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Macrophages play a unique role in the plaque calcification by enhancing the osteogenic signals exerted by vascular smooth muscle cells

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ABSTRACT

Vascular calcification is a major risk factor for the cardiovascular disease, yet its underlying molecular mechanisms remain to be elucidated. Recently, we identified that osteogenic signals via bone morphogenetic protein (BMP)-2 exerted by vascular smooth muscle cells (VSMCs) play a crucial role in the formation of atherosclerotic plaque calcification. Here we report a synergistic interaction between macrophages and VSMCs with respect to plaque calcification. Treatment with conditioned medium (CM) of macrophages dramatically enhanced BMP-2 expression in VSMCs, while it substantially reduced the expression of matrix Gla-protein (MGP) that inhibits the BMP-2 osteogenic signaling. As a result, macrophages significantly accelerated the osteoblastic differentiation of C2C12 cells induced by VSMC-CM. In contrast, macrophage-CM did not enhance the osteoblastic gene expressions in VSMCs, indicating that macrophages unlikely induced the osteoblastic trans-differentiation of VSMCs. We then examined the effect of recombinant TNF-α and IL-1β on the VSMC-derived osteogenic signals. Similar to the macrophage-CM, both cytokines enhanced BMP-2 expression and reduced MGP expression in VSMCs. Nevertheless, only the neutralization of TNF-α but not IL-1β attenuated the effect of macrophage-CM on the expression of these genes in VSMCs, due to the very low concentration of IL-1β in the macrophage-CM. On the other hand, VSMCs significantly enhanced IL-1β expression in macrophages, which might in turn accelerate the VSMC-mediated osteogenic signals. Together, we identified a unique role of macrophages in the formation of plaque calcification in coordination with VSMCs. This interaction between macrophages and VSMCs is a potential therapeutic target to treat and prevent the atherosclerotic plaque calcification.

1. Introduction

Vascular calcification is one of the most important risk factors for cardiovascular disease, and is also a significant risk for all cause mortality [1,2]. Vascular calcification had been considered as a result of passive precipitation of circulating calcium and phosphate onto blood vessels, but many recent evidence has shown that vascular calcification is actively regulated through the machinery involving the genes that regulate bone calcification [3–8]. In the bone calcification, osteoblasts play a central role through the concomitant production of extracellular matrix and alkaline phosphatase [9]. Alkaline phospahtase breaks pyrophosphates that inhibit mineral precipitation, leading to the accelerated mineral deposition into the extracellular matrix. Osteoblasts are differentiated from mesenchymal precursor cells, and this differentiation is potently induced by bone morhogenetic protein (BMP)-2 [7,8]. On the other hand, matrix Gla protein (MGP) inhibits both mineral deposition and BMP-2 osteogenic signals, and therefore MGP is a crucial anti-calcification factor [10,11]. In fact, genetic deletion of MGP caused severe vascular calcification, and the $MGP^{-/-}$ mice die in 2 months due to the aortic rupture [12]. Of note, vascular smooth muscle cell (VSMC)-specific expression of MGP completely reversed the vascular calcification in $MGP^{-/-}$ mice, indicating that VSMC-derived MGP is essential to protect blood vessels from ectopic calcification [13].

Recently, we identified that dedifferentiated VSMCs play a crucial role in the formation of atherosclerotic plaque calcification [14]. Dedifferentiated VSMCs expressed higher BMP-2 and less MGP than differentiated VSMCs, and thus they efficiently induced the osteoblastic differentiation of precursor cells in vitro. Moreover, VSMC-specific overexpression of BMP-2 significantly enhanced the plaque calcification in vivo in apoE-deficient mice. Therefore, VSMC-derived osteogenic signals in the atherosclerotic intima are crucially involved in the formation of plaque calcification. On the other hand, macrophages play a significant role in the progression of atherosclerosis, and in the plaque calcification

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as well [15,16]. However, detailed functions of macrophages in the formation of plaque calcification remain to be elucidated. Here we investigated a possible interaction between macrophages and VSMCs, and found that macrophages potently accelerated the osteogenic signals exerted by VSMCs via soluble factors including TNF-α. Our data revealed a synergistic interaction between macrophages and VSMCs, which potentially play an important role in the formation of plaque calcification.

