of an advanced stage of atherosclerosis and is seen in the aorta, coronary arteries, and other large arteries. VC of the medial layer reduces aortic and arterial elasticity, thus impairing cardiovascular hemodynamics contributing to hypertension, aortic stenosis and cardiac hypertrophy. VC is an independent risk factor for cardiovascular morbidity and mortality. Furthermore, VC is tightly associated with aging and arterial remodelling, including intima-media thickening, as well as changes of the geometry and function of aortic valves (decreased aortic valve surface area and smaller valve opening).

VC is a dynamic regulated process sharing many features with bone formation. Bone is a specialized connective tissue consisting of cells and mineralized extracellular matrix. Organic and inorganic components of the matrix are mainly formed by collagen I fibers and spindle- or plate-shaped crystals of hydroxyapatite, respectively. Bone tissue contains osteoblastic bone forming cells and osteoclastic bone resorbing cells. Osteoblasts (OTBs) originating from local mesenchymal stem cells are responsible for the production of the calcified matrix. Osteoclasts (OTCs) are large multinucleated cells originating from monocyte/macrophage lineage precursors. OTBs hydrolyze both ATP and inorganic pyrophosphate (PPi), providing phosphate to promote mineralization. Development of mature osteoclasts is closely co-regulated by Macrophage Colony-Stimulating Factor (MCSF) and Receptor Activator of Nuclear Factor κB Ligand (RANKL), which are mainly produced by osteoblastic stromal cells in bone and by vascular smooth muscle cells (VSMC) under specific conditions in the arterial wall. RANKL is either a membrane-bound protein on the surface of OTBs or can be shedded upon cleavage by osteoclast-derived matrix metalloproteinase (MMP)7. RANKL binds to its receptor RANK present on the membranes of macrophage precursors thus driving OTC differentiation by increasing tartrate-resistant acid phosphatase (TRAP) expression. A current hypothesis for VC relies on the balance between potential osteoblast-like and osteoclast-like activities, the latter preventing calcium phosphate deposition in the vasculature.

The key event in bone resorption is the adhesion of osteoclasts to the bone extracellular matrix. Indeed, mature osteoclasts migrate to different bone regions and attach to the bone surface through interaction of integrin αV/β3 heterodimers with bone matrix proteins such as osteopontin (OPN) or vitronectin. Osteoclastic bone resorption initially involves mineral dissolution, followed by a degradation of the organic phase. Bone demineralization involves acidification of the extracellular microenvironment, mediated by an H+-ATPase in the cell’s ruffled membrane that pumps H+ ions into the resorption pit. Carbonic anhydrase type II (CA2) that catalyzes the production of H+ from carbon dioxide and water and sodium-bicarbonate co-transporter (NBCn1) are required in this process. H+ are released via H+-ATPase pumps to create an acidic environment that dissolves the mineral constituents of bone and provides an optimal environment for organic matrix degradation mainly by the lysosomal protease Cathepsin K (CTSK) and MMP-9 and heparanase-1 (HSPE-1). Osteoclasts derived from CTSK-deficient mice display an impaired bone resorption activity, supporting the notion that CTSK is of major importance in bone remodelling. The presence of both monocyte-macrophages that are able to differentiate directly into osteoclasts, and VSMC that have an osteoblast-like phenotype and secrete factors involved in osteoclast differentiation (such as RANKL, MCSF or pro-inflammatory cytokines) in the calcified vascular wall, strongly suggest the existence of osteoclastogenesis in the arterial beds. Indeed, TRAP positive multinucleated giant OTC-like cells have been detected in human atherosclerotic lesions. Moreover, co-localization of CA2 staining with the macrophage marker CD68 has been reported in human atherosclerotic plaques. In addition, CTSK expression and activity within the atherosclerotic lesions are mainly detected in macrophages. Furthermore, CTSK expression is increased...
in advanced plaques leading to excessive proteolytic tissue-remodelling thus contributing to plaque rupture 22. However, the presence of osteoclast-like cells in the vascular wall is rather limited, possibly due to factors that inhibit/modulate the differentiation of monocyte/macrophages into osteoclast-like cells 23. Monocyte-derived macrophages present different functional phenotypes depending on their micro-environment. While Th1 cytokines, such as IFNγ, or LPS lead to a “classical” M1 activation phenotype, Th2 cytokines, such as interleukin (IL)-4 or IL-13, induce an “alternative” M2 activation program characterized by high expression of the mannose receptor (MR). Moreover, in vivo, several macrophage subpopulations have been identified in human atherosclerotic plaques 24. We previously reported the presence of CD68⁺MR⁺ alternative macrophages in human atherosclerotic plaques in areas surrounding the lipid-rich area 25 and in iron-rich neo-vascularized zones 26. Since previous studies reported the presence of CD68⁺ macrophages in zones surrounding the calcified areas of human atherosclerotic plaques 27, the objective of this study was to characterize the functional phenotype of these macrophages.

METHODS

Immunohistology and laser-capture microdissection (LCM). Human atherosclerotic plaques were collected during carotid endarterectomy procedures at the University Hospital of Lille (France) after the approval of the local ethics committee. Informed consent was obtained from all patients. Samples were snap-frozen in liquid nitrogen directly after the surgery. For immunohistochemical staining, endogenous peroxidase activity was quenched. VSMC were identified with anti-α-smooth muscle actin (α-SMA), endothelial cells with anti-CD31/PECAM-1 (Novus Biological) and macrophages with anti-CD68 antibodies (Dako), using N-Histofine Simple Stain (Nichirei Biosciences Inc.). α-actin was revealed as a grey precipitate (Vector SG), CD31 by blue staining and CD68 by red staining (Vector Nova Red). Adjacent sections were stained with goat polyclonal anti-human MR (Santacruz biotechnology), rabbit anti-CA2 (Abcam) or mouse monoclonal anti-RANK (LSBio) or anti-CTSK (Santacruz biotechnology) antibodies. Alizarin red S (Sigma-Aldrich, France) staining was performed to detect calcium deposits. Sections of atherosclerotic plaques containing CD68⁺MR⁺ (located near calcification or neo-vascularized areas) as well as CD68⁺MR⁻ macrophages were submitted to laser capture microdissection (LCM) (ArcturusXT MDS Analytical Technologies) 25 and macrophage-enriched areas were captured from 4 adjacent 8 µm-sections and pooled for RNA extraction.

