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Reduced calcification and osteogenic features in advanced atherosclerotic plaques of mice with macrophage-specific loss of TRPC3

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Abstract

Background and aims—Recent *in vitro* studies have showed that in macrophages deletion of the non-selective Ca^{2+}-permeable channel TRPC3 impairs expression of the osteogenic protein BMP-2. The pathophysiological relevance of this effect in atherosclerotic plaque calcification remains to be determined.

Methods—We used *Ldlr*$^{-/-}$ mice with macrophage-specific loss of TRPC3 (*MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$) to examine the effect of macrophage Trpc3 on plaque calcification and osteogenic features in advanced atherosclerosis.

Results—After 25 weeks on high fat diet, aortic root plaques in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice showed reduced size, lipid and macrophage content compared to controls. Plaque calcification was decreased in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice, and this was accompanied by marked reduction in BMP-2, Runx-2 and phospho-SMAD1/5 contents within macrophage-rich areas. Expression of Bmp-2 and Runx-2 was also reduced in bone marrow-derived macrophages from *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice.

Conclusions—These findings show that, in advanced atherosclerosis, selective deletion of TRPC3 in macrophages favors plaque regression and impairs the activity of a novel macrophage-associated, BMP-2-dependent mechanism of calcification.

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CONFLICT OF INTEREST

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Keywords

TRPC3; macrophage; vascular calcification

INTRODUCTION

Calcification is a recurrent feature of advanced atherosclerotic plaques in humans and in mouse models of the disease[1, 2]. To some extent, the mechanism mediating plaque calcification recapitulates the osteogenic process encompassing bone formation, and involves key regulators of osteogenesis such as bone morphogenetic protein-2 (BMP-2) and Runt-related transcription factor 2 (Runx-2)[3], and a yet to be fully understood interplay between endothelium, smooth muscle cells and macrophages [4–6].

Macrophages are believed to promote calcification by stimulating the osteogenic program in smooth muscle cells through the release of cytokines [5, 6] and also by becoming and investing themselves as osteoclast-like cells in the calcifying plaque [5]. *In vitro*, macrophages from human and murine origin are known to produce BMP-2[7]. Yet, the relevance of any of these mechanisms in plaque ossification in atherosclerosis remains unclear.

Transient Receptor Potential Canonical 3 (TRPC3) is a non-selective Ca^{2+}-permeable channel that belongs to the TRPC family (TRPC1-7) of cation channels [8]. In recent studies, we showed that TRPC3 is an obligatory signaling component of endoplasmic reticulum stress-induced apoptosis in macrophages[9]. The pathophysiological relevance of this is evident in atherosclerotic mice with macrophage-selective deletion of *Trpc3*, in which advanced plaques have reduced necrosis and number of apoptotic macrophages compared to control animals, indicative of more stable plaques[10]. In a more recent study, we observed that *Trpc3* deletion in macrophages impairs the expression of Bmp-2 and Runx-2 in these cells[11]. All these effects led us to speculate that TRPC3 might be an important component of a novel macrophage-associated mechanism that stimulates plaque calcification. To test this, in the present work, we used low-density lipoprotein receptor knockout ($Ldlr^{-/-}$) mice with macrophage-specific loss of TRPC3 to examine the effect of macrophage Trpc3 on calcification and osteogenic features of advanced atherosclerotic lesions. Our findings indicate that in this setting, TRPC3 favors plaque regression and is an obligatory component of a novel macrophage-associated, BMP-2-dependent osteogenic mechanism that stimulates calcification. In addition, this work provides the first *in vivo* evidence supporting the notion that macrophages retain their ability to produce osteogenic mediators in the plaque setting.

MATERIALS AND METHODS

Experimental animals

All animal studies described in this work conform to the Guide for Care and Use of Laboratory Animals published by the NIH and have been approved by the University of Toledo IACUC. Generation and characterization of LysMCre $^{+/-}$/Trpc3$^{lox/lox}$ mice on C57BL/6 background (for simplicity, *MacTrpc3*$^{-/-}$) was described in detail [9]. *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice were generated by crossing *MacTrpc3*$^{-/-}$ with low density lipoprotein receptor

knockout ("*Ldlr*$^{-/-}$", B6.129S7-Ldlr$^{tm1Her/J}$, Jackson Labs, ME) for 10 generations, as we described [10]. Colonies were maintained in our animal facility (Division of Laboratory Animal Research, University of Toledo). Total plasma cholesterol and triglycerides were determined using Cholesterol-E and L-Type Triglyceride-M (Wako Chemicals USA, Inc.) and lipoprotein profiles were evaluated by FPLC using Superose 6 column (Pharmacia, Piscataway, NJ), followed by cholesterol assay of the fractions [12].