2. Material and methods

2.1. Materials

Neutralizing antibodies for TNF-α and IL-1β were obtained from R&D Systems (Minneapolis, MN). Recombinant TNF-α and IL-1β, and control rat IgG1 were also obtained from R&D Systems.

2.2. Cell culture

Human coronary artery vascular smooth muscle cells (HCASMCs) were obtained from Cambrex (Charles City, IA), and cultured in SmGM-2 medium (Cambrex). HCASMCs in passage 5–6 were used in all the experiments. RAW 264.7 and C2C12 cells were regularly cultured in DMEM supplemented with 10% FBS. To prepare the conditioned medium (CM) of macrophages, RAW 264.7 cells were incubated in the fresh DMEM supplemented with 5% FBS for 24 h, and thereafter culture medium was collected followed by the centrifugation to remove remaining cells. For the stimulation of HSASMCs with macrophage-CM, conditioned culture medium of RAW 264.7 cells was mixed with the fresh SmGM-2 medium at 1:1 ratio and the mixed culture medium was given to HCASMCs. Cells were incubated for 48 h before the RNA extraction. Cells incubated in the mixture of fresh SmGM-2 medium and fresh DMEM supplemented with 5% FBS were used as a control. To prepare the CM of VSMCs, HCASMCs were incubated in the fresh DMEM supplemented with 5% FBS for 24 h, and thereafter culture medium was collected followed by the centrifugation to remove remaining cells. For the stimulation of RAW 264.7 cells with VSMC-CM, conditioned culture medium of HCASMCs cells was mixed with the fresh DMEM supplemented with 5% FBS at 1:1 ratio and the mixed culture medium was given to RAW 264.7 cells. Cells were incubated for 48 h before the RNA extraction.

To prepare the CM of VSMCs pretreated with macrophages, HCASMCs were incubated in macrophage-CM for 24 h, and then washed with PBS for three times followed by incubation in the fresh DMEM supplemented with 5% FBS for 24 h. C2C12 cells were incubated either in VSMC-CM, CM of VSMCs pretreated with macrophage-CM, or fresh DMEM supplemented with 5% FBS (control) to analyze the osteoblastic differentiation.

For the neutralization of TNF-α and IL-1β, macrophage-CM was incubated with neutralizing antibody for TNF-α (10 μg/ml) or IL-1β (4 μg/ml) for 1 h at 37 °C, and thereafter the treated macrophage-CM was given to HCASMCs. Cells cultured in the macrophage-CM incubated with negative IgG at the same concentration as TNF-α or IL-1β were used as a control.

2.3. Quantitative PCR

Total RNA was extracted by using Trizol (Invitrogen, Carlsbad, CA) followed by purification with RNeasy MiniElute cleanup kit (Invitrogen). cDNA was synthesized from 2–5 μg of total RNA using SuperScript first-strand synthesis system (Invitrogen). PCR reac-

