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

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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 proinflammatory 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–positive, expressing carbonic anhydrase type II, but relatively low levels of cathepsin K. In vitro interleukin-4-polarization of human primary monocytes into macrophages results in lower expression and activity of cathepsin K compared with resting unpolarized macrophages. Moreover, interleukin-4 polarization lowers expression levels of the osteoclast transcriptional activator nuclear factor of activated T cells type c-1, associated with increased gene promoter levels of the transcriptional repression mark H3K27me3 (histone 3 lysine 27 trimethylation). Despite higher expression of the receptor activator of nuclear factor xB receptor, receptor activator of nuclear factor xB ligand/macroage colony-stimulating factor induction of nuclear factor of activated T cells type c-1 and cathepsin K expression is defective in these macrophages because of 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. (Circ Res. 2017;121:19-30. DOI: 10.1161/CIRCRESAHA.116.310262.)

Key Words: atherosclerosis ■ cathepsin K ■ macrophages ■ phenotype ■ vascular calcification

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, whereas 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 remodeling, 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...
rent hypothesis for VC relies on the balance between potential tartrate-resistant acid phosphatase (TRAP) expression. A cur-
precursors thus driving osteoclast differentiation by increasing to its receptor RANK present on the membranes of macrophage
multinucleated cells originating from monocyte/macrophage lin-
produced by osteoblastic stromal cells in bone and vascu-
\( \kappa \)
macrophage colony-stimulating factor (MCSF) and receptor ac-
Development of mature osteoclasts is closely coregulated by
for the production of the calcified matrix. Osteoclasts are large
originating from local mesenchymal stem cells are responsible
Type c-1 in alternative macrophages.
This phenotype is due to impaired RANKL-induced expression of the osteoclast transcriptional activator nuclear factor of activated T cells type c-1 in alternative macrophages.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CA2</td>
<td>carbonic anhydride type II</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>ChIP-seq</td>
<td>ChIP followed by high-throughput sequencing</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LCM</td>
<td>laser capture microdissection</td>
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<tr>
<td>MR</td>
<td>mannose receptor</td>
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<tr>
<td>NFATc-1</td>
<td>nuclear factor of activated T cells type c-1</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor ( \kappa )-ligand</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase</td>
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<tr>
<td>VC</td>
<td>vascular calcification</td>
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<td>VSMC</td>
<td>vascular smooth muscle cells</td>
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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 originating from local mesenchymal stem cells are responsible for the production of the calcified matrix. Osteoblasts are large multinucleated cells originating from monocyte/macrophage lineage precursors. Osteoblasts hydrolyze both ATP and inorganic pyrophosphate, providing phosphate to promote mineralization. Development of mature osteoclasts is closely coregulated by macrophage colony-stimulating factor (MCSF) and receptor activator of nuclear factor \( \kappa \)-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 osteoblasts or can be shedded by cleavage by osteoclast-derived matrix metalloproteinase-7. RANKL binds to its receptor RANK present on the membranes of macrophage precursors thus driving osteoclast 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.

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 subpopulations have been identified in human atherosclerotic plaques including alternative macrophages that 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\(^{+}\)-alternative macrophages. In vitro interleukin-4-polarized macrophages display an impaired bone resorption activity because of altered nuclear factor of activated T cells type c-1 expression and signaling, resulting in a lower expression and activity of cathepsin K. 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\(^{+}\)-macrophages in vascular micro- and macrorcalcification areas and whether they modulate plaque stability positively or negatively.
presence of osteoclast-like cells in the vascular wall is rather limited, possibly because of factors that inhibit/modulate the differentiation of monocyte/macrophages into osteoclast-like cells. 21 Monocyte-derived macrophages present different functional phenotypes depending on their microenvironment. Although Th1 cytokines, such as IFNγ, or lipopolysaccharide, 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 22 and in iron-rich neovascularized zones. 26 Because 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

Immunohistochemistry and Laser-Capture Microdissection

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 (platelet endothelial cell adhesion molecule-1; Novus Biological) and macrophages with anti-CD68 antibodies (Dako), using N-Histofine Simple Stain (Nichirei Biosciences Inc.), α-actin was revealed as a gray 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 (Santa Cruz Biotechnology), rabbit anti-CA2 (Abcam), mouse monoclonal anti-RANK (LSBio), or anti-cathepsin K (Santa Cruz 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 neovascularized areas) and CD68+MR- macrophages were submitted to laser capture microdissection (ArcturusXT MDS Analytic Technologies) 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 (Roswell Park Memorial Institute) 1640 medium (Gibco, Invitrogen) supplemented with gentamicin (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. 26 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 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 αMEM medium (Gibco, Invitrogen) with RANKL/MCSF (25 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.