Preparation of bone marrow-derived macrophages

Culture of bone marrow-derived macrophages was performed as in [9].

Aortic root sectioning and plaque analysis

Aortic root sections were prepared as described in [10, 12]. Briefly, euthanized mice were perfused through the left ventricle (4% paraformaldehyde followed by PBS) and the heart was cut, keeping all three aortic valves in the same geometric plane. The upper portion of the heart was embedded in O.C.T., frozen in the Peltier stage of the cryostat (Thermo-Scientific Allan-HM550) and sections (10 μm) collected onto Fisher-Superfrost-Plus-coated slides, starting from where aorta exits the ventricle and moving towards the aortic sinus according to the scheme that we described [13]. Lesion analysis, Oil Red O (ORO) and hematoxylin and eosin stainings were as described [10, 12]. Alizarin Red staining to evaluate presence of calcium-hydroxyapatite deposits was performed as described [5]. Stained areas were captured (Micropublisher 3.3 Megapixel Cooled-CCD Color Digital Camera) and measured (NIS-Elements D).

Laser capture microdissection (LCM)

$CD68^+$ cells in plaques from aortic root sections (6 μm) were isolated by LCM (Arcturus-PixCell II Instrument) following protocols that we described [10]. Two pools of RNA per group (3 aortas each; ~1,300 $CD68^+$ cells per aorta) were used to measure expression of Trpc3, markers of M1- (iNOS, Arg2, TNFα, IL-12) and M2-like (Arg1, CD206, Ym1, Fizz1) macrophages, netrin-1 and Ccr7 by qRT-PCR. Primers used were as in[9, 12]. LCM-derived RNA was highly enriched (~35–40 fold) in CD68 mRNA compared to whole-section RNA, whereas *α-Sma* mRNA (smooth muscle cell) was negligible; *cyclophilin-A* was the control gene.

Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was performed as previously described[9]. Total RNA was prepared from bone marrow-derived macrophages (Rneasy Mini-Kit, Qiagen) or from aortic lysates (Rneasy-Fibrous Tissue Mini-Kit, Qiagen) according to manufacturer's instructions. cDNA was synthesized with oligo dT primers and reverse transcriptase (Applied Biosystems cDNA-RT kit #4368814) and used for qRT-PCR using TrueAmp SYBR-green qPCR supermix (Applied Biosystems, CA). The relative amount of mRNA was normalized relative to *Gapdh*. The primers used were as follows: *BMP-2* (F:AACTGGCTAGAATATTAAGCACTGCA; R:AGTGATTTCCTAACTGCCCAGG); *Runx-2* (F:GGGCACAAGTTCTATCTGGAAAA; R:CGGTGTCACTGCGCTGAA); *ALP*

(F:GTGACTACCACTCGGGTGAAC; R:CTCTGGTGGCATCTCGTTATC); *Gapdh* (F:AGGTCGGTGTGAACGGATTTG; R:TGTAGACCATGTAGTTGAGGTCA).

Immunohistochemistry

Immunohistochemistry was performed using Vectastain Elite-ABC kit (Vector Labs) following manufacturer's instructions. Sections were fixed in acetone and after quenching endogenous peroxidase processed for immunostaining for CD68 (#MCA1957GA, Bio-Rad), phospho-Smad1/5 (#9516, Cell signaling), Runx-2 (#ab102711, Abcam), or BMP-2 (#ab14933, Abcam). After treatment with secondary antibodies (for CD68: biotinylated goat anti-rat antibody #BA-9401, Vector laboratories; for p-smad1/5, Runx-2 and BMP-2: biotinylated goat anti-rabbit antibody, Vectastain Elite ABC kit, Vector laboratories), sections were incubated with Vectastain ABC reagent and peroxidase substrate solution (Vector lab). Counterstaining was done with hematoxylin. Negative controls: primary antibodies were substituted with non-immune IgG (same species and at the same concentration). Nonspecific immunostaining was not detected. Stained areas were captured (Micropublisher 3.3 Megapixel Cooled-CCD Color Digital Camera) and measured (NIS-Elements D).