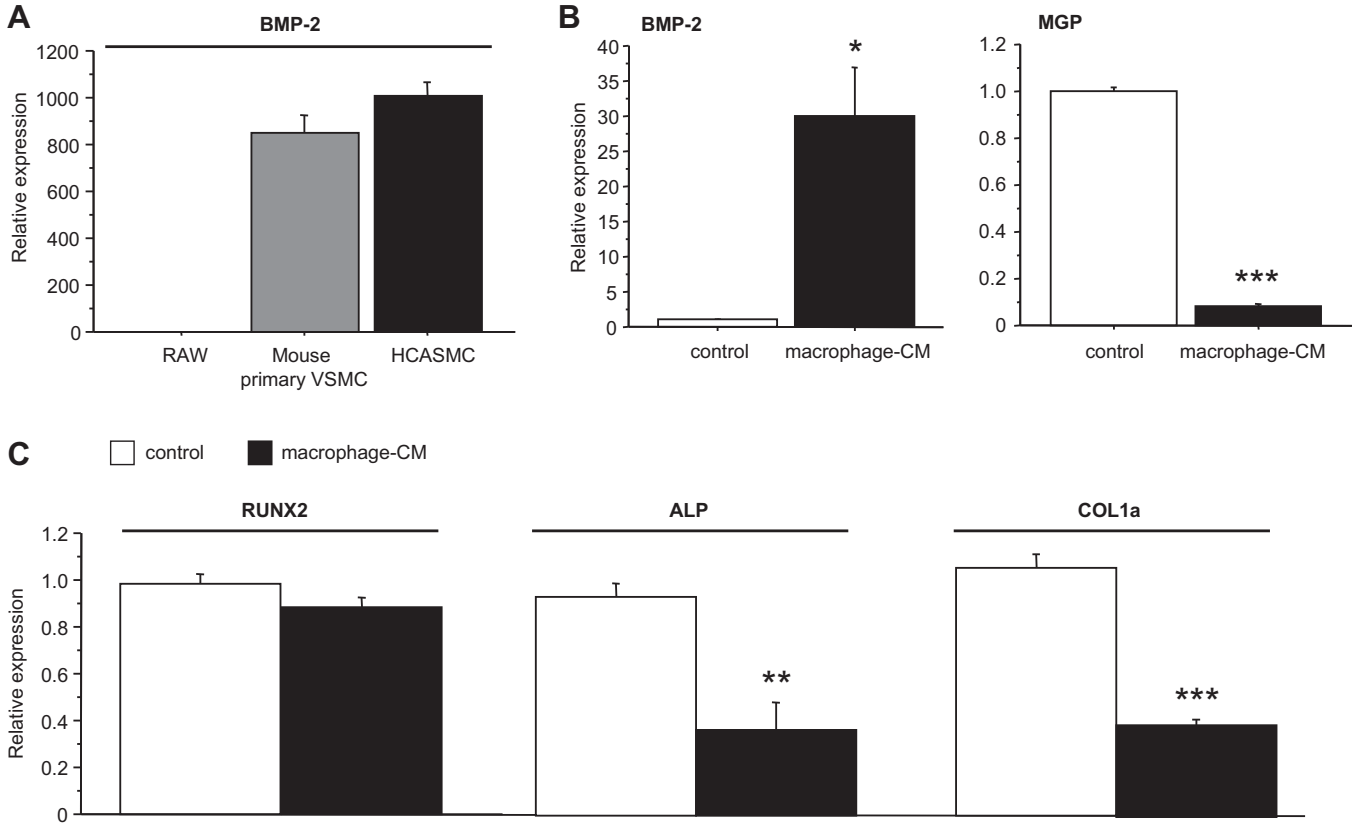

Fig. 1. Macrophages enhance the osteogenic signals exerted by VSMCs. (A) Quantitative analysis of BMP-2 mRNA in mouse macrophages (RAW), mouse primary VSMC or human coronary artery smooth muscle cell (HCASMC). (B) Expression of BMP-2 or MGP in VSMCs cultured in either control medium or conditioned medium of macrophages (macrophage-CM) ($^{*}p < 0.05$, $^{***}p < 0.0001$ versus control). (C) Osteoblastic gene expressions in VSMCs cultured in either control medium or macrophage-CM ($^{**}p < 0.001$, $^{***}p < 0.0001$ versus control). RUNX2: runt-related transcription factor 2, ALP: alkaline phosphatase, COL1a: type-I collagen alpha chain.

tions were prepared by using LightCycler FastStart DNA Masterplus SYBR Green I (Roche Applied Science, Auckland, New Zealand) followed by the real time PCR using LightCycler (Roche Applied Science) or by using CYBR Premie Ex TaqII (TaKaRa, Japan) followed by the real time PCR using Thermal Cycler Dice (TaKaRa). Target genes mRNA levels relative to β-actin or GAPDH was shown. Nucleotide sequence of each primer is shown in the Supplementary Table 1. For the comparison of BMP-2 expression in RAW 264.7 cells, mouse primary aortic VSMCs and HCASMCs, primers designed at the identical nucleotide for mouse and human BMP-2 were used. For all experiments, 2–3 independent cultured cells were used, and the results were regularly confirmed by 2–3 independent experiments.

