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Targeting vascular endothelial growth factor ameliorates PMMA-particles induced inflammatory osteolysis in murine calvaria

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ABSTRACT

Cytokines and growth factors mediate inflammatory osteolysis in response to particles released from bone implants. However, the mechanism by which this process develops is not entirely clear. Blood vessels and related factors may be required to deliver immune cells and soluble factors to the injury site. Therefore, in the current study we investigated if, vascular endothelial growth factor (VEGF), which is required for angiogenesis, mediates polymethylmethacrylate (PMMA) particles-induced osteolysis. Using bone marrow derived macrophages (BMMs) and ST2 stromal cell line, we show that PMMA particles increase VEGF expression. Further, using a murine calvarial osteolysis model, we found that PMMA injection over calvaria induce significant increase in VEGF expression as well as new vessel formation, represented by von Willebrand factor (vWF) staining. Co-treatment using a VEGF-neutralizing antibody abrogated expression of vWF, indicating decreased angiogenesis. Finally, VEGF neutralizing antibody reduced expression of Tumor necrosis factor (TNF) and decreased osteoclastogenesis induced by PMMA particles in calvariae. This work highlights the significance of angiogenesis, specifically VEGF, as key driver of PMMA particle-induced inflammatory osteolysis, inhibition of which attenuates this response.

1. Introduction

Inflammatory osteolysis continues to pose a skeletal health concern especially in chronic settings fueled by the presence of bacteria or particles released from orthopedic implants. In this regard, it has been suggested that wear-debris particles at the bone-cement interface causes an inflammatory osteolytic reaction. Attempts to address these osteolytic processes have mainly targeted osteoclasts due to their bone-resorbing abilities [1]. To understand their role in aseptic loosening, research has turned to the removed implants, which have been associated with the formation of pseudo-membranes at the cement-bone interface. These membranes have been shown to have elevated levels of interleukin-1, tumor necrosis factor-α and prostaglandin E2 [2,3], both activate the NF-κB pathway and exacerbate osteoclasts. [4,5] [6–8].

Vasculature has long been known to play a role in bone metabolism by providing nutrients, minerals, structure, and a desirable environment for hematopoietic stem cell (HSC) growth and differentiation [9]. Recently, blood vessels have been ascribed to angiocrine functions as well, providing paracrine signaling to promote bone growth [10]. Therefore, it is thought that angiogenesis is required for the development, remodeling, and repair of bone tissue. Vascular endothelial growth factor (VEGF) has been recognized as the most important mediator of angiogenesis [11], and has been shown to directly enhance osteoclastic bone resorption and survival of mature osteoclasts [12,13]. Furthermore, many have shown that VEGF was detected in osteoblasts, osteoclasts, endothelial cells, fibroblasts and macrophages adjacent to bony inflammation [12,14–16]. This also lends confidence to the thought that VEGF, and therefore angiogenesis, are tied closely to bone metabolism.

It is well established that PMMA debris particles are major players in the induction of implant-induced osteolysis. Further, the close apparent relationship between angiogenesis and osteoclast activity in inflammatory osteolysis suggest that polymethylmethacrylate (PMMA) particles may induce expression of VEGF in osteoclast progenitors and mesenchymal cells to facilitate and enhance the inflammatory microenvironment through promotion of vascular infiltration.

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Moreover, it has been demonstrated that when bone marrow osteoclast precursors were treated with VEGF, this directly enhanced their differentiation, survival, and resorptive activities [13,17]. We have also seen that osteoclasts not only react to VEGF, but also express it, suggesting potential autocrine and paracrine mechanisms. Other studies have demonstrated that osteoclasts express VEGF in a size dependent manner, such that cells with a larger number of nuclei have an elevated level of expression compared to those with a smaller number of nuclei, and that patients with increased osteoarthritis (OA) severity had increased VEGF expression [18,19]. Furthermore, expression of VEGF was mediated by HIF-1, which in turn mediates RANKL and NF-κB [18]. In addition, several studies have shown that by blocking the VEGFR using various methods, growth differentiation and survival of osteoclasts are attenuated, hence restricting the extent of osteolysis. In one report, the use of a VEGFR-targeted tyrosine kinase inhibitor attenuated osteoclast differentiation and cancer-induced bone destruction [20]. In another, intra-articular injections with bevacizumab (a monoclonal IgG1 antibody targeting VEGF) in rabbits with OA showed a reduction in articular cartilage damage, osteophyte formation, and pain [21–23]. These reports highlight the role of VEGF in bone and joint pathology.