Statistical analysis

Values are shown as mean ± SEM. Statistical analysis of qRT-PCR data was determined with Student's *t*-test, and that of histomorphometric data with non-parametric Mann-Whitney test (Prism GraphPad v.6 for Windows 2007, GraphPad Software, San Diego, CA). p values <0.05 were considered significant.

RESULTS

Six week-old female $Ldlr^{-/-}$ mice with macrophage-specific loss of TRPC3 ($MacTrpc3^{-/-}/Ldlr^{-/-}$, n=12) or controls ($Ldlr^{-/-}$, n=12) were placed on high-fat diet (HFD) for 25 weeks. At sacrifice, body weight, total cholesterol and triglycerides, and lipoprotein profiles were similar in both groups (not shown). Analysis of aortic root sections showed that, compared to control animals, $MacTrpc3^{-/-}/Ldlr^{-/-}$ mice had significant reductions in plaque size (~40%) [986,772 ± 53,341 μm^2 *vs.* 690,364 ± 56,466 μm^2, for $Ldlr^{-/-}$ and $MacTrpc3^{-/-}/Ldlr^{-/-}$ mice, respectively, $p = 0.03$], lipid content (~70%) [505,200 ± 48,730 μm^2 *vs.* 163,100 ± 16,370 μm^2, for $Ldlr^{-/-}$ and $MacTrpc3^{-/-}/Ldlr^{-/-}$ mice, respectively, $p = 0.0002$; Fig. 1A] and macrophage content [CD68-positive area as percent of total lesion area: 29.60 ± 1.80% *vs.* 18.19 ± 1.1%, for $Ldlr^{-/-}$ and $MacTrpc3^{-/-}/Ldlr^{-/-}$ mice, respectively, p= 0.007; Fig. 1B]. To gain insights into potential mechanisms associated to these regressive features of plaques in $MacTrpc3^{-/-}/Ldlr^{-/-}$ mice, we isolated CD68$^+$ cells (macrophages) from aortic root plaques from both groups of mice using laser capture microdissection (LCM), and evaluated expression of markers of M1- and M2-like macrophage subsets, and netrin-1 and the chemokine receptor Ccr7, which play key roles in macrophage retention in and egress from plaques[14, 15], respectively. Compared to $Ldlr^{-/-}$ mice, LCM-captured CD68$^+$ cells from plaques of $MacTrpc3^{-/-}/Ldlr^{-/-}$ mice were enriched in the M2-like markers Arg1, CD206, Ym1 and Fizz1 (Fig. 1C). Netrin-1 and Ccr7 were, respectively, reduced and increased. The ~16-fold reduction in Trpc3 in LCM-captured macrophages

from *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice confirms efficient Cre-mediated deletion of *Trpc3* in the plaque, as previously reported[10]. Calcification was clearly visible in aortic root plaques from both groups of mice, although it was markedly decreased in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice (Fig. 2A). The mRNA levels of the osteogenic proteins Bmp-2 and Runx-2 and the osteogenic marker alkaline phosphatase (ALP) were greatly reduced in aortic lysates from *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice compared to controls (Fig. 2B). In agreement with this, plaques from *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice showed more than 60% reduction in immunoreactivity for BMP-2 and Runx-2 compared to *Ldlr*$^{-/-}$ mice (Fig. 3A and B). Phospho-SMAD1/5, an early indicator of BMP-2 related signaling, was decreased by more than 80% in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice (Fig. 3C). Interestingly, most of the immunoreactivity for BMP-2, Runx-2 and phospho-SMAD1/5 was contained within macrophage rich (CD68^{+}) areas. mRNA levels of Bmp-2, Runx-2 and ALP were also reduced in bone marrow-derived macrophages prepared from *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice compared to controls (Fig. 3D).

DISCUSSION

In a recent *in vitro* study aimed at examining the osteogenic potential of polarized macrophages, we reported that deletion of the TRPC3 channel in macrophages impaired expression of the osteogenic regulators Bmp-2 and Runx-2[11]. Also important, these studies revealed the existence of a prominent constitutive autocrine/paracrine BMP-2 osteogenic signaling in macrophages, which prevented *in vitro* examination of the true effects of macrophage TRPC3 on calcification[11]. In the present work, we maintained *Ldlr*$^{-/-}$ mice with macrophage-specific deletion of Trpc3 (*MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$) on a HFD for 25 weeks, to promote calcification of advanced atherosclerotic plaques. *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice had markedly reduced calcification and this was clearly associated with reduced plaque content of the osteogenic proteins BMP-2 and Runx-2 and of phospho-SMAD1/5, suggesting impaired BMP-2 associated signaling in the lesion environment. Remarkably, bone marrow-derived macrophages prepared from *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice also exhibited a marked decrease in Bmp-2 and Runx-2 expression, similar to our observations in polarized macrophages[11]. That macrophages from human or murine origin can produce BMP-2, at least *in vitro*, is not new[7]. What our findings reveal, however, is that their ability to produce osteogenic proteins seems to be retained in the plaque setting, and that such property has an obligatory dependence on TRPC3 function.