2.4. Statistical analysis

All data are presented as mean ± S.E. Differences between groups were analyzed by Student's *t*-test or one-way ANOVA with post hoc multiple comparison by Bonferroni/Dunn test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Macrophages enhance the VSMC-derived osteogenic signals

We previously reported that VSMC-mediated osteogenic signals via BMP-2 play a crucial role in the formation of atherosclerotic plaque calcification both in vitro and in vivo [14]. Macrophages are profoundly involved in the progression of atherosclerosis, and have reported to modify the vascular calcification. Nevertheless, little is known about a possible interaction between macrophages and VSMCs with respect to the plaque calcification. We first examined the BMP-2 expression in macrophages and VSMCs. Mouse aortic VSMCs as well as human coronary artery SMCs abundantly expressed BMP-2 comparing with macrophages (RAW 264.7 cells) (Fig. 1A). This implied that major source of BMP-2 in the atherosclerotic plaque is VSMCs, and therefore we investigated the effect of macrophages on the osteogenic signals via BMP-2 exerted by VSMCs. When treated with conditioned medium (CM) of macrophages, BMP-2 expression was dramatically increased, while BMP-2 antagonist, MGP expression was substantially reduced in VSMCs (Fig. 1B). Of note, expressions of osteoblastic genes such as runt-related transcription factor (RUNX)-2, alkaline phosphatase and type-I collagen in VSMCs were not changed or rather reduced by the treatment wih macrophage-CM, indicating that macrophages unlikely induce the osteoblastic trans-differentiation of VSMCs (Fig. 1C). Moreover, VSMCs pre-treated with macrophage-CM induced osteoblastic differentiation of C2C12 cells more efficiently than VSMCs without the treatment (Fig. 2). These collectively indicate that macrophages enhance the osteogenic signals exerted by VSMCs through soluble factors.

3.2. TNF-α plays a major role in the enhanced BMP-2 expression in VSMCs treated with the macrophage-CM

Since macrophage-derived TNF-α and IL-1β are cytokines of major importance in inflammation and immunity, we examined