Cell culture. Human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll density gradient centrifugation. Resting macrophages were obtained from adherent monocytes cultured for 6 days in RPMI 1640 medium (Gibco, Invitrogen) supplemented with gentamicin (40 μg/mL), l-glutamine (2 mmol/L) (Sigma-Aldrich) and 10% pooled human serum (Abcys). To yield IL-4-polarized macrophages, recombinant human IL-4 (15 ng/mL, Promocell) was added at the beginning of differentiation 25. In some experiments, monocytes were differentiated during 6 days in αMEM medium (Gibco, Invitrogen) containing IL-4 or not (resting macrophages), in the absence or in the presence of RANKL/MCSF (50 ng/ml and 30 ng/ml respectively, R&D Systems) to promote osteoclastogenesis. Culture medium was changed every 3 days. For bone resorption experiments, monocytes were differentiated in MEM medium (Gibco, Invitrogen) with RANKL/MCSF (25 ng/ml and 30 ng/ml, respectively) in the absence or in the presence of IL-4 during 14 days. Culture medium was changed twice a week.

siRNA-mediated RNA interference. Smart-pool siRNA oligonucleotides corresponding to human NFATc-1 and Blimp1 (Dharmacon Thermo Scientific) and scrambled control RNA (Ambion) were used. After six days of differentiation in the presence of RANKL/MCSF, resting macrophages were transfected using Dharmafect4 reagent
(Dharmacon Thermo Scientific) in serum-free αMEM medium for 16 hours. Transfection medium was then replaced with fresh serum-free αMEM medium and the incubation continued for 24 hours before RNA extraction.

**RNA extraction and analysis.**

RNA from macrophages and osteoclasts was extracted using Trizol (Life Technologies, France) and RNeasy kits (Qiagen), respectively. RNA extraction from LCM-isolated samples was performed using the Picopure RNA extraction kit (MDS Analytical Technologies). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Only samples displaying a RNA Integrity Number (RIN) ≥ 6 were used for further analyses. RNA was amplified in two rounds using the ExpressArt TRinucleotide mRNA amplification Nano kit (AmpTec GmbH). For quantitative PCR (qPCR), RNA was reverse transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies, France). Reverse transcribed cDNAs were quantified by Brilliant III Ultra-Fast SYBR green based q-PCR using specific oligonucleotides (Online Table I) on an Mx3000 apparatus (Stratagene, La Jolla, CA). mRNA levels were normalized to TFIIB and cyclophilin as internal control for in vitro and ex vivo experiments, respectively.

**Protein extraction and western blot analysis.**

Cells were lysed in phosphate saline buffer containing Triton X100 1%, sodium deoxycholate 5 mg/ml (Sigma-Aldrich) supplemented with 2 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride, 10 mmol/L sodium diphosphate (Sigma-Aldrich) and protease inhibitor cocktail (Roche Diagnostics, France). Proteins were transferred onto a nitrocellulose membrane (GE Healthcare, France). After incubation for 1 hour in 5% non-fat dried milk (Dutscher, Brumath, France), membranes were incubated overnight with rabbit polyclonal CA2 (Abcam), mouse monoclonal CTSK (Santacruz biotechnology), phospho-p38 MAP Kinase (Thr180/Tyr182, Cell Signaling), p38 MAPK (Cell Signaling), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204, Cell Signaling), p44/42 MAPK (Erk1/2) (Cell Signaling) or polyclonal anti-β-actin (Santacruz biotechnology) antibodies. After washes with Tris 100 mmol/L, NaCl 150 mmol/L, Tween 0.1%, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (1/5000, GE Healthcare) and quantified by densitometry using the Image J software.

To quantify protein expression levels of NFATc-1, c-fos and phospho-c-fos, a capillary-based western analysis (ProteinSimple) was used according to the manufacturer’s protocol, using primary antibodies against NFATc-1, c-fos (Santacruz biotechnology) and phospho-c-fos (GeneTex). A HSP90 antibody (Santacruz biotechnology) was used to loading control. Mouse and rabbit secondary antibodies and chemiluminescent substrates provided by the manufacturer were used for immunoprobing. Chemiluminescent signals were detected, quantified and analyzed by Compass Software (ProteinSimple).

**Flow cytometry.**

Cells were washed with PBS containing 0.1% BSA and 2 mmol/L EDTA during 30 min at room temperature (RT) followed by scraping of the adherent cells. Then, cells were incubated for 1 hour at RT in blocking buffer (PBS containing 0.1% BSA and non-specific goat IgG at 1 μg/ml) and consecutively incubated with antibodies in autoMACS running buffer (Miltenyi Biotec, SAS) containing 0.1% BSA for 45 min at 4°C. Polyclonal primary rabbit anti-CA2 (Abcam) or monoclonal primary mouse anti-RANK (Abcam) antibodies were used followed by incubation with phycoerythrin (PE)-labeled goat anti-rabbit and anti-mouse secondary antibodies (Abcam). To perform MR staining, a mouse anti-MR antibody coupled with APC (BD Pharmingen™) was used. For negative controls, cells were incubated without primary antibody for CA2 and RANK or with isotype control antibody of MR coupled with APC (eBioscience). Data were analyzed using FACScalibur™ (BD Biosciences). Data were processed using the FlowJo xV software.
ChIP-qPCR and ChIP-seq analyses. 
Chromatin immunoprecipitation (ChIP) assays were performed essentially as described in 28 using an antibody directed against H3K27me3 (Abcam). Immunoprecipitated DNA was analyzed by q-PCR using (Supplemental Table 2). Fold enrichments were calculated based on background DNA recovery defined using the average of 5 control regions. See the Supplemental Methods for details.