Currently, insufficient evidence exists to explain the relationship and effects of PMMA on VEGF expression, osteoclastogenesis, and inflammatory osteolysis in an *in vivo* model. Therefore, the present study seeks to answer several questions: 1) Do PMMA particles promote VEGF expression? 2) Do PMMA particles promote angiogenesis? 3) Does neutralization of VEGF inhibit inflammatory osteolysis? Thus, this work examines the effect of PMMA treatment on VEGF expression in murine bone marrow macrophages and ST2 mesenchymal cells *in vitro*. These studies are paired with studies in a well-established murine calvaria osteolysis model, which mimics the interactions of wear particles and various bone cells seen during aseptic loosening of orthopedic implants in humans. In this model we investigated the *in vivo* effects of PMMA on the expression of VEGF, and other angiogenic factors in the calvaria and the utility of VEGF neutralizing antibody to inhibit PMMA-mediated osteolysis.

2. Material and methods

2.1. Polymethylmethacrylate (PMMA) particles

Microsphere PMMA particles (Polysciences) 1–10 μm in diameter were used for all experiments as previously reported. PMMA particles, were first washed in ethanol four times, followed by overnight incubation in ethanol for sterilization, and then washed four times with PBS. Particles were later resuspended in serum-free MEM and stored at 4 °C. For *in vitro* experiments the cells were treated with 100 μg/ml of PMMA particles.

2.2. Cell culture

ST2 cells were cultured in RPMI 1640 culture media supplemented with 100 units/ml penicillin/streptomycin and 10% FBS (v/v) (Gibco, Thermo Fisher Scientific). Bone marrow macrophages (BMMs) were, differentiated from bone marrow cells (BMCs) isolated from tibiae and femurs of 2-month-old mice. Bone marrow cells were cultured in α-MEM supplemented with 100 units/ml penicillin/streptomycin and 10% FBS (v/v) with 10 ng/ml M-CSF for 16 h to separate adherent cells from non-adherent cells. Non-adherent cells were collected and further cultured with M-CSF (20 ng/ml) for 3 to 4 days to generate bone marrow macrophages.

2.3. Immunoblotting

ST2 cells and BMM were treated with PMMS (0.1% w/v) different time points. Total cell lysates were prepared in cell lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors (Thermofischer). Equal amount of proteins as determined by BCA kit were subjected to western blotting. Membranes were probed with VEGF primary antibody (R&D) and Actin (Sigma).

2.4. Animals

8–10 weeks old C57BL/6 wild type male and female mice (at approximate equal ratios) were, housed at the Washington University School of Medicine barrier facility. The mice were kept at 12 h day and night cycle with free access to water and food. All experimental protocols were carried out in accordance with the animal ethical guidelines approved by the Washington University School of Medicine Institutional Animal Care and Use Committee (IACUC). To induce calvarial osteolysis mice ($n = 10$ were injected with 100 μl of PMMA particles (1 mg/100 μl of PBS) over the calvarium. Corresponding control mice were injected with PBS only. At the same time mice were randomly divided and were administered with or without IgG ($n = 5$) or VEGF neutralizing antibody (AF-493-NA; R&D) (SC, 0.1 mg/kg BW/daily) ($n = 5$). The murine model of calvarial osteolysis is a well-established model to study aseptic loosening of joints with no adverse effect to the animals over a short period of PMMA administration. After 8 days, mice were sacrificed using CO_2 asphyxiation and the calvariae were collected from different groups for histological analysis.

2.5. Histology

At the end of the experiment the mice were sacrificed and the calvariae were surgically removed, preserved in 10% buffered formalin (24 h), and decalcified using 10% EDTA, pH 7.0 for 7 days. Calvariae were then dehydrated in graded alcohol, cleared through xylene and embedded in paraffin. Paraffin blocks were sectioned longitudinally. Five-micron sections were then stained for either tartrate-resistant acid phosphatase (TRAP) or for TNF, VEGF and von Willebrand Factor (vWF).