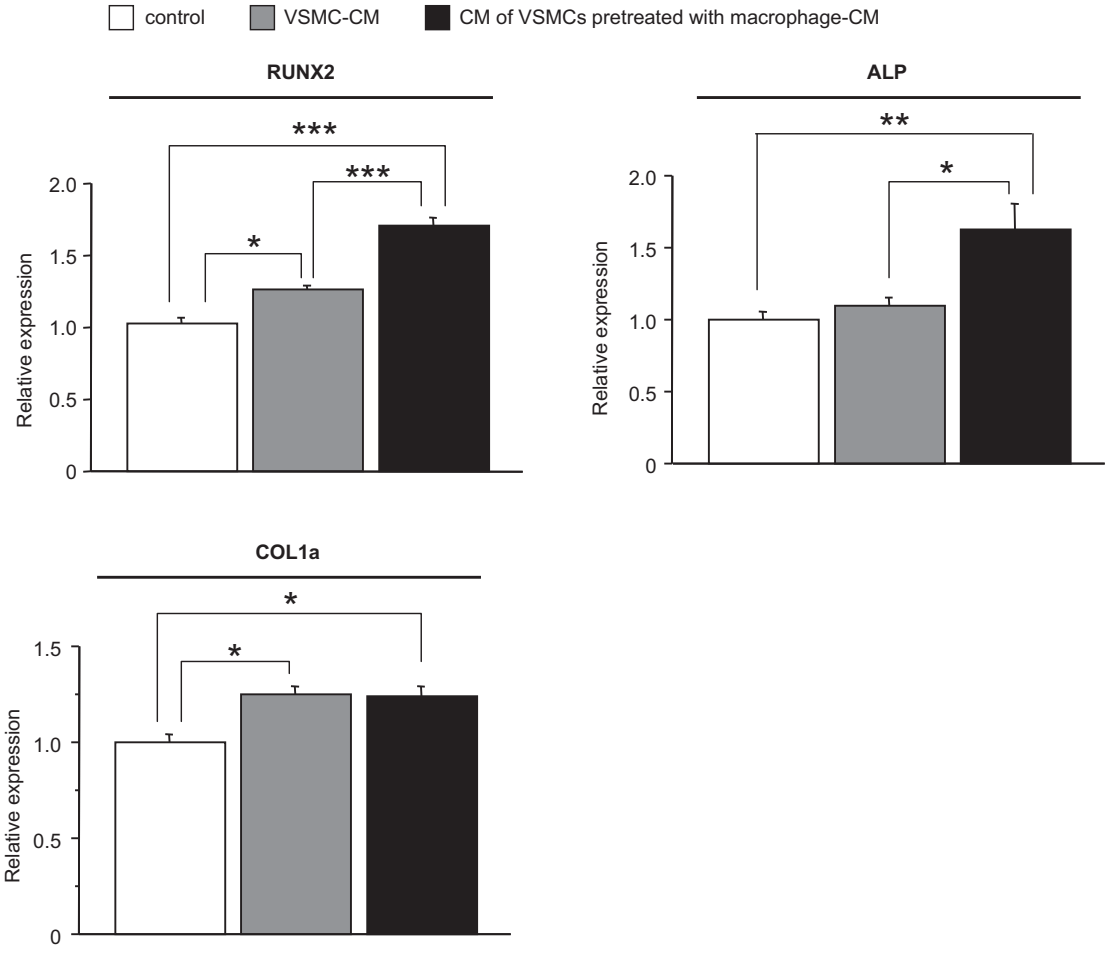

Fig. 2. Macrophages stimulate the VSMC-mediated osteoblastic differentiation of precursor cells. Expression of osteoblastic genes was quantitatively analyzed in C2C12 cells cultured in either control medium, VSMC-CM or CM of VSMCs pretreated with macrophage-CM (*$p < 0.005$, **$p < 0.001$, and ***$p < 0.0001$).