In a recent study on *Ldlr*$^{-/-}$ mice fed a HFD for 14 weeks, we found that deletion of *Trpc3* in macrophages resulted in reduced plaque necrosis at the expense of decreased macrophage apoptosis[10]. Since apoptotic bodies are important nucleation centers for passive mineral deposition, it is conceivable that the reduced calcification in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice is in part due to reduced presence of apoptotic macrophages[10]. The studies conducted after 14 weeks on HFD also showed that despite the beneficial effect on plaque stability, i.e., less necrosis and apoptotic macrophages, loss of TRPC3 function in macrophages was not accompanied by reductions in plaque size or macrophage content[10]. In the present work, mice were kept on HFD for eleven more weeks for a total of 25 weeks, and notably, plaque volume, lipid content and cellularity were all markedly reduced in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ animals. Our findings on LCM-captured macrophages from plaques of *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice showing enrichment in markers of M2 macrophages are consistent with the regressive

features of plaques in these animals. The decrease in netrin-1 levels and increased Ccr7 expression suggest that reduced plaque $CD68^+$ cell content could be mediated by macrophage egress. Noteworthy, we previously showed that M1 macrophages deficient in Trpc3 are enriched in processes linked to motility and exhibit increased *in vitro* migration[16]. Although awaiting experimental validation, it is likely that this to some extent contributes to the plaque regression features observed here.

Studies on the role of macrophages as part of cell-driven processes that stimulate vascular calcification have traditionally been focused on their capacity to stimulate the osteogenic program in smooth muscle cells *via* release of tumor necrosis factor-α or interleukin-6 [5, 6], their ability to invest themselves in the ossification process as osteoclast-like cells [5], or producing –unknown- factors that regulate BMP-2-dependent calcification in the plaque milieu[17]. Yet, the significance of these mechanisms in atherosclerotic plaque ossification remains unclear. Our findings confirm the relevance of the BMP-2 signaling in controlling calcification in atherosclerosis, but most notably, point to the existence of a macrophage-associated BMP-2 process that stimulates plaque ossification.

The novelty of the present work is thus threefold. First, it indicates that in the setting of advanced atherosclerosis, TRPC3 is an obligatory component of a macrophage associated, BMP-2-dependent mechanism that stimulates calcification. Second, it shows for the first time that at advanced stages of atherosclerosis, loss of TRPC3 function in macrophages favors plaque regression. Third, it provides the first *in vivo* evidence supporting the notion that macrophages can produce osteogenic mediators, e.g. BMP-2, and that this ability is retained in the plaque setting. These findings offer novel opportunities to explore strategies aimed at alleviating plaque calcification.

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HIGHLIGHTS

- TRPC3 is a component of a novel mechanism that stimulates plaque calcification.
- A macrophage-associated, BMP-2 dependent process stimulates plaque calcification.
- First evidence suggesting that macrophages retain ability to produce BMP-2 *in vivo*.