H3K9ac ChIP followed by high-throughput sequencing (ChIP-seq) was performed to monitor H3K9ac levels in both resting and IL-4-polarized macrophages from 2 independent donors using an antibody against H3K9ac (17-658 from Millipore) 28. Public ChiP-seq data 29,30 were downloaded from Gene Expression Omnibus and mapped to hg19. Reads were extended to 200 bp and signal intensities (wig files) were defined as the number of uniquely mapped sequenced reads normalized to the total number reads within 25 bp windows as in 31. ChIP-seq signals were normalized to the total number of tags before visualization using the Integrated Genome Browser (IGB) 32.

CTSK activity measurement.
CTSK activity was measured in cellular extracts using a fluorescence-based assay (Abcam) following the manufacturer’s instructions.

Tartrate-Resistant Acid Phosphatase (TRAP) osteoclast staining and bone resorption activity measurement.
Monocytes were differentiated into osteoclasts by growth on cortical bone slices obtained from bovine femora 23, in the absence or in the presence of RANKL/MCSF, with or without IL-4. Mature osteoclasts were stained for TRAP expression using a commercially available kit (leukocyte acid phosphatase staining kit, Sigma). Multinucleated (> 3 nuclei) TRAP-positive cells were counted as osteoclasts under microscopic examination with a Leica DM 2500 (Leica) coupled to a CCD video system (Sony). Pictures were acquired with Bonoscan (Microvision Instruments). To evaluate bone resorption activity, resorption lacunae (pits) were visualized by staining bone slices with haematoxylin red and a toluidine blue solution containing 1% sodium borate after osteoclast removal. The percentage of resorbed bone surface areas was quantified using Bonoscan (Microvision Instruments).

Statistical analysis.
Statistical significance was analyzed by ANOVA and Student t-test. Differences were considered significant when $P < 0.05$. 

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RESULTS

CD68⁺MR⁺ alternative macrophages are located adjacent to calcified areas in human atherosclerotic lesions.

Given the existence of different macrophage sub-populations in human atherosclerotic lesions, as well as the presence of macrophages in areas surrounding the calcium deposits, we characterized the phenotype of the macrophages resident near the calcified areas. These CD68⁺ macrophages located in the proximity of calcium deposits, positively stained by alizarin red, express the alternative macrophage marker MR as well as CA2, an osteoclast marker. Surprisingly, CTSK gene expression is lower in CD68⁺MR⁺ compared to CD68⁺MR⁻ macrophage-enriched areas, suggesting the existence of a functional osteoclast defect in these calcified zone adjacent macrophages.

To determine whether calcification area-adjacent CD68⁺MR⁺ macrophages differ from CD68⁺MR⁺ macrophages located in other plaque zones, CD68⁺MR⁺ macrophages located in CD31⁻-vascularized and in calcified Alizarin Red positive areas were isolated by LCM, CTSK gene expression levels measured by q-PCR analysis and samples ranked based on CTSK expression levels. Then, the phenotypic characteristics of the analyzed plaque zones were compared on the basis of their relative localization in CD31 or calcium-positive areas respectively. The results indicate that CD68⁺MR⁺ macrophages with low CTSK expression locate primarily in CD31⁻ areas, while CD68⁺MR⁺ macrophages expressing higher CTSK levels are predominantly localized in calcification areas, as also illustrated by histological analysis. These results indicate that the phenotype of CD68⁺MR⁺ macrophages differs depending on their localization.

In vitro IL-4 macrophage polarization induces osteoclast-like cells with defective CTSK expression and activity.

Since CD68⁺ MR⁺ macrophages locate in vivo in the proximity of calcium deposits, we investigated whether the osteoclast profile could be recapitulated in vitro in macrophages differentiated in the absence (resting) or presence of IL-4. IL-4-polarized macrophages, which, as expected, display high MR expression levels, are characterized by higher mRNA levels of the osteoclast marker CA2. In line, expression of CA2 protein, measured by flow cytometry and western blot analysis, as well as NBCn1, necessary for CA2 activity, was elevated in IL-4-polarized macrophages. Conversely, IL-4 polarization resulted in reduced CTSK gene expression levels. ChIP-seq analysis showed lower histone H3 lysine-9 acetylation (H3K9ac) levels at the CTSK promoter in IL-4-polarized macrophages, strongly suggesting that the low CTSK mRNA levels are due to reduced gene transcription. Furthermore, CTSK protein expression and enzyme activity were significantly lower in IL-4-polarized macrophages. Moreover, mRNA levels of clusterin (CLU), a specific stabilizer of extra-cellular CTSK, were significantly lower in IL-4-polarized macrophages. Finally, expression of genes modulating structure and degradation of extra-cellular matrix, such as MMP-9, HSP-E-1 and osteopontin (OPN), was lower in IL-4-polarized macrophages. Additionally, mRNA levels of the Tartrate-Resistant Acid Phosphatase (TRAP)/acid phosphatase 5 (ACP5), a glycosylated monomeric metalloenzyme, was higher in IL-4-polarized macrophages. Taken together, these data suggest that IL-4-polarized macrophages display a particular osteoclast phenotype with low expression and activity of CTSK.