Small Interfering RNA–Mediated RNA Interference

Smart-pool small interfering RNA oligonucleotides corresponding to human nuclear factor of activated T cells type c-1 (NFATc-1) and Blimp1 (Dharmacon Thermo Scientific) and scrambled control RNA (Ambion) were used. After 6 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 laser capture microdissection–isolated samples was performed using the Picopure RNA extraction kit (MDS Analytic Technologies). RNA quality was assessed using the Agilent 2100 Bioanalyser (Agilent Technologies). Only samples displaying a RNA Integrity Number ≥6 were used for further analyses. RNA was amplified in 2 rounds using the ExpressArt TRinucleotide mRNA amplification Nano kit (AmpTec GmbH). For quantitative polymerase chain reaction (qPCR), RNA was reverse transcribed using random hexamer primers and Superscript reverse transcriptase (Life Technologies). Reverse-transcribed cDNAs were quantified by Brilliant III Ultra-Fast SYBR green-based qPCR using specific oligonucleotides (Online Table I) on an Mx3000 apparatus (Stratagene, La Jolla, CA). mRNA levels were normalized to TFIIB (transcription factor IIB) 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% nonfat dried milk (Dutscher, Brumath, France), membranes were incubated overnight with rabbit polyclonal CA2 (Abcam), mouse monoclonal cathepsin K (Santa Cruz Biotechnology), phospho-p38 MAP (mitogen-activated protein) 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 (Santa Cruz 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) for 1 hour at room temperature. Immunoreactive bands were detected with an enhanced chemiluminescence Western blotting detection reagent (ECL plus; 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 (Santa Cruz Biotechnology) and phospho-c-fos (GeneTex). An HSP90 (heat shock protein 90) antibody (Santa Cruz 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 minutes at room temperature followed by resuspension of the adherent cells. Then cells were incubated for 1 hour at room temperature in blocking buffer (PBS containing 0.1% BSA and nonspecific goat IgG at 1 μg/mL) and consecutively incubated with antibodies in auto-MACS running buffer (Miltenyi Biotec, SAS) containing 0.1% BSA for 45 minutes at 4°C. Polyclonal primary rabbit anti-CA2 (Abcam) or monoclonal primary mouse anti-RANK (Abcam) antibodies were used followed by incubation with phycoerythrin-labeled goat anti-rabbit and anti-mouse secondary antibodies (Abcam). To perform MR staining, a mouse anti-MR antibody
coupled with APC (allophycocyanin; 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.

Chromatin Immunoprecipitation-qPCR and Chromatin Immunoprecipitation-Seq Analyses

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described by Chinetti-Gbaguidi et al. using an antibody directed against H3K27me3 (histone 3 lysine 27 trimethylation; Abcam). Immunoprecipitated DNA was analyzed by qPCR using (Online Table II). Fold enrichments were calculated based on background DNA recovery defined using the average of 5 control regions. See the Methods in the Online Data Supplement 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). Public ChIP-seq data 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 Dubois-Chevalier et al. ChIP-seq signals were normalized to the total number of tags before visualization using the Integrated Genome Browser.

Cathepsin K Activity Measurement

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

Tartrate-Resistant Acid Phosphatase Osteoclast Staining and Bone Resorption Activity Measurement

Monocytes were differentiated into osteoclasts by growth on cortical bone slices obtained from bovine femora, 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 hematoxylin 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 \( P < 0.05 \).

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 (platelet endothelial cell adhesion molecule-1; blue), \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA, gray) in human atherosclerotic lesions. A, Right, and D, Higher magnification of calcium deposits by Alizarin Red (red), \( \alpha \)-SMA, CD68, CD31, mannose receptor (MR), carbonic anhydrase type II (CA2), and cathepsin K (CTSK) stainings. Scale bars are indicated.