2.6. Data analysis

The *in vitro* western blot images represent three independent experiments with similar findings. For *in vivo* experiments, 5 animals were used in each treatment group. At the end of the treatment, all the animals were sacrificed and the calvariae were used for histology. The images were analyzed double blinded for qualitative assessment of staining and osteolysis. The photomicrographs represents the analysis of five calvariae sections from different animals.

3. Results

3.1. PMMA particles induce expression of VEGF by myeloid and stromal cells

PMMA particles have been shown to stimulate osteoclastogenesis *in vitro*, at least in part by induction of RANKL and TNF, and by activation of NF-κB and MAP kinases [24,25]. However, the osteolytic response *in vivo* comprises a wide range of cell types and circulating factors considered to be active constituents of inflammatory osteolysis. The flux of inflammatory and immune cells in response to PMMA particles involves robust secretion of chemokines and other factors by mesenchymal, myeloid and endothelial cells. We hypothesized that angiogenesis and angiogenic factors play a key role in the development and resolution of the inflammatory and osteolytic responses by promoting influx of inflammatory cells.

To investigate this, we first examined the expression of VEGF in myeloid and mesenchymal lineage cells in response to treatment with PMMA particles, which have been shown to evoke inflammatory and osteoclastogenic responses [8,24,26]. As seen in Fig. 1, both ST2 cells and BMMs expressed very little VEGF at baseline, but treatment with

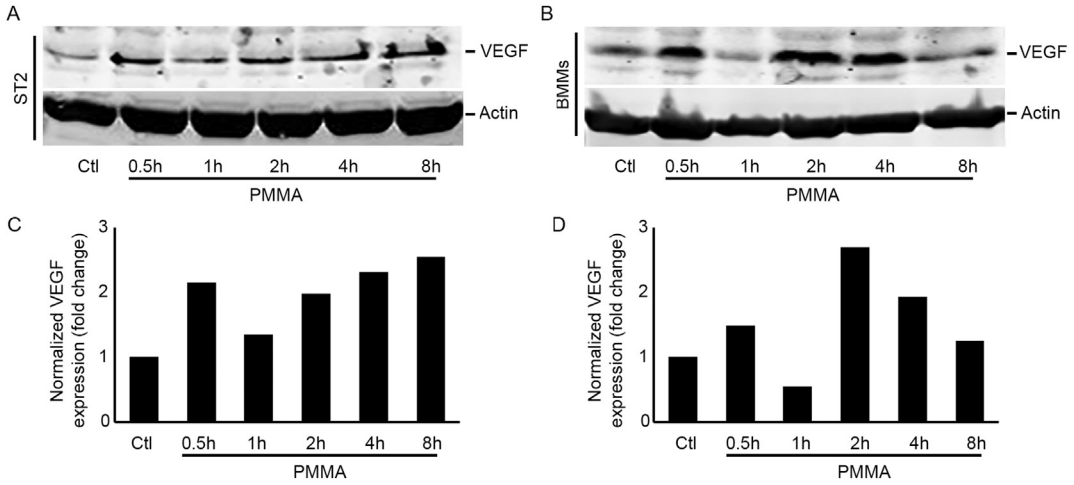

Fig. 1. VEGF expression in myeloid and stromal cells is induced by PMMA particles. ST2 stromal cells and primary bone marrow macrophages were treated in culture with PMMA particles (100 μg/ml of media) for the time points indicated. ST2 (A) and BMM (B) lysates were collected and subjected to Western blot analysis with VEGF and Actin antibodies. Quantification of VEGF expression normalized to actin is presented for ST2 cells (C) and BMM's (D); $n = 3$.

PMMA particles induced higher levels of VEGF expression, indicating that the PMMA-induced inflammatory cascade stimulates bone marrow macrophages and ST2 mesenchymal cells to produce VEGF. Notably, VEGF expression in ST2 lineages appeared to increase with time upon treatment with PMMA, signifying a possible dose responsiveness of stromal cells to PMMA.

