Macrophage-derived TGF-βand VEGF promote the progression of trauma-induced heterotopic ossification

Bing Tu
Shanghai Jiaotong University Affiliated Sixth People's Hospital
Juehong Li
Shanghai Jiaotong University Affiliated Sixth People's Hospital
Ziyang Sun
Shanghai Jiaotong University Affiliated Sixth People's Hospital
Tongtong Zhang
Shanghai Jiaotong University Affiliated Sixth People's Hospital
Hang Liu
Shanghai Jiaotong University Affiliated Sixth People's Hospital
Feng Yuan
Shanghai Jiaotong University Affiliated Sixth People's Hospital
Cunyi Fan (fancunyi888@163.com)
Shanghai Jiaotong University Affiliated Sixth People's Hospital

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Abstract

Objective

Heterotopic ossification (HO) is a pathological bone formation process caused by musculoskeletal trauma. Our previous studies have indicated that macrophage inflammation is involved in traumatic HO formation. In this study, we validated the functional roles of M2 macrophages in HO.

Methods

Macrophage Infiltration and activation of TGF-β/Smad2/3 signaling were examined in human HO tissues. By using a mice traumatic HO model, the effect of macrophages on HO progression was tested. Then the roles of angiogenesis and TGF-β signaling were elevated following macrophages depletion. Finally, the subtypes of macrophages were determined by FACS.

Results

We found that macrophage infiltration and TGF-βsignaling activation are presented in human HO. Depletion of macrophages effectively suppressed traumatic HO formation in a mice model, and macrophage depletion significantly inhibited the activation of TGF-β/Smad2/3 signaling. In addition, the TGF-β blockade created by a neutralizing antibody impeded ectopic bone formation *in vivo*. Notably, endochondral ossification and angiogenesis are attenuated following macrophage depletion or TGF-β inhibition. Furthermore, our observations on macrophage polarization revealed that M2 macrophages, rather than M1 macrophages, play a critical role in supporting HO development by enhancing the osteogenic and chondrogenic differentiation of mesenchymal stem cells.

Conclusion

Our findings on ectopic bone formation in HO patients and the mice model indicate that M2 macrophages are an important contributor for HO development, and that inhibition of M2 polarization or TGF-β activity may be a potential method of therapy for traumatic HO.

Introduction

Heterotopic ossification (HO) is the abnormal formation of mature bones in extraskeletal tissues. Traumatic HO is a frequent complication caused by trauma, such as fractures, severe burns, and total hip or knee arthroplasty [1]. Clinical treatment is now limited to Nonsteroidal anti-inflammatory drugs (NSAIDs), radiation therapy, and surgical resection. However, performing an operation is risky because HO often reoccurs after surgical excision. Under certain special circumstances, the HO tissues may be nonresectable due to their sensitive location [2]. HO lesions are preceded by inflammatory events, including innate immune response and adaptive immunity [3, 4]. It is still not clear how inflammatory cells affect HO development. Our recent studies have implicated that macrophages play a critical role in

the progression of HO [5, 6]. However, *in vivo* studies on the contribution made by macrophages for HO progression and its underlying mechanisms involved in HO are limited.

Tissue injury leads to an inflammatory response that recruits a variety of cells, such as monocytes and lymphocytes, at different stages [7]. Previous research studies have indicated that the aberrant expression of monocytes/macrophages are essential for wound healing, including that of HO [8, 9]. Studies also have demonstrated the participation of macrophages in HO caused by endochondral ossification [10], but have not identified the cell population that responds to HO progression. Macrophages are characterized by high functional plasticity in different environments [11]. They can be polarized into two opposing functional states, known as anti-inflammation (M1) macrophages and pro-inflammation (M2) macrophages [12]. M1 macrophages produce a variety of cytokines, such as IL-12, IFN-γ, and TNF-a, which lead to a pro-inflammation pattern. On the contrary, M2 macrophages may promote tissue remodeling, enhance angiogenesis, and regulate immune responses by secreting anti-inflammatory cytokines [13].

M2 macrophages, which express the cell surface marker, CD206, and produce IL-10 and TGF-β, have been described as a reparative and anti-inflammatory factor in trauma [14]. Aberrant activation of TGF-βhas been observed in many diseases. In the skeletal system, elevated TGF-βexpression is coupled with bone resorption and remolding [15]. It is well known that TGF-β is an important regulator of chondrogenic differentiation, and that endochondral ossification is a common feature of HO [16]. In addition, osteogenesis is a metabolic process that requires angiogenesis. Recent studies have shown that abundant blood vessel formation is a common feature during the development of HO [17].

Given the role of TGF-β and VEGF during normal and ectopic bone development and the infiltration of macrophages throughout the formation of HO, we hypothesized that aberrant macrophages activation is a significant source of TGF-β and VEGF. We found that TGF-β and angiogenesis are enhanced in human and mice HO. The inhibition of macrophages and TGF-β activity effectively mitigates HO formation. In addition, our findings identified that M2 macrophages play a central role in regulating TGF-β and VEGF expression during HO development.

Materials And Methods

Patients and specimens

This study was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, and written informed consent was obtained from all patients or their legal guardians. Traumatic HO was identified using X-ray and CT radiography conducted on 24 patients (14 males and 10 females, previously healthy, age ranging from 24 to 67 years) who had previously suffered an elbow fractures and were treated using internal fixation, or revision after hip/knee arthroplasty (8 patients per group). The muscle tissues were used as baseline controls. Blood samples (5 ml per person) were collected 1 day before clinical surgery and blood samples obtained from 8 healthy individuals were

used as baseline controls. All the samples were processed immediately to collect serum, which was then stored in a -80°C freezer. The serum specimens were processed for ELISA.

Mice

Male 6 to 8 week old BALB/c mice were housed under specific pathogen-free conditions at the Animal Experimental Center of Shanghai Sixth People's Hospital, and all experiments conducted were approved by the Animal Research Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Trauma-induced HO was created using a murine tenotomy model, as we previously described [18]. After anesthesia was induced using an intraperitoneal injection of 1% pentobarbital sodium, a skin incision that was 1 cm in length was made on the lateral aspect of the Achilles tendon to expose its full length. Then, the Achilles tendon was transected precisely at its midpoint using a surgical knife. For the sham operation, the incision was made through the skin without touching the Achilles tendon. The incised skin was closed using absorbable sutures. To assess the effect of clodronate liposomes on HO, the animals were randomly divided into 2 groups: Control group (tenotomy surgery with PBS liposomes) and clodronate group (tenotomy with clodronate). The mice were intraperitoneally injected at doses of 1.4 mg/20 g body weight twice a week from the day of surgery. For the TGF-βblockage experiments, the mice were intraperitoneally injected with TGF-βantibodies (5 mg/kg body weight) daily twice a week before being sacrificed to be used for the micro CT analysis.