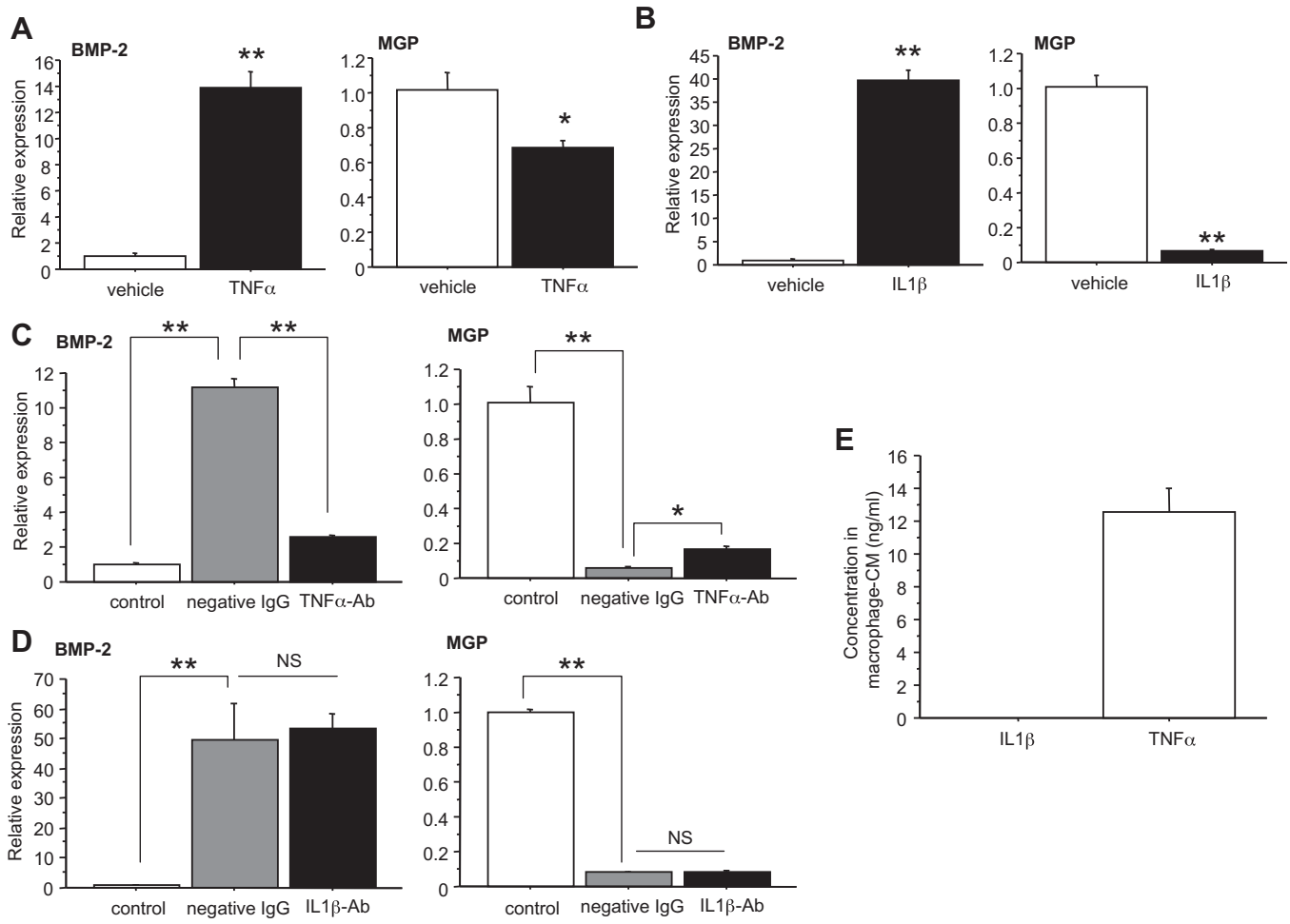

Fig. 3. Inflammatory cytokines modulate the VSMC-derived osteogenic signals. (A) Quantitative analysis of BMP-2 or MGP expression in VSMCs treated with either vehicle or 50 ng/ml recombinant TNF-α ($^{*}p < 0.05$, $^{**}p < 0.0001$ versus vehicle control). (B) Quantitative analysis of BMP-2 or MGP expression in VSMCs treated with either vehicle or 50 ng/ml recombinant IL-1β ($^{**}p < 0.0001$ versus vehicle control). (C) Macrophage-CM was incubated with normal IgG control (negative IgG) or neutralizing antibody for TNF-α (TNF α -Ab) before given to VSMCs. BMP-2 and MGP expressions were quantitatively analyzed in VSMCs cultured in either control medium or the pretreated macrophage-CM ($^{*}p < 0.05$, $^{**}p < 0.0001$ versus control). (D) Macrophage-CM was incubated with negative IgG or neutralizing antibody for IL-1β (IL1 β -Ab) before given to VSMCs. BMP-2 and MGP expressions were quantitatively analyzed in VSMCs incubated in either control medium or the pretreated macrophage-CM ($^{**}p < 0.0001$ versus control, NS; not significant). (E) Concentration of IL-1β or TNF-α in macrophage-CM was measured by enzyme-linked immunosorbent assay.

the effect of these cytokines on the expression of BMP-2 and MGP in VSMCs. Similar to the macrophage-CM, both recombinant TNF-α and IL-1β substantially enhanced BMP-2 expression in VSMCs (Fig. 3A and B). TNF-α modestly reduced MGP expression, while IL-1β caused dramatical reduction in MGP expression in VSMCs (Fig. 3A and B). To clarify whether TNF-α and/or IL-1β is responsible for the enhanced osteogenic signals in VSMCs induced by macrophages, we neutralized either TNF-α or IL-1β in the macrophage-CM. Of note, neutralization of TNF-α significantly reversed the enhanced BMP-2 expression, and modestly canceled the reduction of MGP expression in VSMCs treated with macrophage-CM (Fig. 3C). In contrast, neutralization of IL-1β failed to reverse the expression of these genes in VSMCs treated with macrophage-CM (Fig. 3D). Consistent with these findings, we detected a significant amount of TNF-α (~12.5 ng/ml) in the macrophage-CM, but IL-1β was barely detectable in the CM (~0.01 ng/ml) (Fig. 3E).