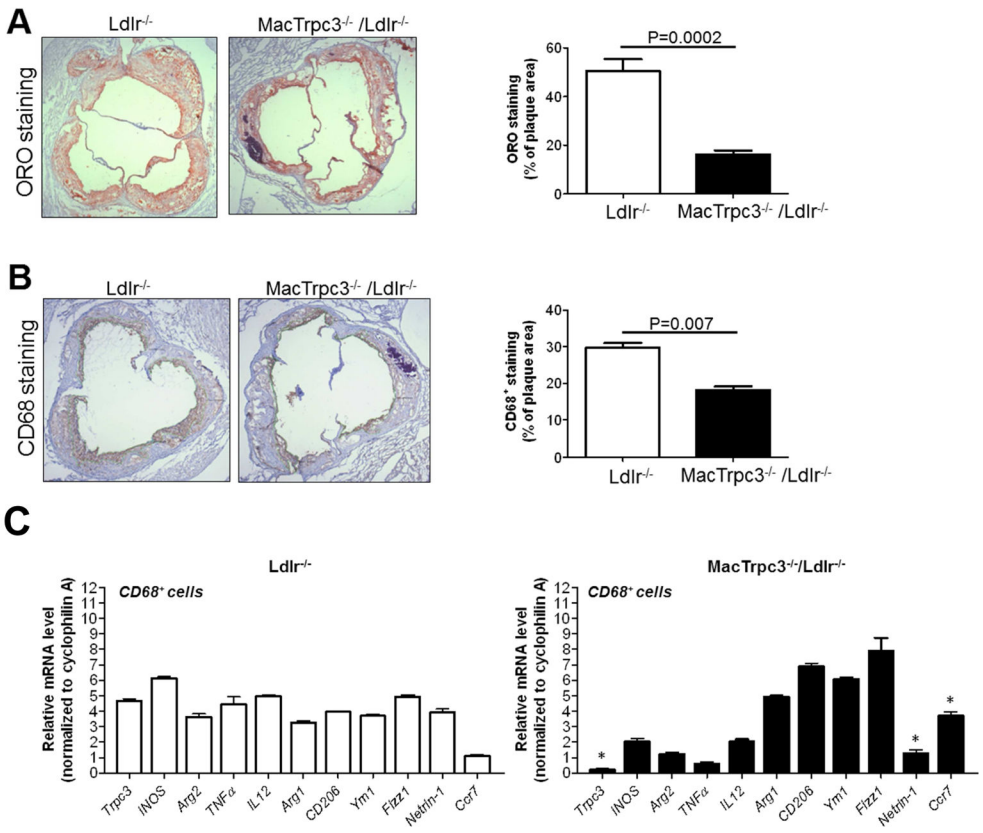

Figure 1. Plaque regression in mice with macrophage-specific deletion of *Trpc3*

Aortic root sections from *Ldlr*$^{-/-}$ or *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice maintained on a high-fat diet for 25 weeks were stained with (A) Oil-Red-O (ORO) for lipid content or (B) anti-CD68 antibody to evaluate macrophage content. Quantitation of stained areas is shown as percent of total plaque area. Magnification: 50x. (C) CD68^{+} cells (macrophages) from aortic root plaques of *Ldlr*$^{-/-}$ or *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice were isolated by laser capture microdissection and expression of the indicated genes was evaluated by qRT-PCR. Differences in levels of M1 (iNOS, Arg2, TNFα, IL-12) *vs.* M2 (Arg1, CD206, Ym1, Fizz1) markers within the *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ group, in M2 markers in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ *vs.* *Ldlr*$^{-/-}$ groups, and in M1 markers in *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ *vs.* *Ldlr*$^{-/-}$ groups, had all p values <0.001.*p<0.0001 compared to netrin-1, Ccr7 and Trpc3 values in *Ldlr*$^{-/-}$ group. averages of results from 2 RNA pools per group (3 aortas each; ~1,300 CD68^{+} cells per aorta) are shown.

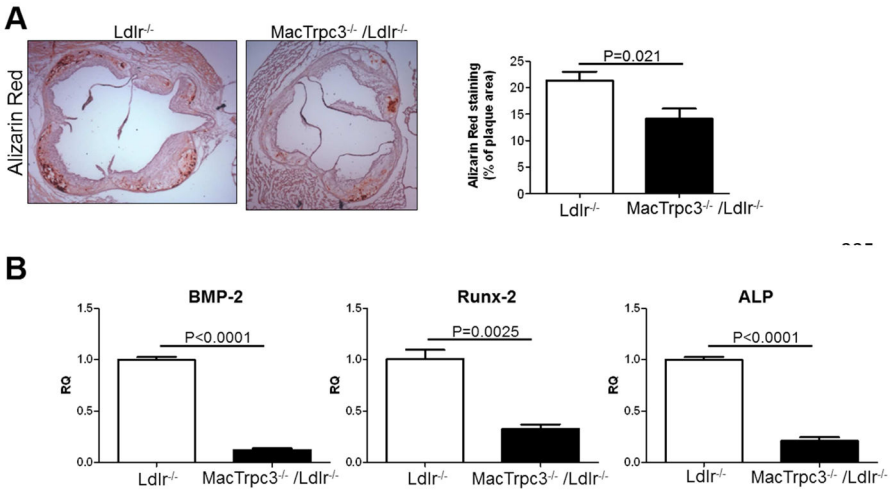

Figure 2. Reduced plaque calcification in mice with macrophage-specific deletion of *Trpc3*

Aortic root sections from *Ldlr*$^{-/-}$ or *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice maintained on a high-fat diet for 25 weeks were stained with (A) Alizarin red to determine calcification. Quantitation of stained areas is shown as percent of total plaque area. Magnification: 50x. (B) mRNA levels for *Bmp-2*, *Runx-2* and alkaline phosphatase (*ALP*) as evaluated by q-RT-PCR in aortic lysates prepared from *Ldlr*$^{-/-}$ or *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice. Graphs represent data (mean ± SEM) of three independent experiments each performed in triplicates.