Histological analysis

Mice were sacrificed using carbon dioxide (CO_2) inhalation for an indicated period to be used for histological observations. The ankles with Achilles tendons were dissected and fixed in 4% paraformaldehyde for 24 h. The HO tissues from patients were collected during surgical operation. Then, the tissues were decalcificated using 10% ethylenediaminetetracetic acid (EDTA) solution for 1 month. The decalcified tissues were processed using graded dehydration and were then embedded in paraffin. 5 μm thick histological sections were obtained using a microtome and were subsequently processed for staining.

For SOFG staining, the sections were stained with 0.1% Safranin-O and 0.02% Fast Green (Sigma-Aldrich, Oakville, ON, Canada) by following the manufacturer's instructions.

Immunohistochemical staining was carried out using primary antibodies against TGF-β, p-Smad2/3, CD68, CD31, and VEGF (Abcam) at a 1:500 dilution of the appropriate secondary antibody. Protein expression was visualized using a DakoCytomation Envision staining kit. The mean density of the positive area was measured using Image-Pro Plus 6.0 (IPP) image analysis software. Three random slides were selected, and the images of five random fields were captured in each sample.

Micro-CT

The tenotomy mice were sacrificed at the durations indicated. The hind limbs of the mice in each group were fixed overnight in 4% paraformaldehyde and were analyzed using a high-resolution Micro-CT scanner Skyscan 1176 (Bruker, Kontich, Belgium). The parameter was set at a resolution of 18 μm and 70 kV voltage. The region of interest (ROI) was set as the entire tibia to ensure that all heterotopic bones were included within the ROI. Three-dimensional images were reconstructed and obtained using NRecon software, and HO bone volumes were analyzed using CTAn software, as previously described [19].

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of TGF-βand VEGF in the serum were determined using the Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), by following the manufacturer's instructions.

Cell Culture

Bone marrow-derived macrophages (BMDMs) were isolated from the bone marrow of 6 to 8 week old mice, as previously described [5]. In brief, both the femur and tibia of the mice were excised and the soft tissue was completely removed. Then, the bone marrow cells were flushed from the marrow cavity using a 26G needle. The harvested cells were used in the experiments described below.

Cell sorting and flow cytometry analysis

After filtration using RBC lysis and washing with 0.1% BSA in PBS, we counted the cells and incubated equal numbers of cells for 45 min at 4°C with the primary antibody. For macrophage identification, we used F4/80, CD115, and CD11b antibodies. For M1 macrophage identification, we used F4/80 and CD86 antibodies. For M2 macrophage identification, F4/80 and CD206 antibodies (BioLegend, San Diego, CA, USA) were used. The stained cells were processed on a BD FACS Calibur flow cytometer and were analyzed using FlowJo software.

Cell culture

The sorted cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. To prepare the conditioned medium (CM), the cells were grown to 80% confluence in 5 cm dishes containing DMEM/10% FBS. The medium was discarded, and the cells were further cultured in serum-free DMEM for 24 h. Then, the medium was collected, centrifuged at 1,000 × g for 10 min, and filtered through 0.22-μm filters (Millipore, Billerica, MA).

RNA isolation and real-time PCR

MSC cells (purchased from the Chinese Academy of Sciences, Shanghai, China) were exposed to a macrophage conditioned medium for 48 h. Total RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific), and cDNA was synthesized using the Mix-X miRNA First-Strand Synthesis Kit (TaKaRa Bio). Subsequently, real-time PCR was performed using SYBR Green Premix Ex Taq (Takara) to quantify the

target gene mRNAs. All procedures were performed by following the manufacturer's instructions. The following primer sequences were used: GAPDH, 5’-ATGGGGAAGGTGAAGGTCG-3’ (forward) and 5’-GGGGTCATTGATGGCAACAATA-3’ (reverse); Runx2: forward 5’-CCGCC TCAGTGATTTAGGGC -3’, reverse 5’- GGGTCTGTAATCTGACTCTG TCC -3’. OCN: forward 5’-CCTCAC ACTCCTCGCCCTATT-3’, reverse 5’-CCCTCCTGCTTGGACACAAA-3’. Sox9: forward 5’-CAGCCCCTTCAACCTTCCTC-3’, reverse 5’- TGATGGTCAGCGTAGTCGTATT-3’. Sp7: forward 5’- ATGGCGTCCTCTCTGCTTG-3’, reverse 5’- TGAAAGGTCAGCGTATGGCTT-3’. All of the reactions were performed in triplicate.

Statistical analysis

GraphPad Prism 8 was used for all statistical analyses of the data obtained. The data are presented as mean ± standard deviation (SD). Comparisons between groups were performed using Student’s t-test, while one-way ANOVA was used for comparisons between multiple groups. All experiments were performed in triplicate, and representative experiments are shown. Statistical significance was set at $P<0.05$.

Results

Macrophage Infiltration and activation of TGF-β/Smad2/3 increased in human HO tissues

The HO patients who had undergone elbow injury or knee/hip arthroplasty, and were identified using X-ray or CT imaging were included in this study. The HO specimens were collected during elbow internal fixation, knee or hip replacement revision surgeries. The HE staining showed thick cartilage layers adjacent to the bone tissues in the HO samples (Fig. 1A). To examine the effects of macrophages on the progression of HO, CD68 expression was detected using immunohistochemistry (IHC). We observed a significant infiltration of $CD68^+$ macrophages into HO tissues during elbow, knee and hip surgeries (Fig. 1B, 1C). Immunostaining showed that TGF-β expression was significantly higher in the HO samples than in the muscle (Fig. 1D, 1E). Additionally, a significant increase in the expression of p-Smad2/3 was observed in HO tissues (Fig. 1F, 1G). Finally, the concentration of TGF-βin HO patient serum was higher than that of the healthy control (Fig. 1H). Together, our data revealed that enhanced macrophages accumulation and TGF-β/Smad2/3 signaling activation are involved in traumatic HO development.