3.3. VSMC enhances IL-1β expression in macrophages

We then explored whether VSMCs affect the expression of inflammatory cytokines or calcification regulatory factors in macrophages. Treatment with VSMC-CM did not show significant effect on BMP-2 expression, while it tended to increase MGP expression ($P = 0.0613$) in macrophages (Fig. 4A). Of note, VSMC-CM significantly enhanced IL-1β expression, whereas it modestly reduced TNF-α expression in macrophages (Fig. 4A). Given that recombinant IL-1β caused substantial increase of BMP-2 and decrease of MGP in VSMCs, VSMC-mediated IL-1β induction in macrophages would in turn accelerate the osteogenic signals in VSMCs.

Together, we identified a synergistic interaction between macrophages and VSMCs in the formation of plaque calcification (Fig. 4B). Our data revealed a unique role of macrophages in the pathogenesis of plaque calcification, which further corroborates the molecular link between inflammation and plaque calcification.

4. Discussion

Vascular calcification is strongly associated with morbidity and mortality of cardiovascular disease [2,10,17,18]. Ectopic calcification largely occurs at two sites of arteries; tunica media where Monckeberg's sclerosis forms, and intima where it is often associated with atherosclerosis. Medial calcification is commonly observed in the elderly population, as well as in patients with diabetes mellitus and chronic kidney disease [19–22]. Although medial calcification does not encroach the vessel lumen, it