B, Quantitative polymerase chain reaction (qPCR) analysis of CTSK mRNA performed on laser capture microdissection (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 statistically significant differences are indicated (t test; *** \( P < 0.001 \)). C, CD68+MR+ macrophage-enriched areas (n=37) were isolated by LCM and CTSK mRNA expression measured by qPCR. The upper and lower tertiles of CTSK expression were then compared based on their respective localization in CD31 or calcium positive zones.
Results
CD68^+MR^+ Alternative Macrophages Are Located Adjacent to Calcified Areas in Human Atherosclerotic Lesions

Given the existence of different macrophage subpopulations in human atherosclerotic lesions,\(^2,4\) as well as the presence of macrophages in areas surrounding the calcium deposits,\(^2,7\) 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 (Figure 1A), express the alternative macrophage marker MR and CA2, an osteoclast marker (Figure 1A). Surprisingly, cathepsin K gene expression is lower in CD68^+MR^+ than in CD68^+MR^- macrophage-enriched areas, suggesting the existence of a functional osteoclast defect in these calcified zone adjacent macrophages (Figure 1B).

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 laser capture microdissection, cathepsin K gene expression levels were measured by qPCR analysis, and samples were ranked based on cathepsin K 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 (Figure 1C). The results indicate that CD68^+MR^+ macrophages with low cathepsin K expression locate primarily in CD31^+ areas, whereas CD68^+MR^- macrophages expressing higher cathepsin K levels are predominantly localized in calcification areas (Figure 1C), as also illustrated by histological analysis (Figure 1D). 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 Cathepsin K Expression and Activity

Because 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 (Figure 2A)\(^2,5\) are characterized by higher mRNA levels of the osteoclast marker CA2 (Figure 2B). In line, expression of CA2 protein, measured by flow cytometry and Western blot analysis (Figure 2C and 2D), as well as sodium bicarbonate cotransporter 1, necessary for CA2 activity (Online Figure 1A), was elevated in IL-4–polarized macrophages.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Osteoclast markers are differently expressed in IL-4–polarized macrophages. Quantitative polymerase chain reaction analysis of mannose receptor (MR; A), carbonic anhydrase type II (CA2; B), cathepsin K (CTSK; E), and tartrate-resistant acid phosphatase (TRAP; H) mRNA levels in interleukin (IL)-4–polarized compared with resting macrophages, representative of 7 different cell preparations. The mRNA levels were normalized to TFIIB (transcription factor IIB) mRNA and expressed relative to the levels in resting set at 1. Protein expression of CA2 by flow cytometry (G) and by Western blot performed in resting and IL-4–polarized macrophages from 4 donors (D). Protein expression of CTSK (27 KDa) and β-actin (42 KDa) analyzed by Western blot performed in resting and IL-4–polarized macrophages from 4 donors (E). 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, ^**^P<0.01, and ^***^P<0.001). MW indicates molecular weight; and ns, a nonspecific band.
macrophages. Conversely, IL-4 polarization resulted in reduced cathepsin K gene expression levels (Figure 2E). ChIP-seq analysis showed lower histone H3 lysine-9 acetylation (H3K9ac, a marker of active regulatory regions\(^3\)) levels at the CTSK promoter in IL-4–polarized macrophages, strongly suggesting that the low cathepsin K mRNA levels are because of reduced gene transcription (Online Figure II). Furthermore, cathepsin K protein expression and enzyme activity were significantly lower in IL-4–polarized macrophages (Figure 2F and 2G). Moreover, mRNA levels of clusterin, a specific stabilizer of extracellular cathepsin K, were significantly lower in IL-4–polarized macrophages (Figure 2F and 2G). Finally, expression of genes modulating structure and degradation of extracellular matrix, such as matrix metalloproteinase-9, heparanase-1, and osteopontin, was lower in IL-4–polarized macrophages (Online Figure IC through IE). In addition, mRNA levels of the TRAP/acid phosphatase 5, a glycosylated monomeric metalloenzyme,\(^3\) was higher in IL-4–polarized macrophages (Figure 1H). Taken together, these data suggest that IL-4–polarized macrophages display a particular osteoclast phenotype with low expression and activity of cathepsin K.

Because 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 osteopontin in resting, but not in IL-4–polarized macrophages (Figure 3A through 3C). In line, RANKL/MCSF induced cathepsin K 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 cathepsin K 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 of IL-4 or not, 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 cathepsin K 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 and 4D). 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 and 4G), 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 cathepsin K-knockout mice, suggesting that reduced cathepsin K expression and activity are driving the impaired bone resorption capacity of IL-4–polarized macrophages.