Since osteoclast differentiation is dependent on the RANKL-signaling pathway, the effect of IL-4 polarization on RANKL/MCSF activation of osteoclast markers was investigated. Human monocytes were differentiated during 6 days in αMEM medium supplemented or not with RANKL/MCSF, in the absence or presence of IL-4. RANKL/MCSF activation significantly increased gene expression of CA2,
TRAP, and OPN in resting, but not in IL-4-polarized macrophages (Figure 3A-C). In line, RANKL/MCSF induced CTSK gene expression (Figure 3D) and activity (Figure 3E) in resting, but not in IL-4-polarized macrophages. Altogether, these data indicate that IL-4 polarization induces an osteoclast-like phenotype observed in calcified areas of human atherosclerotic lesions by impeding acquisition of a functional osteoclast phenotype with low CTSK expression and activity.

**RANKL/MCSF-induced osteoclast activity is impaired by IL-4 polarization.**

To evaluate the functional consequences of the reduced response of IL-4-polarized macrophages to RANKL/MCSF activation, monocytes were seeded on bovine bone slices and differentiated for 14 days in the presence or not of IL-4, with or without RANKL/MCSF (Figure 4). Again, MR expression was increased by IL-4, independent of RANKL/MCSF treatment (Figure 4A). Similar as in Figure 3, RANKL/MCSF treatment induced CTSK mRNA levels in resting but not in IL-4-polarized macrophages (Figure 4B). Moreover, the number of mature multinucleated TRAP positive osteoclasts was strongly increased by RANKL/MCSF in resting, but not in IL-4-polarized macrophages (Figure 4C&D). The ability of these macrophages to degrade bone matrix, evaluated as the number of formed pits and as resorption rate, was significantly enhanced by RANKL/MCSF in resting, but not in IL-4-polarized macrophages (Figure 4E&G), indicating that the latter macrophages are unable to degrade bone matrix. Interestingly, the impaired bone resorption phenotype observed in IL-4-polarized macrophages resembles the one seen in osteoclasts derived from CTSK-KO mice 17, suggesting that reduced CTSK expression and activity are driving the impaired bone resorption capacity of IL-4-polarized macrophages.

**Expression of the CTSK transcriptional regulator NFATc-1 is impaired upon IL-4 polarization.**

To understand the molecular mechanisms underlying reduced CTSK expression upon IL-4 polarization, we assessed whether IL-4 may modulate the expression of the CTSK transcriptional regulator NFATc-1 35,36 using a siRNA-mediated silencing approach. Reducing NFATc-1 mRNA and protein in RANKL/MCSF treated resting macrophages inhibited CTSK gene and protein expression (Figure 5A&B), thus inducing the phenotype of IL-4-polarized macrophages. Moreover, IL-4 polarization lowered NFATc-1 mRNA and protein expression as well as its induction by RANKL/MCSF (Figure 5C). Analyses of ChIP-seq data from human macrophages 28-30 indicated that the NFATc-1 promoter is bivalent, i.e. showing both the activating histone H3 lysine 4 trimethylation (H3K4me3) and repressive H3K27me3 marks (Online Figure III), the latter requiring removal for gene activation 37. Interestingly, the decreased NFATc-1 gene expression upon IL-4 polarization was accompanied by higher promoter H3K27me3 levels as demonstrated by ChIP-qPCR analysis (Figure 5D). Therefore, IL-4 polarization epigenetically impairs NFATc-1 gene expression and subsequent CTSK gene transcriptional activation.

**IL-4 polarized macrophages exhibit higher RANK expression but defective RANKL signaling.**

To further study the mechanism via which IL-4 polarization impairs NFATc-1 gene expression and RANKL-responsiveness, RANK expression was measured. Surprisingly, IL-4-polarized macrophages express higher levels of RANK mRNA and membrane protein (Figure 6A&B) and a significantly higher proportion of cells are MR' RANK' as determined by flow cytometry analysis (Figure 6B&C). In vivo, in human atherosclerotic plaques, RANK was also present in MR' areas (Figure 6D). Moreover, RANK mRNA levels were higher in calcified area CD68'MR' compared to CD68' MR' macrophages (Figure 6E). Since these data rule out a lower expression of RANK as an explanation for the defective RANKL-mediated activation of NFATc-1 expression, we next tested whether IL-4-polarization may alter the response of kinase signaling pathways known to be regulated by RANKL, such as Erk1/2 and p38 38. Stimulation with RANKL/MCSF enhanced Erk1/2 phosphorylation in resting, but not in IL-4-polarized macrophages (Figure 6F). By contrast, p38 phosphorylation was not different (Online Figure IV).
c-fos-deficiency abrogates NFATc-1 mRNA expression and osteoclast differentiation and since Erk1/2 regulates c-fos expression and stability \(^{36,39}\), the effect of RANKL on c-fos activation was tested. In parallel to Erk1/2 phosphorylation, RANKL also increased c-fos activation in resting, but not in IL-4-polarized macrophages, associated with increased NFATc-1 and CTSK protein expression (Figure 6G).

In addition, NFATc-1 may regulate CTSK expression both directly \(^{35}\) and indirectly by controlling the expression of B-lymphocyte-induced maturation protein-1 (Blimp1), another osteoclast activator and repressor of anti-osteoclastogenic genes \(^{40}\). Indeed, Blimp1 expression was also repressed by IL-4 and its knock-down modulated CTSK expression (Online Figure V). Moreover, the transcriptional repressors B-cell lymphoma 6 (Bcl6) and Interferon Regulatory Factor-8 (IRF8) \(^{41,42}\) increased upon IL-4-polarization (Online Figure V). Hence, IL-4-polarization controls osteoclast function by regulating an osteoclastogenic transcriptional regulatory network.