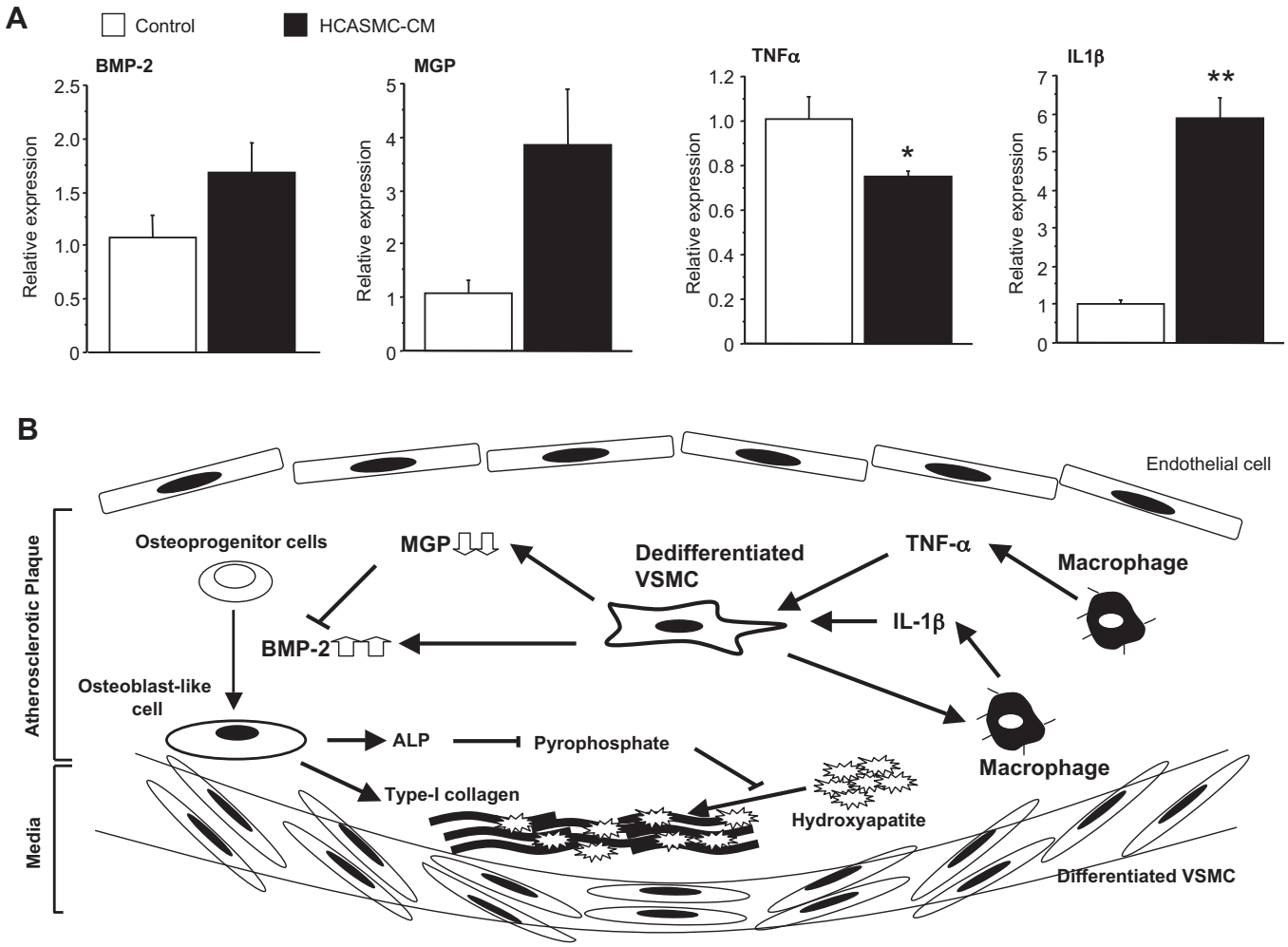

Fig. 4. Synergistic interaction between macrophages and VSMCs in the plaque calcification. (A) Expressions of BMP-2, MGP, TNF-α and IL-1β were quantitatively analyzed in macrophages incubated in either control medium or VSMC-CM ($^{*}p < 0.05$, $^{**}p < 0.0005$ versus control). (B) Proposed molecular mechanisms in the atherosclerotic plaque calcification. Macrophages and VSMCs coordinately and synergistically contribute to the plaque calcification by giving rise to the osteogenic signals in the plaque.

adversely affects the blood perfusion by stiffening the arteries. On the other hand, atherosclerotic plaque calcification is often associated with plaque instability, unsuccessful coronary intervention and balloon-induced coronary artery dissections [23,24]. Therefore, inhibition of vascular calcification is a promising approach to reduce the cardiovascular morbidity and mortality.

We have reported that cellular senescence of VSMCs induced phenotypical transition into osteoblast-like cells, which potentially causes the medial calcification associated with aging [25,26]. Also, we have identified a significant role of dedifferentiated VSMCs in the atherosclerotic intimal calcification by giving rise to the osteogenic signals via BMP-2 [14]. These unique and distinctive roles of VSMCs in the formation of medial and intimal calcification shed light on VSMCs as a key player in the vascular calcification. In the current study, we revealed a previously undescribed interaction between macrophages and VSMCs with respect to the plaque calcification. This newly identified interaction potentially accelerates and exacerbates the atherosclerotic plaque calcification.

VSMCs normally reside in the vessel wall media in a differentiated state wherein their contractile properties regulate the vascular tone. However, VSMCs have a unique feature characterized by the ability to enter a synthetic state of proliferation [27], and they dedifferentiate into synthetic type in response to injury and/or growth factors and cytokines, leading to the migration into the atherosclerotic intima to contribute to the progression of atherosclerosis [28–30]. In all the in vitro experiments, we used the growth medium supplemented with 5% FBS for the culture of VSMCs, and the conditioned medium of macrophages also contained 5% FBS. Therefore, VSMCs we used were in the dedifferentiated state. Since VSMCs localized in atherosclerotic intima are considered to be dedifferentiated, the interaction between macrophages and VSMCs we have shown in this study would take place in the atherosclerotic plaque, and it remains unclear whether similar interaction is applicable for the differentiated VSMCs that reside in the tunica media.

It has been reported that TNF-α is crucially involved in the osteoblastic trans-differentiation of precurcer cells including VSMCs [31,32]. Our data showed a new aspect of inflammatory cytokines, which stimulates the osteogenic signals exerted by VSMCs, in the molecular mechanisms for the plaque calcification. Inflammation is also involved in the process of VSMCs dedifferentiation, and thus it might play a primary role in the formation of atherosclerotic plaque calcification. Therefore, treatments that ameliorate vascular inflammation might effective to prevent plaque calcification, concurrently with the amelioration of atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.045.

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