Macrophage depletion prevents HO progression in mice

Clodronate liposomes has been used as an effective agent to deplete macrophages in various *in vivo* models [20]. To study the effect of liposomal clodronate on HO, we used a HO mice model with dissection at the midpoint in the Achilles. We treated the mice with intraperitoneal injections of clodronate liposome twice a week until they were euthanized. At week 4 and 8, the mice were sacrificed, tissue and blood samples were subsequently obtained and analyzed. We first examined HO formation, and the micro-CT results showed that treatment with clodronate liposomes almost entirely inhibited HO formation at week 4 and 8 (Fig. 2A, 2B). The efficacy of the clodronate liposomes on the depletion of macrophages was confirmed using CD68 staining. Treatment with clodronate liposomes for 8 weeks eliminated CD68+ cells

from the HO tissue (Fig. 2C, 2G). The Safranin O and fast green (SOFG) staining revealed that hardly any cartilage or bone could be observed following macrophage depletion (Fig. 2D). Additionally, immunostaining demonstrated that the expression of the osteogenic differentiation marker, osteocalcin (Ocn, Fig.2E, 2H), had decreased significantly after clodronate liposome treatment. A similar result was observed with the chondrogenic differentiation marker, Sox9 (Fig. 2F, 2I). Altogether, these data indicate that macrophage depletion prevents HO formation by inhibiting endochondral ossification.

Macrophage depletion reduces angiogenesis in HO

It is well known that angiogenesis is essential for endochondral ossification [21, 22]. We investigated the impact of clodronate liposomes on angiogenesis in HO. X-ray photography confirmed that clodronate liposomes significantly inhibited HO formation at week 2, 4, and 8 (Fig. 3A). HE staining demonstrated that blood vessels were present in the control HO group (treated with PBS liposomes, Week 8), whereas fewer blood vessels were observed in the clodronate liposome treated groups (Fig. 3B). These results were confirmed using CD31 immunohistochemistry staining. Compared with the control HO group, microvessel density was significantly lower following clodronate liposomal treatment (Fig. 3C, 3E). Macrophages are a major source of secreted vascular endothelial growth factor (VEGF) that can mediate angiogenesis [23]. Therefore, we determined VEGF expression in HO tissues and confirmed that the reduction in blood vessel density was associated with a significant decrease in VEGF levels upon treatment with clodronate liposomes ((Fig. 3D, 3F). Furthermore, a marked reduction in circulating VEGF levels were observed after treatment with clodronate liposomes (Fig. 3G). To examine whether clodronate liposome directly affect VEGF expression in macrophages, bone marrow-derived macrophages (BMDMs) were isolated from the bone marrow and were treated with clodronate liposomes for 2 hours. The VEGF expression levels decreased following clodronate liposome treatment (Fig. 3H). Collectively, these results indicate that depletion of macrophages attenuates angiogenesis and HO formation.

Macrophage depletion suppresses TGF-βsignaling

To investigate the role played by TGF-βin the progression of HO, we examined TGF-βexpression using the traumatic HO mice model. Ectopic bone was formed in the Achilles tendon of the mouse model 4 weeks post puncture (Fig. 4A). The immunohistochemistry staining showed that the expression of TGF-βincreased 2 weeks post puncture and was maintained at a high level at 8 weeks post puncture (Fig. 4B, 4D). Meanwhile, the serum TGF-βconcentration in the mice increased 2 weeks after trauma (Fig. 4E). In addition, a similar result was observed with p-Smad2/3 staining of the HO tissues (Fig. 4C, 4F). The HO mice were treated by administrating clodronate liposome injection. Our data demonstrated that TGF-βlevels in the tissue (Fig. 4G, 4I) and serum (Fig. 4J) were significantly inhibited upon treatment with clodronate liposomes. Finally, the number of pSmad2/3+ cells decreased following macrophage depletion (Fig. 4H, 4K). Taken together, the results demonstrate that macrophage depletion effectively suppressed the activation of TGF-β signaling, which plays an important role in triggering HO formation.

TGF-β antibody inhibits the formation of trauma-induced HO

We investigated whether TGF-β is directly involved in traumatic HO progression. A TGF-β neutralizing antibody or IgG (used as control) was injected into the mice twice a week. To examine the change in HO signaling, the mice were sacrificed at week 2, 4, and 8.μ-CT analysis showed that HO was not formed following TGF-β antibody treatment at week 2 and 4. However, HO was formed at week 8. We observed a significantly reduced HO volume after administration of the TGF-β neutralizing antibody injection, compared with the controls (Fig. 5A, 5F). SOFG staining demonstrated that endochondral ossification formation was reduced following TGF-β antibody treatment (Fig. 5B). Immunostaining revealed that the number of pSmad2/3 positive cells had decreased significantly after the HO mice were injected with a TGF-β antibody (Fig. 5C, 5G). The immunostaining of CD31 showed that the number of blood vessels had decreased significantly in TGF-β antibody treatment mice (Fig. 5D, 5H). In addition, we observed a similar change in Ocn expression in the mice (Fig. 5E, 5I). Collectively, our data demonstrates that TGF-β signaling inhibition attenuates the progression of HO.

M2 macrophages promote osteogenic and chondrogenic differentiation

To determine the type of macrophage involved in HO progression, an Achilles tendon mice model was used and bone marrow-derived macrophages were collected at week 0, 2, 4 and 8. M1 and M2 macrophages from the BMDMs were examined using flow cytometry analysis. The results showed that the percentage of M2 macrophages increased significantly after trauma, whereas the percentage of M1 macrophages was maintained at a stable level during the whole process (Fig. 6A, 6B and 6C). These results indicate that M2 macrophages may be responsible for HO development. Then, M1 and M2 macrophages were sorted and isolated using FASC. The 4 and 8-week M1 and M2 cells were cultured in DMEM medium for 24 h and the conditioned medium was collected. Then Mesenchymal stem cells (MSCs) were incubated in the M1 or M2 conditioned medium for another 48 h, the expression levels of osteogenic and chondrogenic differentiation makers were examined using real-time PCR. The 4-week and 8-week M2 conditioned medium enhanced the expression of level of the osteogenic markers, Runx2 and OCN, and the chondrogenic markers, Sox9 and Sp7. However, the M1 conditioned medium did not affect the expression levels of these genes (Fig. 6D). Taken together, these results suggest that M2 macrophages promote endochondral ossification during HO progression.