DISCUSSION

The process leading to vascular calcification (VC) in human atherosclerotic lesions, an inappropriate deposition of calcium phosphate mineral, follows an osteochondrogenic pathway resembling the process of bone formation. Growing evidence suggests that VC is an actively regulated process, implicating both inducible and inhibitory factors, mediated by osteoblast-like and osteoclast-like cells, respectively. Furthermore, VC is tightly associated with aging and arterial remodelling, such as intima-media thickening as well as changes in the geometry and function of aortic valves (decreased aortic valve surface area and smaller valve opening). VC is thus an independent risk factor for cardiovascular morbidity and mortality \(^{43}\). Nevertheless, within atherosclerotic lesions, calcification can have dual effects, depending on the pattern and type of calcium deposition. While spotted and granular calcifications, due to pro-inflammatory processes, are associated with plaque progression toward an unstable rupture prone phenotype, larger laminated macro-calcifications are often observed in fibrotic lesions and are suggested to support plaque stabilization \(^{44}\).

Osteoclasts originate from hematopoietic lineage cells derived from bone marrow myeloid precursors or circulating monocytes. However, whether mature osteoclasts are present in the arterial wall is still controversial and cells with potential osteoclast properties have been named osteoclast-like cells \(^{18}\). Thus, in atherosclerotic lesions, macrophages which express both the MCSF and RANKL receptors \(^{45}\) can be activated by their ligands produced by endothelial cells, VSMC as well as monocyte/macrophages themselves and can thus potentially differentiate into osteoclast-like cells. Cells positive for TRAP have been observed in human atherosclerotic lesions \(^{46}\). CA2 staining co-localizes with the macrophage marker CD68 in human atherosclerotic plaques \(^{20}\). Moreover, CTSK is expressed in macrophages of advanced atherosclerotic plaques \(^{21}\) where it contributes to proteolytic tissue-remodelling and possibly plaque rupture \(^{22}\). Interestingly, we found that macrophages located in areas surrounding intimal calcium deposits in human atherosclerotic lesions display an alternative phenotype, since they express both CD68 and MR \(^{25,47}\). This macrophage sub-population is characterized by lower gene expression levels of CTSK, a phenotype also observed in vitro upon IL-4 polarization, accompanied by a reduced protein expression and activity.

These alternative macrophages display a particular phenotype with regard to VC: while they express some of the canonical osteoclast markers, such as CA2, together with high levels of RANK receptor, their response to RANKL/MCSF activation is severely altered. Consequently, alternative macrophages are functionally unable to degrade bone matrix upon RANKL/MCSF activation. Altogether these data indicate that the capacity of bone degradation is impaired in alternative macrophages. Interestingly this phenotype resembles the one observed in osteoclasts derived from CTSK-deficient mice.
suggested that the altered CTSK expression and activity observed in these macrophages is sufficient to compromise their osteoclast-like activity.

The effects of IL-4 on osteoclast maturation have been studied mainly using mouse cells where several mechanisms have been proposed. IL-4 was found to suppress osteoclast development and mature osteoclast functions from mouse bone marrow precursors by suppressing RANK gene expression. IL-4 was also shown to inhibit mouse osteoclast formation by inhibiting RANKL induction via a STAT6 dependent pathway. Furthermore, it has been suggested that IL-4 abrogates osteoclastogenesis in mouse cells by inhibiting NF-κB activation and several MAP kinase signalling pathways. These studies contrast with the results obtained in our study using human macrophages, which are characterized by higher RANK expression in IL-4-polarized macrophages. Moreover, we found that Erk1/2, but not p38 phosphorylation is selectively impaired in IL-4-polarized macrophages upon MCSF/RANKL stimulation, leading to lower activation of c-fos, an activator of NFATc-1 expression. Moreover, in addition to impeding RANKL-mediated activation of NFATc-1 transcription, we show that IL-4-polarization has an intrinsic negative impact on NFATc-1 gene expression through epigenetic impairment of NFATc-1 gene expression via increased promoter H3K27me3 levels (Figure 7).

NFATc-1 and c-fos are key transcription factors in RANKL-induced activation of osteoclastic genes including CTSK. During osteoclast differentiation, c-fos-mediated amplification of NFATc-1 sustains NFATc-1 expression and function. In IL-4-polarized macrophages, the expression and activation of both NFATC-1 and c-fos is impaired leading to lower CTSK expression and activity.

The fact that CD68+MR+ macrophages accumulate around calcified areas could be the consequence of their incapacity to resorb pre-existing calcium phosphate crystals, derived, for example, from apoptotic death of VSMC which have acquired an osteogenic profile. On the other hand, this calcification might occur spontaneously due to the absence of osteoclastic activity of the surrounding CD68+MR+ macrophages. CD68+MR+ macrophages have now been located in particular areas of the plaque, such as neo-vascularized and haemorrhagic zones. Thus, it appears important to understand the specific signals that induce macrophage accumulation and phenotypic polarization in particular plaque areas, such as those associated to calcification. However, the very low expression and activity of CTSK, a protease also involved in plaque destabilization, in these CD68+MR+ macrophages, can suggest a role for these macrophages in plaque stability. Indeed, CTSK-deficiency in atherosclerosis mouse models reduced the number of lesions and decreased the area of individual plaques, an effect accompanied by increased cholesterol accumulation in macrophages and augmented deposition of extra-cellular matrix. However, given the potential different effects of micro- and macro-calcifications with respect to plaque stability, further extensive studies will be necessary to explore whether CD68+MR+ macrophages preferentially accumulate around a given type of calcium depot, what their exact role is with respect to vascular calcification in such depots and whether they modulate the plaque stability phenotype positively or negatively.