3.2. PMMA particles induce angiogenesis and expression of angiogenic factors in the calvarium, a process attenuated by VEGF neutralizing antibody

Next, we examined the expression of VEGF and the angiogenic Von Willebrand factor (vWF) by immunostaining of calvarial sections obtained from control and PMMA-implanted mice. Our results indicate a significant increase in VEGF expression (Fig. 2A) and angiogenesis as evident by increased vWF staining (Fig. 2B) in calvariae from PMMA-treated animals compared to controls. These findings validate the *in vitro* results that PMMA particles can induce VEGF expression.

We further investigated if this activity of VEGF was due to signaling downstream of the VEGFR. To this end, we concomitantly treated PMMA-implanted mice with a VEGF neutralizing antibody or IgG control antibody. VEGF neutralization attenuated angiogenesis as seen by the lack of vWF staining compared with positive staining evident in Fig. 2 (Fig. 3). This demonstrates that the PMMA-induced pro-angiogenic properties of VEGF within bone were a result of direct interaction of VEGF with a VEGFR.

3.3. Neutralizing VEGF abrogates PMMA-induced inflammation and calvarial osteoclast mediated osteolysis in mice

We then sought to determine if VEGF-mediated angiogenesis was critical for the infiltration of inflammatory and immune cells and osteolysis in response to PMMA, and if VEGF-neutralization was sufficient to prevent this process. To address this proposition, we injected control and PMMA-implanted mice with either a VEGF neutralizing Ab or control IgG for 8 days. Histological analysis shows that PMMA particles induce inflammatory and osteolytic response when compared with control mice. PMMA treatment results in increase in TNF staining (Fig. 4A; middle panel), inflammatory and immune cell infiltration (Fig. 4B, top panel; asterisk), bone resorbing TRAP-positive osteoclast (Fig. 4B, top panel; arrows) and large area of focal bone loss. Interestingly VEGF neutralization significantly abrogated PMMA-induced increase in TNF expression (Fig. 4A; bottom panel), plummeted inflammatory responses and halted osteoclast mediated bone loss (Fig. 4B; bottom panel). These findings further confirm that VEGF acts as a key factor to mediate PMMA particles-induced osteolysis. Thus, regulating VEGF bioavailability or activity can be a highly effective method for preventing osteolysis associated with aseptic joint loosening.

4. Discussion

Over the past 20 years, research into osteolysis and inflammation has shed light on many crucial signaling molecules and their relationships to macrophages and other cells involved in bone metabolism. Some of these, such as RANKL and TNF, have been studied extensively and their mechanisms are now much better understood. However, the crucial role of other factors such as VEGF has been also described. In 1999, Gerber et al. demonstrated that the process of endochondral ossification and subsequent bone lengthening is dependent on VEGF expression causing angiogenesis and blood vessel invasion [27]. They also found that VEGF expression seemed to recruit chondroclasts to the growth plate, and when VEGF activity was blocked with a VEGF receptor chimeric protein, cartilage resorption and bone growth/growth plate lengthening did not occur. This demonstrated that VEGF and angiogenesis are necessary for bone generation and remodeling, establishing the role of VEGF as a growing area of interest. Further, it was shown that under inflammatory conditions, exposure to orthopedic particles lead to increased VEGF expression both at the mRNA and protein levels [5,28] and regulation of osteoclasts. These studies lend credence to the critical role of VEGF in homeostatic bone generation and regeneration as well as its contribution to inflammatory, particle-induced, osteolysis.

This study sheds further insight into the role of VEGF in inflammatory osteolysis. We provide evidence that PMMA particles induce both angiogenic and pro-inflammatory responses *via* expression of VEGF and TNF, respectively, in murine osteoclast progenitors and mesenchymal cells. As we demonstrated, treatment of murine calvariae with PMMA particles lead to a significant increase in vWF expression, indicating a strong angiogenic response to PMMA. Based on our findings, the angiogenic environment is initiated not only through VEGF expression in bone marrow macrophages, but also by mesenchymal cells, which go on to compose a significant proportion of the non-hematopoietic cells in the marrow space. Multiple groups have previously demonstrated the variety of cells involved in the production of and reaction to VEGF as it relates to osteolysis. Yang et al. were able to show that bone marrow-derived mesenchymal stem cells produced VEGF in response to inflammatory cytokines [29]. Other groups have investigated and shown that osteoblasts, osteoclasts, and neutrophils all