Discussion

It has been found that the immune system and macrophages are closely related to the HO formation. However, we still have limited knowledge about the mechanisms that link them. The recruitment of circulatory monocytes initiates a regenerative milieu at the site of injury by secreting a number of cytokines. However, monocytes and macrophages are heterogeneous cells and the specific functions of each of their subpopulations remain unclear. Previously, we demonstrated that macrophages are involved in HO progression. In this study, we identified M2 macrophage and TGF-βinhibition as a promising therapeutic method for traumatic HO prevention in a preclinical mice model.

Previous studies have provided evidence that macrophages are an important regulator of HO formation [9, 24]. Depletion of macrophages is shown to significantly impair the development of genetic and trauma-induced HO [8, 25], while it has been shown to exert a promotional effect on HO in other studies [26]. To better understand the role of macrophages during the initiation and development of HO, we analyzed HO tissues from different injury sites of clinical patients. We found that an increase in macrophage numbers were accompanied traumatic HO development, as observed in patients. Our observations provide clinical insights into the contribution made by activated macrophages for the pathophysiological regulation of ectopic bone formation. In addition, we found that the ablation of macrophages caused by clodronate liposomes significantly suppressed the development of HO *in vivo*. Meanwhile, our data showed that the depletion of macrophages inhibited the endochondral ossification and the expression of the Ocn and Sox9 genes.

Monocyte-macrophage lineage cells are characterized by the diverse functions they exert, which play a crucial role in regulating normal tissue homeostasis, including bone development and repair, wound recovery, and the regulation of angiogenesis [27-29]. Clodronate liposomes have been used often for macrophage depletion in several disease models, including cancer, autoimmune disorders, and diseases of the skeletal system [30, 31]. Although it is well established that clodronate liposomes can effectively inhibit macrophages *in vitro* and *in vivo*, its potential effects on other cell types cannot be ruled out. In traumatic HO, the phenotypic heterogeneity of macrophages remains poorly understood. Our findings showed that the number of M2 macrophages increased during HO development, and provided evidence that M2 macrophages could produce a large amount of VEGF and TGF-β. These data demonstrate that the aberrant activation of M2 macrophages may be an important contributor to traumatic HO.

HO development is an energy-consuming metabolic process that requires blood vessels to deliver nutrients for chondrogenesis and osteogenesis [32]. Angiogenesis and osteogenic processes are tightly coupled during skeletal development [33, 34]. Recent research studies have shown that human HO exhibits a consistent pattern of vascularization[35], which involves coordination between osteogenesis and angiogenesis [36]. Our data demonstrated that neovascularization is inhibited following macrophage depletion, indicating that the inhibition of neovascularization may attenuate traumatic HO. It is well known that VEGF plays a critical role in angiogenesis and chondrogenesis [37, 38], a process that is essential for HO formation as it occurs primarily through endochondral ossification [39, 40]. In this study, we found that VEGF levels in HO and the serum decreased after clodronate liposome treatment. These results suggest that macrophages promote neovascularization and facilitate HO formation.

TGF-β is considered to be a specific marker of regenerative macrophages and can regulate the biological functions of monocytes and macrophages [41]. It is known that TGF-β also plays a critical role in chondrogenic differentiation [42, 43]. Several recent studies have implicated TGF-β signaling in genetic and neurogenic HO [44, 45]. Our findings demonstrated that TGF-βpresent at the injury sties appears to play a critical role in aberrant bone formation. Blockage of active TGF-β by a specific antibody significantly inhibited HO development *in vivo*. Depletion of macrophages effectively reduced the

expression of TGF-β. Our data indicates that TGF-β producing macrophages form a critical link between inflammation and aberrant ectopic bone generation.

Our previous study showed that quercetin, a natural flavonoid class of polyphenols, attenuated trauma-induced HO by restraining monocyte-to-macrophage transition, indicating that macrophages infiltration plays a pivotal role in HO [5]. In this study, our findings revealed the critical role played by M2 macrophages in the regulation of aberrant ectopic bone formation by secreting excessive amounts of TGF-βand VEGF. Using clinical sample analysis, we found a remarkable level of macrophage infiltration and TGF-βactivation in human HO tissues. Further, we showed that TGF-β and VEGF derived from macrophages play an important role in driving traumatic HO formation through endochondral ossification. In addition, we identified M2 macrophages as an important source of TGF-β and VEGF following trauma. Our study proposes a paradigm that can be used to understand the functional impact of macrophage polarization on HO progression.

Abbreviations

HO: Heterotopic ossification

TGF-β: Transforming growth factor-β

NSAIDs: Nonsteroidal anti-inflammatory drugs

IL: Interleukin

IFN: Interferon

TNF: Tumor necrosis factor

VEGF: Vascular endothelial growth factor

CT: Computed tomography

EDTA: Ethylenediaminetetracetic acid

SOFG: Safranin-O and Fast Green

ROI: Region of interest

ELISA: Enzyme-Linked Immunosorbent Assay

BMDMs: Bone marrow-derived macrophages

MSCs: Mesenchymal stem cells

Declarations

Conflict of Interest

The authors declare no conflicts of interest.