Despite its clinical relevance, research on the role of macrophages in resorbing mineral deposition in arteries has been limited and remains at an early stage. Since VC rarely regress, the major goals are prevention of formation and stabilization of existing calcifications. Because intimal calcification relates to atherosclerosis, the general treatment approach is non-specific: lipid lowering therapies, use of aspirin, treatment of obesity and hypertension, physical activity, smoking cessation, and treatment of diabetes. Our data identify, depending on their localization, distinct phenotypes of alternative macrophages in advanced atherosclerotic lesions, which may not always result in beneficial effects on atherosclerosis progression. Therefore, approaches to modulate the activity of macrophage subpopulations, involved in VC may provide future pharmacological approaches targeting these macrophages in order to modulate vascular calcium deposition.
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DISCLOSURES
None.

REFERENCES


DOI: 10.1161/CIRCRESAHA.116.310262


FIGURE LEGENDS

**Figure 1.** Macrophages surrounding calcified areas of human atherosclerotic lesions are CD68⁺MR⁺ alternative macrophages. (A left) Representative immunostaining for CD68 (red), CD31/PECAM-1 (blue), α-smooth muscle actin (α-SMA, grey) in human atherosclerotic lesions. (A right and D) Higher magnification of calcium deposits by Alizarin Red (red), αSMA, CD68, CD31, MR, CA2 and CTSK stainings. Scale bars are indicated. (B) q-PCR analysis of CTSK mRNA performed on LCM-isolated CD68⁺MR⁺ and CD68⁺MR⁻ macrophage-rich areas from atherosclerotic plaques. CTSK mRNA levels were normalized to cyclophilin mRNA and expressed relative to the levels in CD68⁺MR⁻ areas set at 1. Each point corresponds to a single atherosclerotic plaque. The mean value and statically significant differences are indicated (t-test; ***p< 0.001). (C) CD68⁺ MR⁺ macrophages-enriched areas (n=37) were isolated by LCM and CTSK mRNA expression measured by q-PCR. The upper and lower tertiles of CTSK expression were then compared based on their respective localization in CD31 or calcium positive zones.

**Figure 2.** Osteoclast markers are differently expressed in IL-4-polarized macrophages. q-PCR analysis of MR (A), CA2 (B), CTSK (E), and TRAP (H) mRNA levels in IL-4-polarized compared to resting macrophages, representative of 7 different cell preparations. The mRNA levels were normalized to TFIIB mRNA and expressed relative to the levels in resting set at 1. Protein expression of CA2 by flow cytometry (C) and by western blot performed in resting and IL-4-polarized macrophages from 3 donors (D). Protein expression of CTSK (27 KDa) and β-actin (42 KDa) analysed by western blot performed in resting and IL-4-polarized macrophages from 4 donors (E). ns indicates a non specific band. MW: molecular weight. Intracellular CTSK activity was measured in resting and IL-4-polarized macrophages normalized to cellular protein content (G). The results are expressed as mean ± SD of triplicate determinations relative to the levels in resting set at 1. Statistically differences are indicated (t-test, *p<0.05 and ***<0.001).

**Figure 3.** IL-4-polarized macrophages respond less to MCSF/RANKL activation. q-PCR analysis of CA2 (A), TRAP (B), NFATc-2 (C), OPN (C) and CTSK (D) mRNA levels measured in resting and IL-4-polarized macrophages differentiated for 6 days with or without RANKL/MCSF. mRNA levels were normalized to TFIIB and results expressed as mean ± SD of triplicate determinations relative to the levels in resting set at 1, representative of 7 donors. (E) Intracellular CTSK activity measured in resting and IL-4-polarized macrophages cultured in the presence or in the absence of RANKL/MCSF, normalized to cellular protein content. The results are expressed as mean ± SD from 3 independent donors relative to the levels in resting set at 1. Statistically significant differences are indicated (t-test, *<0.05, **<0.01 and ***<0.001).

**Figure 4.** IL-4-polarized macrophages fail to degrade bone matrix in response to RANKL/MCSF activation. q-PCR analysis of MR (A) and CTSK (B) mRNA measured in resting and IL-4-polarized macrophages grown on dentin slices, treated or not with RANKL/MCSF during 14 days. mRNA levels were normalized to TFIIB and results expressed as mean ± SD of triplicate determinations relative to the levels in resting set at 1, representative of 3 independent donors. (C) TRAP staining (x100 magnification, arrows point multinucleated cells) and quantification of TRAP-positive osteoclasts (OTC) in resting and IL-4-polarized macrophages treated with RANKL/MCSF (D). Bone slice staining with haematoxylin red and toluidine blue to visualize the resorption lacunae (pits) (x200 magnification, arrows point the pits) (E). The number of pits was counted (F) and the resorption rate calculated and expressed as the “resorbed surface / bone surface” ratio (G). The results represented the mean ± SD of 3 independent experiments. Statistically significant differences are indicated (t-test; *<0.05, **<0.01 and ***<0.001).

**Figure 5.** NFATc-1 expression is altered in IL-4-polarized macrophages and controls CTSK expression. NFATc-1 mRNA and protein levels measured in RANKL/MCSF differentiated resting
macrophages transfected with scrambled siRNA or specific NFATc-1 siRNA (A) or measured in resting and IL-4-polarized macrophages differentiated for 6 days with or without RANKL/MCSF (C). CTSK mRNA and protein levels in RANKL/MCSF differentiated resting macrophages transfected with scrambled siRNA or specific NFATc-1 siRNA (B). mRNA levels were normalized to TFIIB and results expressed as mean ± SD of triplicate determinations relative to the levels in resting set at 1 or to the levels of scrambled transfected cells set at 1, representative of 3 cell preparations. Statistically significant differences are indicated (t-test; *<0.05, ***<0.001). (D) ChIP experiments were performed on H3K27me3 immunoprecipitated chromatin from 2 donors using primers covering the NFATc-1 promoter and enhancers. Fold enrichment are shown.