Expression of the Cathepsin K Transcriptional Regulator NFATc-1 Is Impaired on IL-4 Polarization

To understand the molecular mechanisms underlying reduced cathepsin K expression on IL-4 polarization, we assessed whether IL-4 may modulate the expression of the cathepsin K transcriptional regulator NFATc-1 using a small interfering RNA–mediated silencing approach. Reducing NFATc-1 mRNA and protein in RANKL/MCSF-treated resting macrophages inhibited cathepsin K gene and protein expression (Figure 5A and 5B), 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 indicated that the NFATc-1 promoter is bivalent, that is, showing both the activating histone H3 lysine 4 trimethylation (H3K4me3) and repressive H3K27me3 marks (Online Figure III), the latter requiring removal for gene activation. Interestingly, the decreased NFATc-1 gene expression on 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 and 6B) and a significantly higher proportion of cells are MR+RANK+ as determined by flow cytometry analysis (Figure 6B and 6C). 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+ than in CD68+MR- macrophages (Figure 6E). Because 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. 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 because Erk1/2 regulates c-fos expression and stability, the effect of RANKL on c-fos activation was tested. In parallel to Erk1/2 phosphorylation in resting, but not in IL-4–polarized macrophages (Figure 6F). By contrast, p38 phosphorylation was not different (Online Figure IV).

In addition, NFATc-1 may regulate cathepsin K expression both directly and indirectly by controlling the expression of Blimp1 (B-lymphocyte–induced maturation protein-1), another osteoclast activator and repressor of antiosteoclastogenic genes. Indeed, Blimp1 expression was also repressed by IL-4 and its knockdown modulated cathepsin K expression (Online Figure V). Moreover, the transcriptional repressors B-cell lymphoma 6 (Bcl6) and interferon regulatory factor-8 increased on 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 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 remodeling, such as intima-media thickening and 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. Nevertheless, within atherosclerotic lesions, calcification can have dual effects, depending on the pattern and type of calcium deposition. Although spotted and granular calcifications, because of proinflammatory processes, are associated with plaque progression toward an unstable rupture-prone phenotype, larger laminated macrocalculations...
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 that express both the MCSF and RANKL receptors\(^ {45}\) can be activated by their ligands produced by endothelial cells, VSMC, and monocyte/macrophages themselves and can thus potentially differentiate into osteoclast-like cells. Cells positive for TRAP have been observed in human atherosclerotic plaques.\(^ {46}\) CA2 staining colocalizes with the macrophage marker CD68 in human atherosclerotic lesions.\(^ {20}\) Moreover, cathepsin K is expressed in macrophages of advanced atherosclerotic plaques where it contributes to proteolytic tissue-remodeling 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 because they express both CD68 and MR.\(^ {25,47}\) This macrophage subpopulation is characterized by lower gene expression levels of cathepsin K, a phenotype also observed in vitro on IL-4 polarization, accompanied by a reduced protein expression and activity.

These alternative macrophages display a particular phenotype with regard to VC: although 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 on 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 cathepsin K–deficient mice,\(^ {47}\) suggesting that the altered cathepsin K expression and activity observed in these macrophages are 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. Furthermore, it has been suggested that IL-4 abrogates osteoclastogenesis in mouse cells by inhibiting nuclear factor-kB activation and several MAP kinase signaling 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 on 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).

The fact that CD68+MR+ macrophages accumulate around calcified areas could be the consequence of their incapacity to resorb preexisting calcium phosphate crystals, derived, for example, from apoptotic death of VSMC that have acquired an osteogenic profile. On the contrary, this calcification might occur spontaneously because of 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 neovascularized and hemorrhagic zones. Thus, it seems 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 cathepsin K, a protease also involved in plaque destabilization, in these CD68'MR' macrophages, can suggest a role for these macrophages in plaque stability. Indeed, cathepsin K 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 extracellular matrix. However, given the potential different effects of micro- and macrocalcifications 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 VC 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. Because VC rarely regresses, the major goals are prevention of formation and stabilization of existing calcifications. Because intimal calcification relates to atherosclerosis, the general treatment approach is

![Figure 7](http://circres.ahajournals.org/)

**Figure 7.** Schematic summary of main findings from this study (see Discussion of this article for details). CA2 indicates carbonic anhydrase type II; CTSK, cathepsin K; ERK, extracellular signal-regulated kinases; IL, interleukin; MITF, microphthalmia transcription factor; NFATc-1, nuclear factor of activated T cells type c-1; P38, RANK, receptor activator of nuclear factor kB; RANKL, RANK ligand; and TRAF, TNF receptor-associated factor.
nonspecific: lipid-lowering therapies, use of aspirin, treatment of obesity and hypertension, physical activity, smoking cessation, and treatment of diabetes mellitus. 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 to modulate vascular calcium deposition.