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Figures

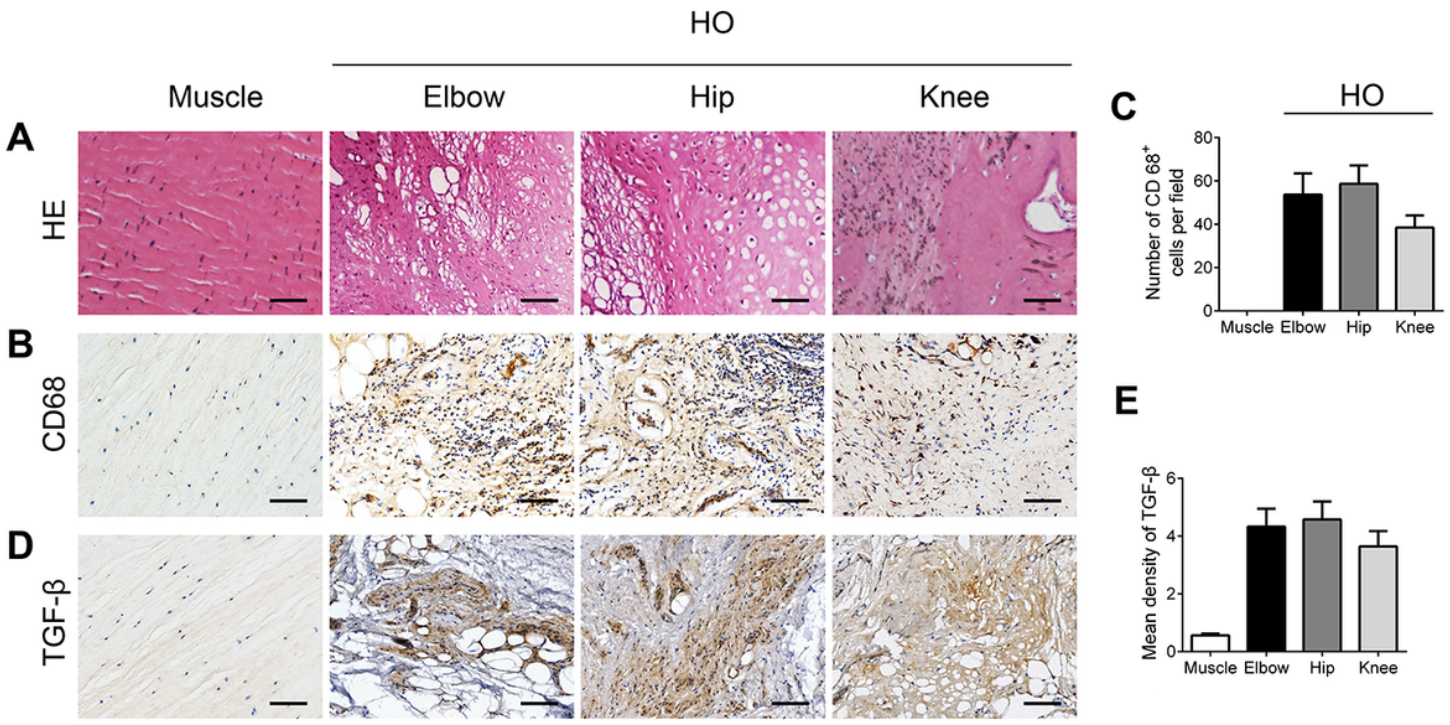

Figure 1

Macrophage infiltration was enhanced in HO patients. (A) H&E staining of the normal muscle, and HO samples from elbow fracture, hip or knee revision arthroplasty patients. (B) Immunohistochemistry staining of CD68 in normal muscle and HO. (C) Quantification of CD68+ cells. (D) Immunohistochemistry staining of TGF-β. (E) Quantification of TGF-β expression. (F) Immunohistochemistry staining of p-Smad2/3. (G). Quantification of p-Smad2/3 expression. (H) TGF-β levels in the serum of patients were determined using ELISA. Serum samples obtained from healthy individuals were used as the control. n=8 per group. The data are shown as mean ± SD. *, $p<0.01$. Scale bar, 50 μm.

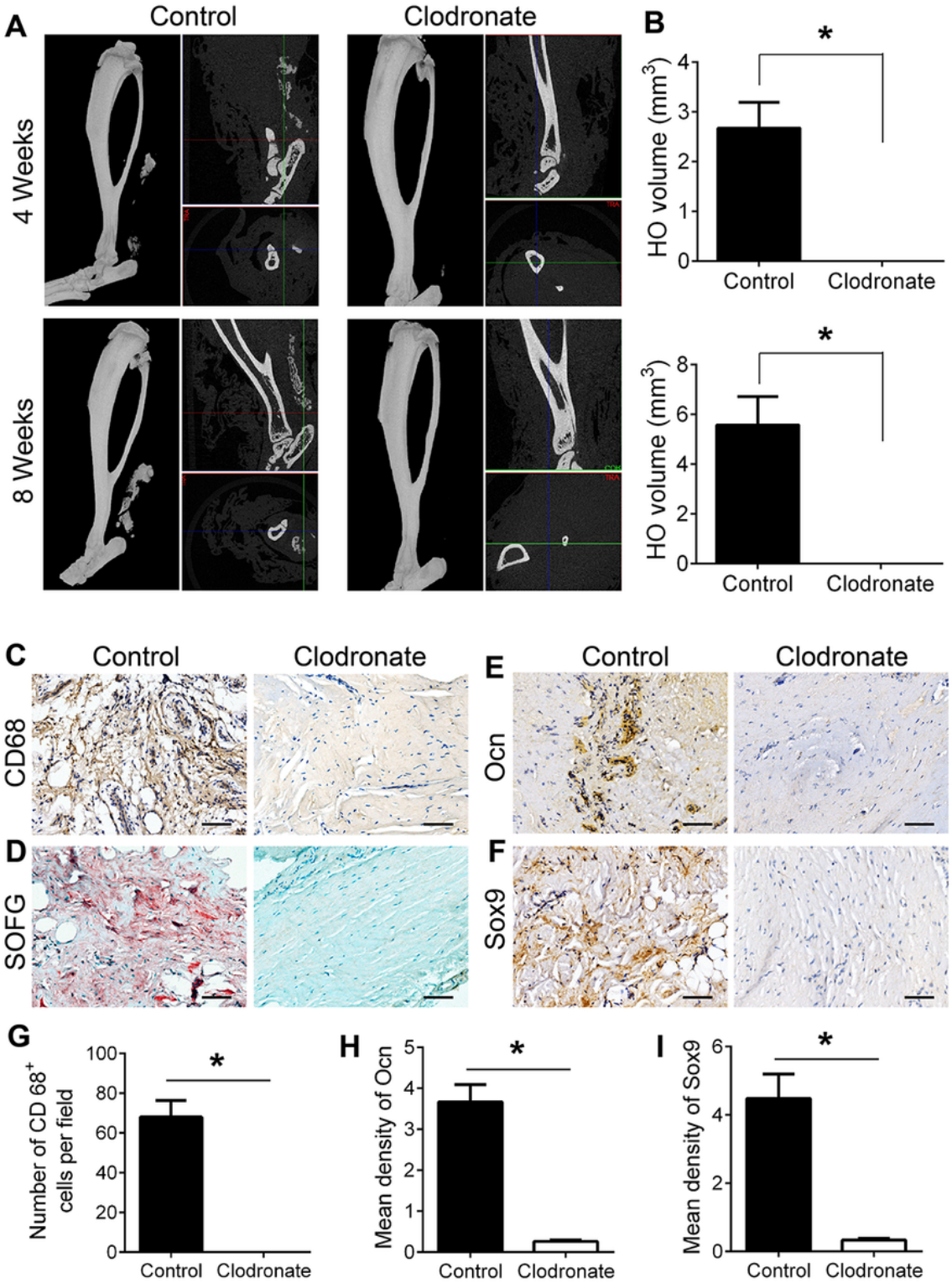

Figure 2

Macrophage depletion prevents HO progression. (A) The mice were subjected to a tenotomy to generate a traumatic HO model. Then, the mice were intraperitoneally injected with clodronate liposomes or PBS liposomes (used as control). Micro-CT images of HO in the Achilles tendon at 4 and 8 weeks. (B) Quantitative analysis of HO volume. (C) Immunohistochemistry staining of CD68. (D) SOFG staining. (E)

Immunohistochemistry staining of Ocn. (F) Immunohistochemistry staining of Sox9. Histomorphometry analysis of (G) CD68+ cells, (H) Ocn, and (I) Sox9 expression. *, p<0.01. Scale bar, 50 μm.