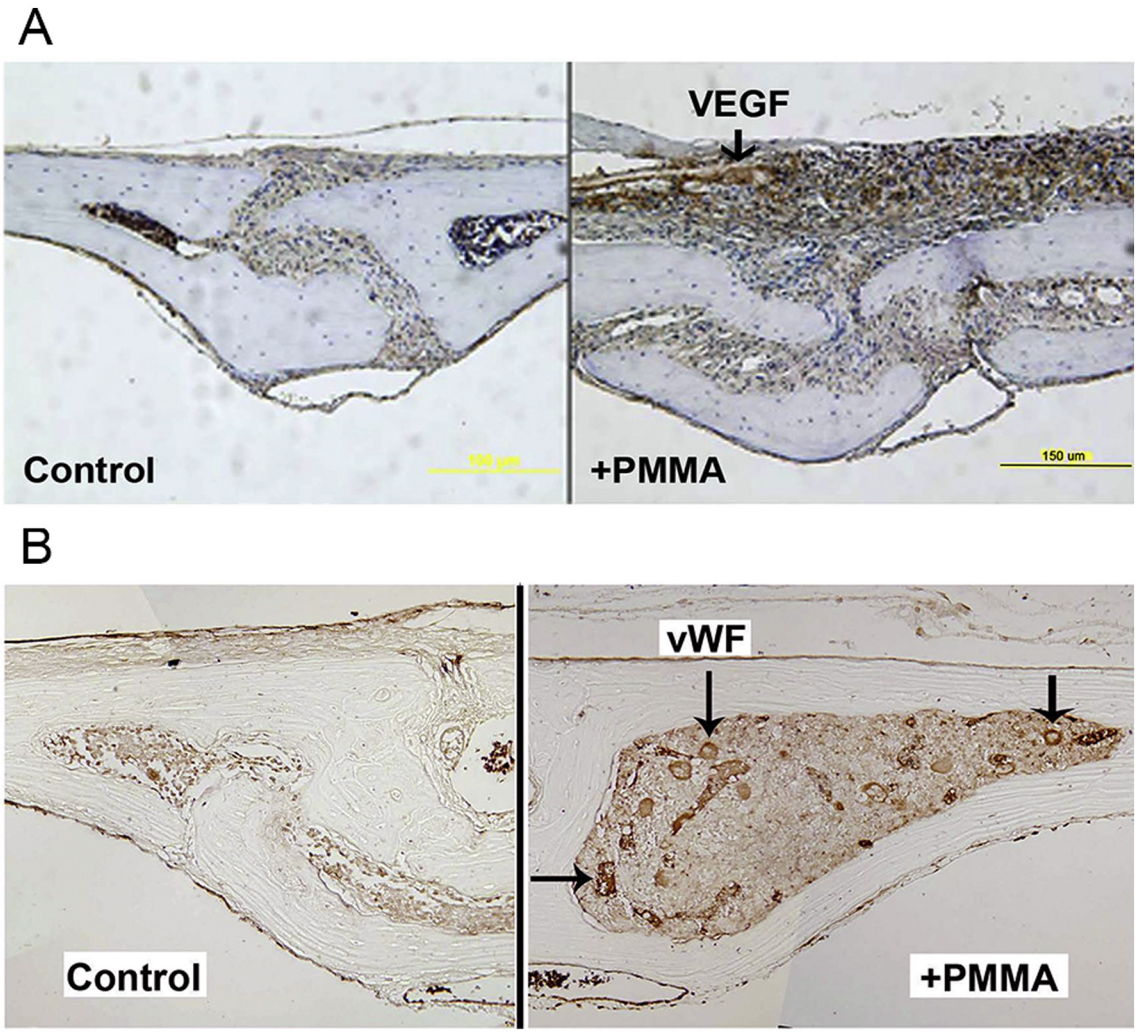

Fig. 2. VEGF-A expression and angiogenesis are elevated in calvarial sections of mice exposed to PMMA particles. PMMA particles (1 mg in 100 μl of PBS) or vehicle were injected over calvaria of 6-week old C57BL6 mice ($n = 5$). After 1 week, calvariae were collected, sectioned and stained with IgG, VEGF and vWF antibody. Representative images of VEGF (A) and vWF (B) expression are indicated by arrows.