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Disclosures

None.

References


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

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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, **p<0.01 and ***p<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).
### Online Table I. Primers used for qPCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>sequence (5' to 3')</th>
<th>Gene name</th>
<th>sequence (5' to 3')</th>
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<tbody>
<tr>
<td>Mannose Receptor (MR)</td>
<td>Forward: GCA ATC CCG GTT CTC ATC GC&lt;br&gt;Reverse: CGA GGA AGA GGT TCG GTT CAC C</td>
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<td></td>
<td>Reverse: GGA AGA GGT TCG GTT CAC C&lt;br&gt;Reverse: CGA GGA AGA GGT TCG GTT CAC C</td>
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<td>Cathepsin K (CTSK)</td>
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<td>Carbonic anhydrase II (CA2)</td>
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<td>RANK</td>
<td>Forward: TGC CTT GCC GGC TAC TTC CTG&lt;br&gt;Reverse: TGG AAA CCG CAT CCG ATT CCG AG</td>
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<tr>
<td>TRAF5</td>
<td>Forward: CAC TGC ATC CTG TCC CTG AG&lt;br&gt;Reverse: GAA GGT GAT CCT GGT ACC GG</td>
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<tr>
<td>NFATc-1</td>
<td>Forward: GTG CAA GCC GAA TTC TCT GGT G&lt;br&gt;Reverse: ACT GGC TTC GCT TCC TCC C</td>
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<td>Bcl6</td>
<td>Forward: CAG CCA CAA GAC AGT CCA TAC C&lt;br&gt;Reverse: CGC AGG TTT CCG ATT TGT AGG G</td>
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<tr>
<td>Blimp-1</td>
<td>Forward: CTG AAG GCC CAT CCG AGA GTG C&lt;br&gt;Reverse: TGG CTT TCC TCT CCA GTG CAC C</td>
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<td>IRF8</td>
<td>Forward: CAC ACC ATC TGG GAG CAA AGA&lt;br&gt;Reverse: CTT ATC ACC CAC TCG GCC GTC AG</td>
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<td>NFATc-2</td>
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<tr>
<td>Osteopontin (OPN)</td>
<td>Forward: AGG CTC ATT CCG GAA GGT ACT CTG&lt;br&gt;Reverse: ACT CCT CGC TTT CCA TGT GTG AGG</td>
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<td>TRAP/ACP5</td>
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<td>MMP-9</td>
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<td>NBc1</td>
<td>Forward: AGA GGG GGT TAC AGG GCT AA&lt;br&gt;Reverse: TGG TTA GCA GGC ATT CCA AA</td>
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<tr>
<td>HSPE-1</td>
<td>Forward: GAC GGC TAA GAT GCT GAA GAG C&lt;br&gt;Reverse: CTT GCT AGC AGT CCG TCC ATT C</td>
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<td>Clusterin (CLU)</td>
<td>Forward: GAG ATC TGG TCT GTG GAC TG&lt;br&gt;Reverse: GAC TTT AGC AGG TGG TGG TA</td>
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<td>Forward: ACC AGG CAG GTG CAT GTC C&lt;br&gt;Reverse: CAC ACA TCA ATA ACC CCG TCA</td>
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<td>Cyclophilin</td>
<td>Forward: GCC TAC GGG TCG CAT CTT GTC C&lt;br&gt;Reverse: ATG GTG ATC TTC TGG TGG TGC TTG C</td>
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Online Table II. Primers used for ChIP-qPCR assays

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<tr>
<td><strong>NFATC1 Enhancer 1</strong></td>
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<td>ACTAGAGCCAGATACCGGA</td>
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<td>GCCGAGGATCCTGTATGG</td>
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<td>ATACCGGTAGTTTACAGCCCCGA</td>
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<tr>
<td><strong>NFATC1 Enhancer 3</strong></td>
<td>GCTTGTGCTGTGGAAGAGTGT</td>
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<td>GCGGATGACACCTACGTT</td>
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<td>TCTTCATGGACTGTACGTGAA</td>
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