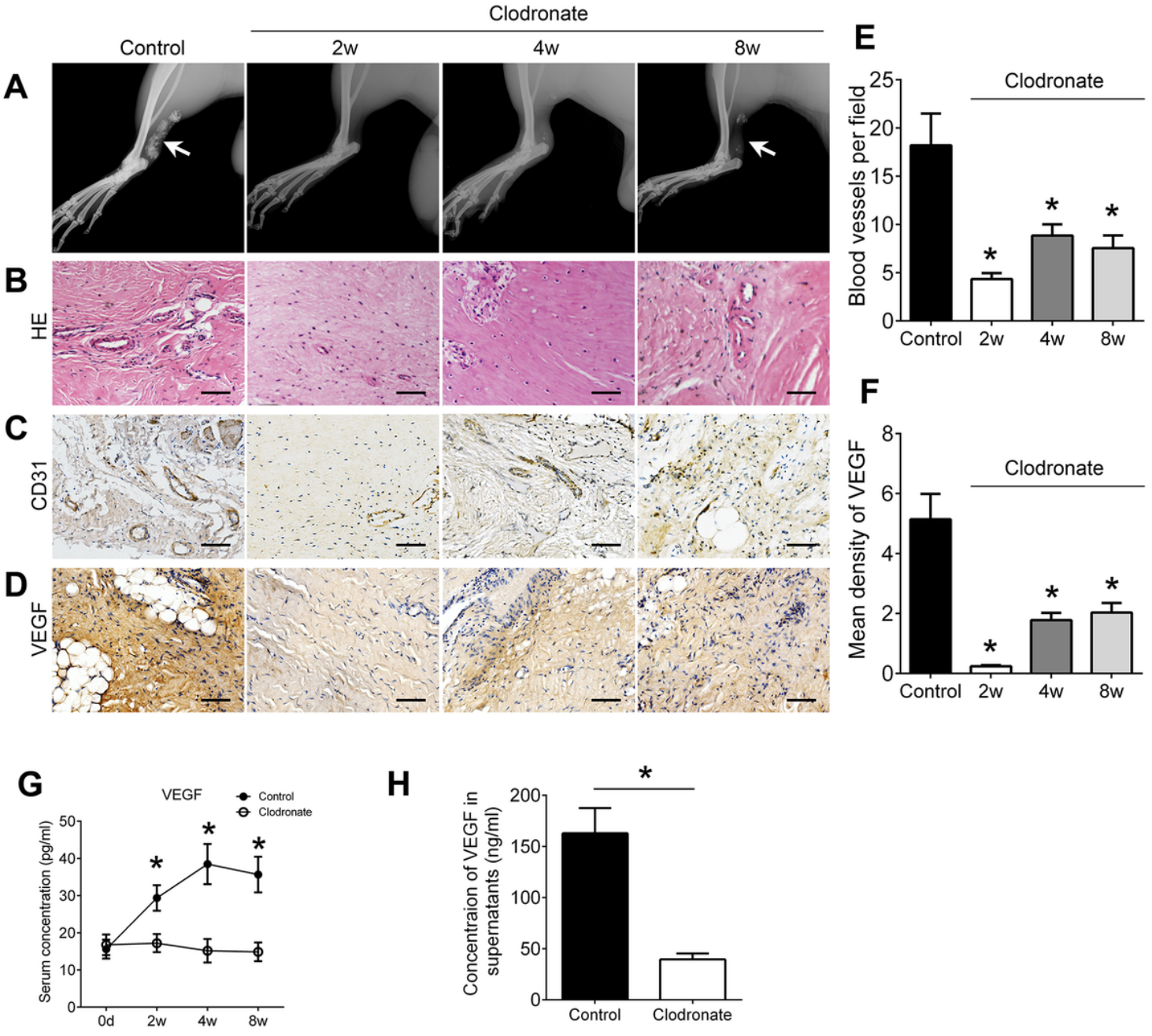

Figure 3

Macrophage depletion reduces angiogenesis in HO. (A) The tenotomy mice were intraperitoneally injected with clodronate liposomes or PBS liposomes. X-ray images of HO in the mice treated with clodronate liposomes at 2, 4, and 8 weeks. Mice treated with PBS liposomes were at week 8 used as control. (B) H&E staining of ectopic bone formation in Achilles tendons. (C) Immunohistochemical staining of CD31. (D) Immunohistochemical staining of VEGF. (E) Quantification of the blood vessels formed in Achilles tendons. (F) Quantification of VEGF expression. (G) Serum VEGF levels in the clodronate liposome or PBS liposome group (control) were detected using ELISA. (H) Bone-marrow derived macrophages were

isolated from the mice and were treated with PBS liposomes or clodronate liposomes for 24 hours. VEGF expression in the cultured cell supernatants was tested by ELISA. n=5 per group. Data is presented as mean ± SD. *, p<0.01. Scale bar, 50 μm.