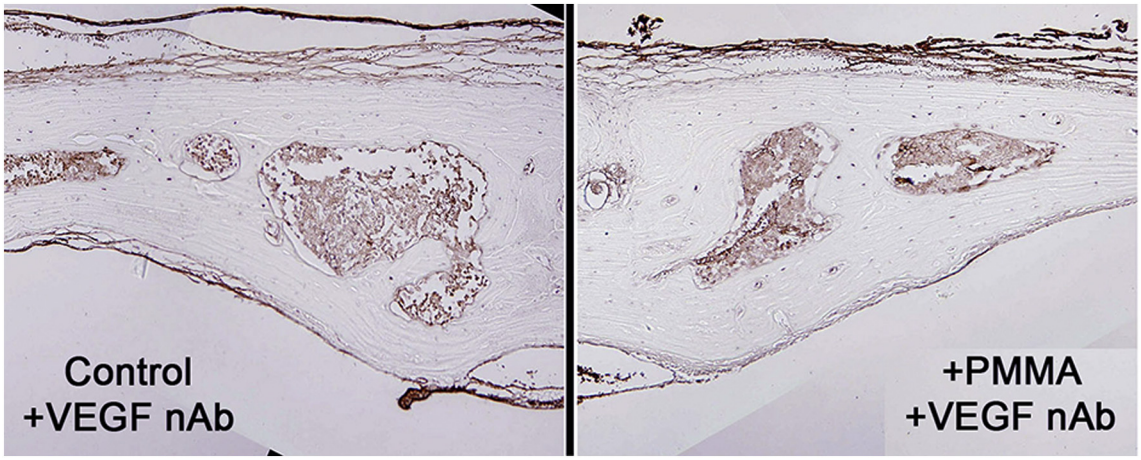

Fig. 3. Administration of VEGF neutralizing antibody (nAb) abrogates expression of angiogenic factors induced by PMMA in mouse calvaria. Control and PMMA-treated mice described in Fig. 2 were injected over the calvaria with wither IgG or VEGF nAb (SC, 0.1 mg/kg BW/daily; $n = 5$ each group) throughout the duration of the experiment (8 days). Mice were then sacrificed and calvarial sections were stained with vWF antibody.