Figure 6. RANK expression is higher in IL-4-polarized macrophages, but RANKL signaling is impaired. (A) q-PCR analysis of RANK mRNA in resting and IL-4-polarized macrophages. mRNA levels were normalized to TFIIB and results expressed as mean ± SD of triplicate determinations relative to the levels in resting set at 1, representative of 7 different cell preparations. (B and C) Flow cytometry analysis of RANK expression in resting and IL-4-polarized macrophages using PE-conjugated anti-RANK monoclonal antibody and APC-MR antibody. The negative controls were performed as indicated in the methods section. Results are representative of 3 independent experiments. (D) Representative immunostaining of MR and RANK in human carotid atherosclerotic lesions. (E) q-PCR analysis of RANK mRNA performed on LCM-isolated CD68⁺MR⁺ and CD68⁺MR⁻ macrophages-rich areas from different atherosclerotic plaques. RANK mRNA levels were normalized to cyclophilin mRNA and expressed relative to the levels in CD68⁺MR⁺ areas set at 1. Each point corresponds to a single atherosclerotic plaque. The mean value is shown. (F) Western blot analysis of total and phosphorylated Erk1/2 in resting and IL-4-polarized macrophages activated in the absence or in the presence of MCSF/RANKL for different time periods. Band intensities were measured and results expressed as a ratio of phosphorylated/total Erk1/2. Statistically significant differences are indicated (t-test, *<0.05 and ***<0.001). (G) Western blot analysis of NFATc-1 and total and phosphorylated c-fos in resting and IL-4-polarized macrophages activated in the absence or in the presence of MCSF/RANKL for the indicated time periods.

Figure 7. Schematic summary of main findings from this study
See discussion for details
NOVELTY AND SIGNIFICANCE

What Is Known?

- CD68+MR+ alternative macrophages are present in human atherosclerotic lesions where they are thought to promote plaque stability.
- CD68+MR+ alternative macrophages exhibit distinct functional phenotypes depending on the atherosclerotic plaque microenvironment.
- Osteoclast-like cells, originating from the monocyte lineage, are present in atherosclerotic lesions.

What New Information Does This Article Contribute?

- CD68+MR+ alternative macrophages surround the calcium depots in human atherosclerotic plaques.
- Alternative macrophages display a dysfunctional osteoclast-like phenotype, characterized by low expression and activity of cathepsin K (CTSK) and defective calcification resorption ability.
- This phenotype is due to impaired RANKL-induced expression of the osteoclast transcriptional activator Nuclear Factor of Activated T cells type c-1 (NFATc-1) in alternative macrophages.

Atherogenesis involves monocytes infiltrating the intima of large arteries and differentiating into macrophages. Plaque macrophages display distinct functional phenotypes depending on their microenvironment. Several macrophage sub-populations have been identified in human atherosclerotic plaques including alternative macrophages which are thought to stabilize atherosclerotic lesions. In this study, we characterized the functional phenotype of macrophages surrounding calcium deposits. Our results indicate that macrophages located around the calcified areas are CD68+MR+ alternative macrophages. In vitro IL-4 polarized macrophages display an impaired bone resorption activity due to altered NFATc-1 expression and signalling, resulting in a lower expression and activity of CTSK. Thus, alternative macrophages around calcified areas are unable to counter calcification. Hence, alternatively polarized macrophages may not always exert atheroprotective activities. Further studies are necessary to determine the exact roles of CD68^MR^ macrophages in vascular micro- and macrocalcification areas and whether they modulate plaque stability positively or negatively.
FIGURE 1

A

[Images of tissue sections labeled α-SMA, CD68, CD31, Calcium, α-SMA, CD68, MR, CA2]

B

[Graph showing CTSK relative mRNA levels (fold over CD68*MR* set at 1) with CD68*MR* on the x-axis and CTSK expression on the y-axis with data points and error bars]

C

[Bar chart showing number of Calcium and/or CD31 positive CD68*MR* areas with lower tertile of CTSK expression and upper tertile of CTSK expression]

D

[Images of neo-vascularized zone and calcified zone with labels: CD68, MR, CD31, Calcium, CTSK]
**FIGURE 2**

**A** MR Relative mRNA levels

**B** CA2 Relative mRNA levels

**C** CtsK Intracellular activity (fold over resting set at 1)

**D** Western blots of CtsK and \( \beta \)-actin in donor 1, donor 2, donor 3, and donor 4.

**E** CtsK Relative mRNA levels

**F** CtsK Intracellular activity (fold over resting set at 1)

**G** TRAP Relative mRNA levels

**H** TRAP Relative mRNA levels

Figure 2.
Figure 3

**Figure 3**

(A) CA2 Relative mRNA levels

(B) TRAP Relative mRNA levels

(C) OPN Relative mRNA levels

(D) CTSK Relative mRNA levels

(E) CTSK intracellular activity (fold over resting set at 1)

- **CON**
- **RANKL**
- **MCSF**

Legend:

- IL-4 -
- IL-4 +

Significance levels:

- *** p < 0.001
- ** p < 0.01
- * p < 0.05
**FIGURE 4**

(A) Relative mRNA levels of MR and CTSK in response to RANKL and MCSF treatment with or without IL-4. Statistical significance is indicated by ***p < 0.001, **p < 0.01, and *p < 0.05.

(B) Relative mRNA levels of MR and CTSK in response to RANKL and MCSF treatment with or without IL-4. Statistical significance is indicated by ***p < 0.001, **p < 0.01, and *p < 0.05.