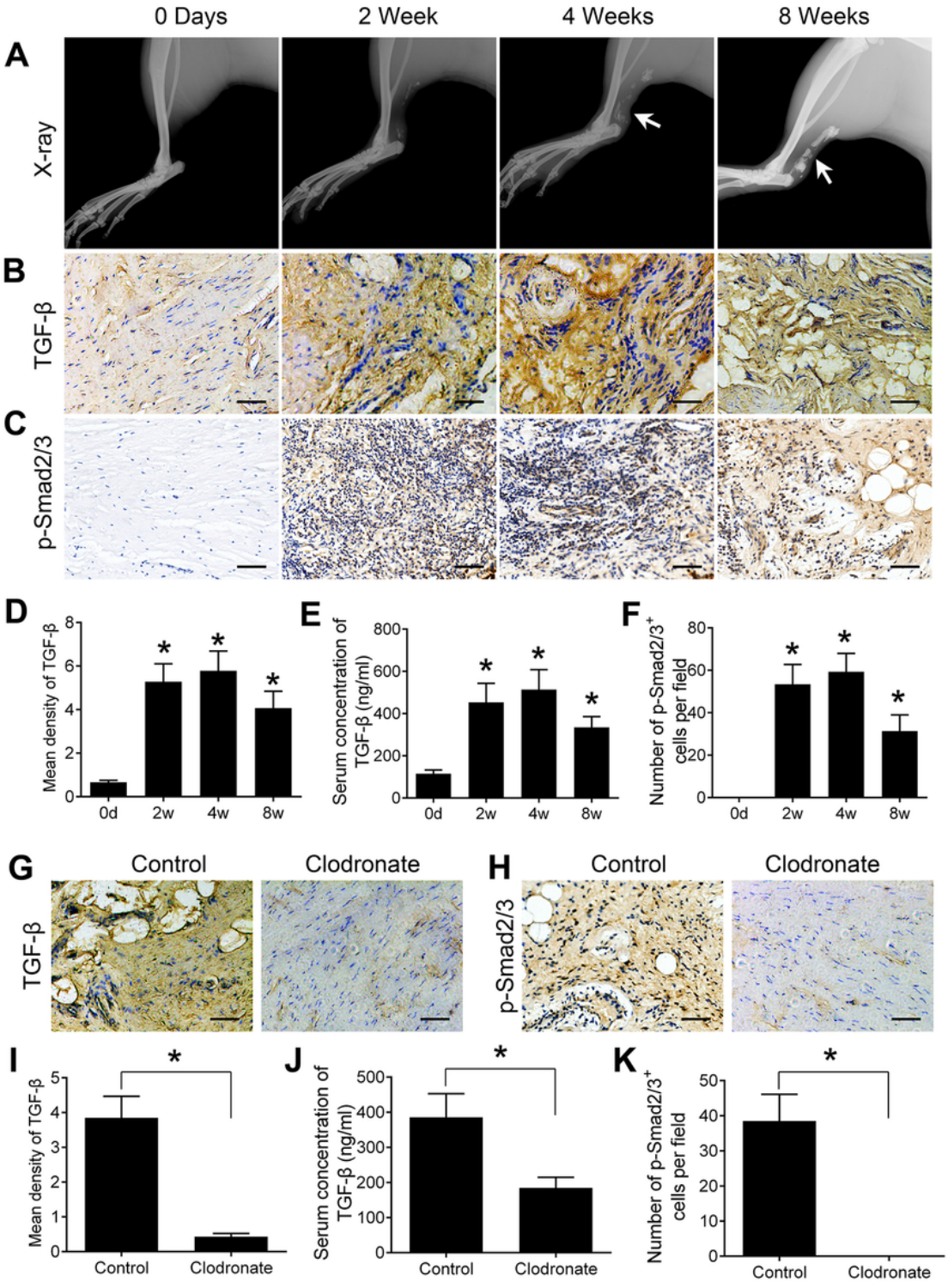

Figure 4

Macrophage depletion suppresses TGF-β signaling. (A) The mice were subjected to a tenotomy to generate a traumatic HO model. Representative X-ray images of HO in the mice at 0 d, 2 weeks, 4 weeks, and 8 weeks. (B) Immunohistochemical staining of TGF-β. (C) Immunohistochemical staining of p-Smad2/3. (D) Quantification of TGF-β expression at the times indicated. (E) Serum TGF-β levels in the mice were detected by ELISA. (F) Quantification of the p-Smad2/3^{+} cells at the times indicated. (G) Immunohistochemical staining of TGF-β in HO mice treated with PBS liposomes or clodronate liposomes. (H) Immunohistochemical staining for p-Smad2/3 in the two groups. (I) Quantification of TGF-β expression in the two groups. (J) Serum TGF-β levels in the two groups. (K) Quantification of p-Smad2/3 expression. n=5 per group. Data are presented as mean ± SD. *, p<0.01. Scale bar, 50 μm.