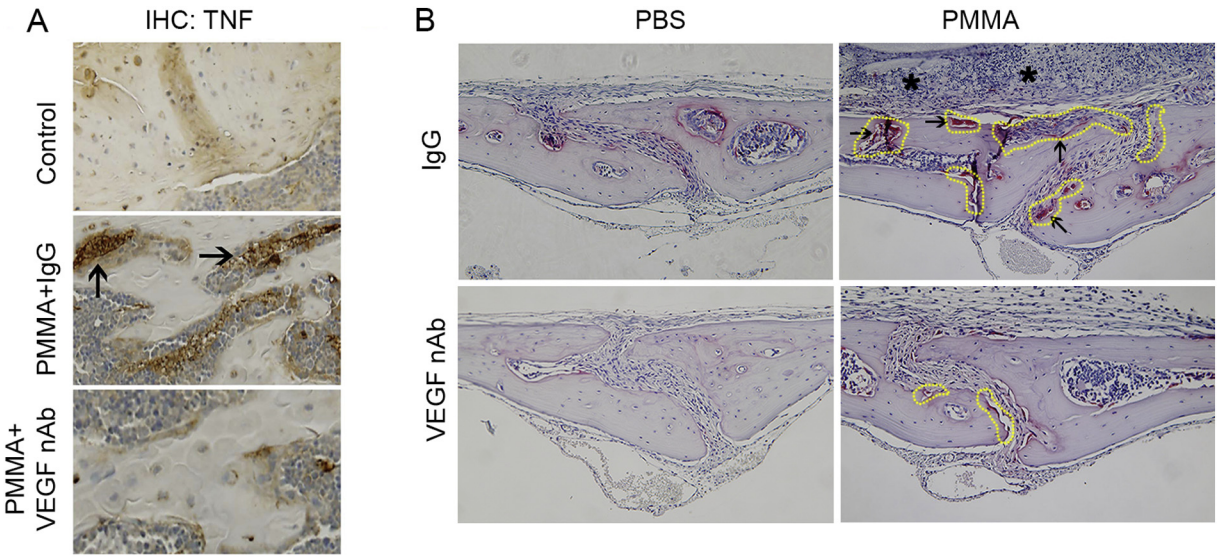

Fig. 4. Administration of VEGF neutralizing antibody inhibits PMMA induced calvarial osteolysis and inflammatory cytokine abundance. VEGF nAb (0.1 mg/kg BW) or IgG were injected daily over the calvaria following PMMA or PBS injection (n = 5). After 8 days, mice were sacrificed and calvariae was collected and processed for immunohistochemistry staining for TNF and TRAP. Representative images of TNF (A), TRAP staining (black arrows), osteolytic zones (yellow dotted lines) and immune infiltrate (asterisk) (B) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

can react to or produce VEGF, leading to osteolysis [5]. These wide cellular responses are expected due to the non-specific nature of implant debris action, supporting the notion that orthopedic particles have the potential to elicit angiogenic and pro-inflammatory responses in numerous cell types around the implant site. The widespread expression of VEGF in response to PMMA throughout the bone microenvironment suggests that targeting of specific cells is likely to be ineffective in treating implant-osteolysis, and requires broad VEGF inhibition to prevent bone loss.

While the role of VEGF in promoting inflammation is well known, the mechanistic details of VEGF effect on bone cells remain vague. We display that angiogenesis is a process central to peri-prosthetic osteolysis since blocking VEGF function using a neutralizing antibody *in vivo* blocked both angiogenesis and osteolysis. Using immunostaining and antibody neutralizing studies, *in vivo*, we provide evidence that the pro-osteolytic effects of VEGF are likely due to a direct physical interaction between VEGF and its receptor on myeloid and stromal cells. Moreover, the fact that blocking VEGF is sufficient to ameliorate the osteolytic response suggests that angiogenic activity in response to PMMA particles is critical for bone loss *via* recruitment of inflammatory cells and mediators (Figs. 3 and 4). This influx of inflammatory cytokines helps promote osteoclast formation and inhibits bone-forming cells.

Our results demonstrate that targeting VEGF using neutralizing antibody provides important intervention advantage and may be highly beneficial slowing implant loosening and the need for revision surgery. However, more studies are required to address potential side effects and utility of such approach in humans. Moreover, the exact mechanism of VEGF-VEGFR interactions is still unclear. In this regard, VEGF-receptors (VEGFR) are known to have at least 3 different isoforms, and VEGF is known to bind to both VEGFR1 and VEGFR2, both of which are present on cells in the bone marrow. VEGFR1 has complex functions in modulating and promoting angiogenesis and VEGFR2 has been shown to be predominantly proangiogenic [11,15,30]. Hence, binding to and blocking VEGF itself rather than targeting VEGFRs affords us the certainty of directly and specifically blocking its effects avoiding uncertain downstream receptor signaling. Nevertheless, elucidating the individual specific effects of each VEGFR on osteolysis stands as an important topic for future research to help us better understand the effects of VEGF on Osteoclasts, BMMs, and other cells involved in the bone resorption process. In addition, knowledge of specific VEGFR signaling in relation to bone loss will allow for the development of more targeted therapeutics for treating implant osteolysis that can spare beneficial functions of VEGF while inhibiting pro-inflammatory properties. Nonetheless, we demonstrate that use of VEGF neutralizing antibody can inhibit a robust, cellular inflammatory response and osteolysis in response to PMMA.

Although the murine calvarial osteolysis model has certain limitations in its representation of human implant-mediated osteolysis, it has been consistently used to study cellular mechanisms of osteolysis. We believe that there is sufficient evidence to suggest that VEGF-mediated angiogenesis plays a vital role in the PMMA-induced inflammatory osteolytic process and poses a potent therapeutic target. However, there still exists the need for further study of PMMA particles in different model systems to better understand the role of angiogenesis in implant failure. In sum, our study highlights the role of angiogenesis in the development of post-implant inflammatory osteolysis and the potential therapeutic benefits of blocking excessive VEGF during inflammation.

Authors contribution

WA, YA and GS designed research; WA, JC and MA performed research and analyzed data; WA, MA, YA and GS participated in writing the manuscript. All authors have read and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

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