(C) Representative images of TRAP+ osteoclasts (OTC) in wells treated with RANKL + MCSF with or without IL-4.

(D) Number of resorption pits in response to RANKL + MCSF treatment with or without IL-4. Statistical significance is indicated by **p < 0.01.

(E) Representative images of staining for osteoclastic activity.

(F) Resorption rate in response to RANKL + MCSF treatment with or without IL-4. Statistical significance is indicated by ***p < 0.001.

Figure 4
Figure 5

NFATc-1 Relative mRNA levels

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CTSK Relative mRNA levels

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H3K27me3 relative fold enrichment

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Figure 5

HSP90

NFATc-1

- IL-4

+ IL-4

RANKL

CON

RANKL / MCSF

- IL-4

+ IL-4

FIGURE 5
Figure 6
Figure 7
Human Alternative Macrophages Populate Calcified Areas of Atherosclerotic Lesions and Display Impaired RANKL-Induced Osteoclastic Bone Resorption Activity

Giulia Chinetti-Gbaguidi, Mehdi Daoudi, Mickael ROSA, Manjula Vinod, Loïc Louvet, Corinne Copin, Melanie Fanchon, Jonathan Vanhoutte, Bruno Derudas, Loïc Belloy, Stephan Haulon, Christophe Zawadzki, Sophie Susen, Ziad A Massy, Jérôme eeckhoute and Bart Staels

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Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2017/04/24/CIRCRESAHA.116.310262.DC1

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SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

ChIP-qPCR
Macrophages were cross-linked with 1% formaldehyde into PBS for 10 minutes at room temperature. Quenching was performed using 1.25mM of glycine and cells were washed and harvested in cold PBS. The chromatin was fragmented using a Bioruptor sonication bath (Diagenode) for 14 min minutes at high intensity (30s ON/OFF cycles) in a lysis buffer comprising 1% SDS, 10mM EDTA, 50mM Tris-HCl pH8.1 and protease inhibitors. Samples were centrifuged at 16 000 g for 10 min at 4°C and the soluble fraction was recovered. Chromatin immunoprecipitation was performed by overnight incubation under rotation at 4°C with 1 µg of an antibody directed against H3K27me3 (Abcam) in a buffer containing 10 mmol/L Tris, pH 7.5, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 100 mmol/L NaCl, 0.1% Na-deoxycholate, 0.5% N-lauroyl sarcosine, 0.08% SDS and 1% Triton X-100. The next day, 10µl of a 50:50 mixture of Protein A/G Dynabeads (Invitrogen), blocked overnight by incubation in PBS-BSA (5mg/ml) and salmon sperm DNA (Invitrogen), was added together with salmon sperm DNA and samples were incubated for an additional 4h under rotation at 4°C. Precipitates were washed 3 times with washing buffer (50mM HEPES pH 7.6, 1mM EDTA, 0.7% Na Deoxycholate, 1% NP-40, 0.5M LiCl and salmon sperm DNA) and once with Tris-EDTA (pH7.6). Elution buffer was added to the beads (1% SDS and 0.1M NaHCO3) and the crosslinking of both ChIPed and input DNA was reversed at 65°C overnight. Immunoprecipitated DNA was purified using the PCR purification kit from Macherey Nagel according to the manufacturer’s instructions for SDS-containing samples.
Online Figure I. Osteoclast markers are differently expressed in IL-4-polarized macrophages.
mRNA levels of NBCn1 (A), CLU (B), MMP-9 (C), HSPE-1 (D) and OPN (E) were measured by q-PCR in IL-4-polarized compared to resting macrophages. The mRNA levels were normalized to TFIIB mRNA and expressed relative to the levels in resting set at 1, representative of 7 healthy donors. Statistically significant differences are indicated (t-test, **<0.01 and ***<0.001).
Online Figure II. H3K9ac ChIP-sequencing data for resting and IL-4-polarized macrophages.
Normalized H3K9ac levels at the CTSK (A) and CA2 (B) promoters in resting and IL-4-polarized macrophages obtained from 2 independent donors. An equivalent scale was used for resting and IL-4 polarized macrophages from a given donor.
Online Figure III. Epigenomic profiles at the *NFATc-1* gene in human macrophages. H3K27me3, H3K4me3 as well as H3K9ac ChIP-seq and input profiles from human macrophages are shown. The NFATc-1 promoter and potential enhancers analyzed in Figure 5D are indicated.
Online Figure IV. p38 phosphorylation by RANKL is not affected in IL-4-polarized macrophages.

Western blot analysis of total and phosphorylated p38 in resting and IL-4-polarized macrophages activated in the absence or in the presence of MCSF/RANKL for different time periods. Band intensities were measured and results expressed as a ratio of phosphorylated/total p38.
Online Figure V. Blimp1 is higher expressed in resting macrophages whereas Bcl6 and IRF8 are higher expressed in IL-4-polarized macrophages.

q-PCR analysis of and Blimp1 (A), Bcl6 (E), IRF8 (F) mRNA in resting and IL-4-polarized macrophages, representative of 7 different cell preparations. q-PCR analysis of Blimp1 (B, C) and CTSK (D) in RANKL/MCSF differentiated resting macrophages transfected with scrambled siRNA or specific NFATc-1 or Blimp1 siRNA, representative of 3 different cell preparations. The mRNA levels were normalized to TFIIB mRNA and expressed relative to the levels in resting (A, E, F) or to the levels in scrambled transfected cells set at 1 (B, C, D). (D) Western blot analysis of CTSK and β-actin in scrambled and siBlimp1 transfected RANKL/MCSF differentiated resting macrophages. ns indicates non specific band. Statistically significant differences are indicated (t-test; *<0.05 and **<0.01).
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Online Table II. Primers used for ChIP-qPCR assays

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