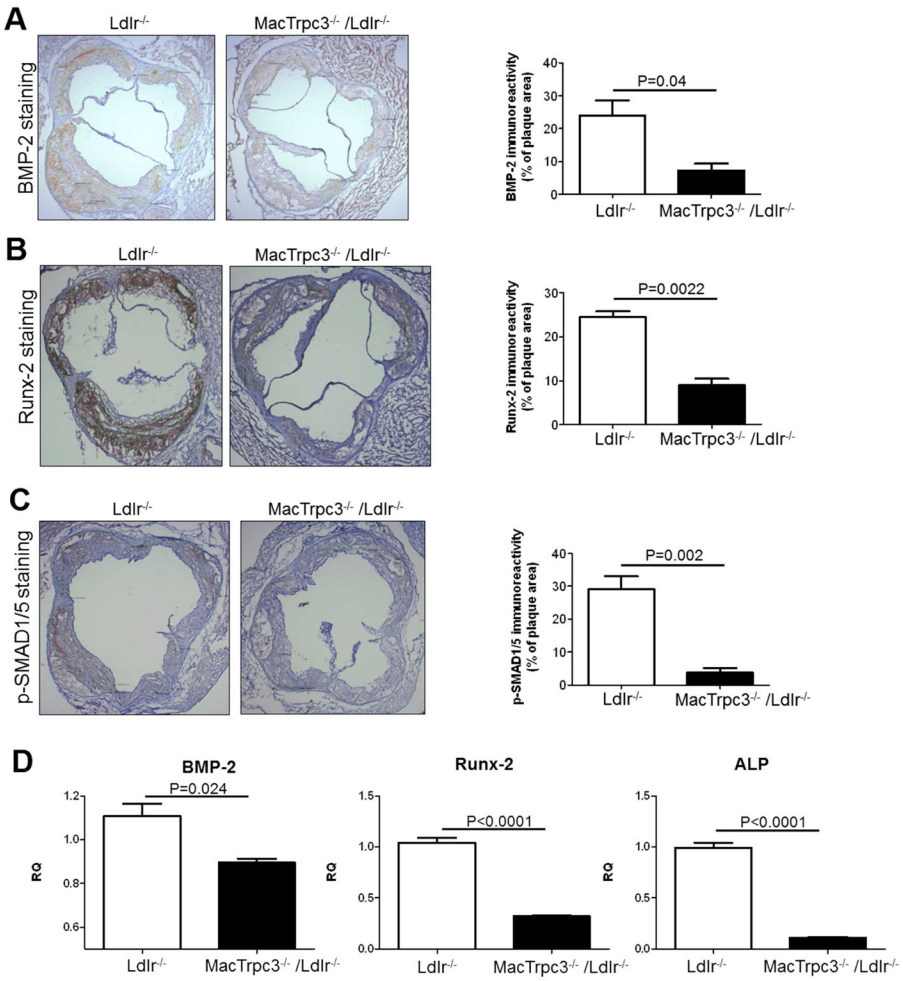

Figure 3. Reduced osteogenic features in plaques of mice with macrophage-specific deletion of *Trpc3*

Aortic root sections from *Ldlr*$^{-/-}$ or *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice maintained on a high-fat diet for 25 weeks were immunostained for (A) BMP-2; (B) Runx-2, or (C) phospho-SMAD1/5. Quantitation of stained areas is shown as percent of total plaque area. Magnification: 50x. (D) mRNA levels for *Bmp-2*, *Runx-2* and alkaline phosphatase (*ALP*) as evaluated by q-RT-PCR in bone marrow-derived macrophages prepared from *Ldlr*$^{-/-}$ or *MacTrpc3*$^{-/-}$/*Ldlr*$^{-/-}$ mice. Graphs represent data (mean ± SEM) of three independent experiments each performed in triplicates.