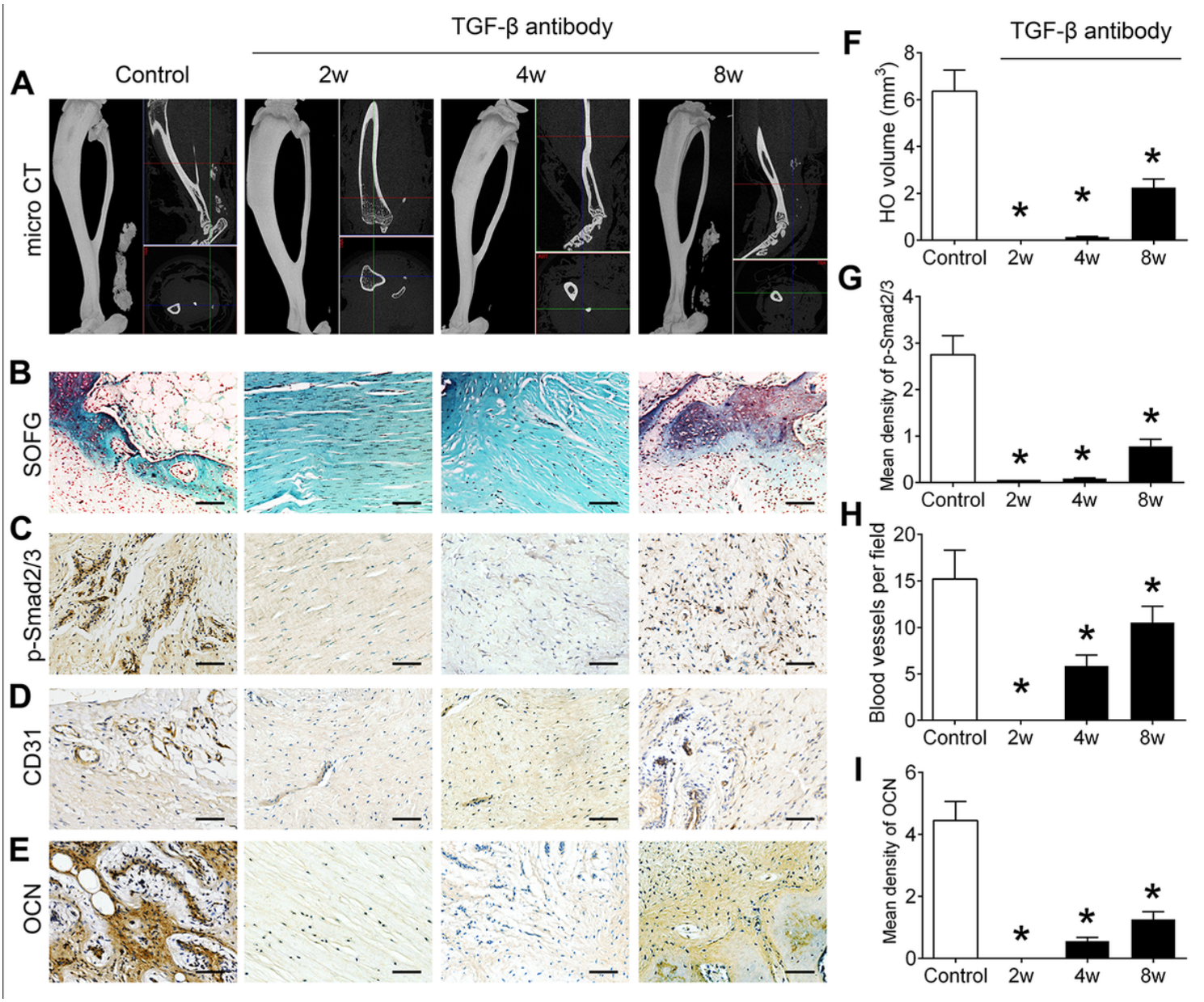

Figure 5

TGF-βantibody inhibits the formation of trauma-induced HO. (A) The tenotomy mice were intraperitoneally injected with a TGF-βantibody. Micro-CT images of the HO in mice treated with the TGF-

βantibody (5 mg/kg) at 2, 4, and 8 weeks after treatment. The mice treated with IgG were used as control at week 8. (B) SOFG staining. (C) Immunohistochemical staining of p-Smad2/3, CD31(D), and Ocn (E). (F) Quantitative analysis of the HO volume. (G) Quantification of p-Smad2/3 expression. (H) Quantification of the number of blood vessels formed. (I) Quantification of Ocn expression. n=5 per group. Data are presented as mean ± SD. *, p<0.01. Scale bar, 50 μm.

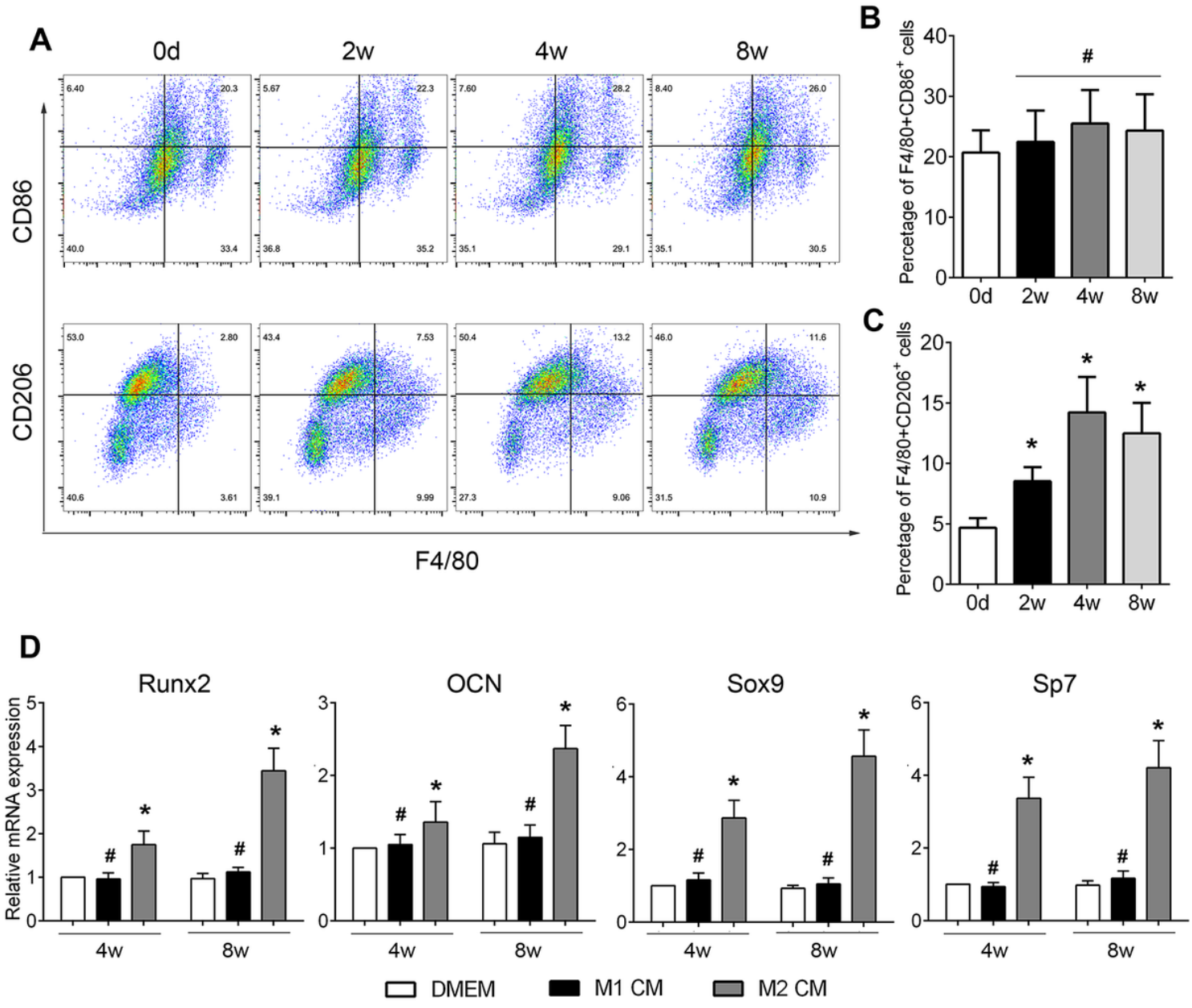

Figure 6

M2 macrophages promote osteogenic and chondrogenic differentiation. (A) The mice were subjected to a tenotomy to generate a traumatic HO model. Bone marrow-derived macrophages were collected from the bone marrow at 0 d, 2 weeks, 4 weeks, and 8 weeks. The M1 (expressing F4/80 and CD86) and M2 (expressing F4/80 and CD206) macrophages were analyzed using flow cytometry. (B) The percentage of

M1 macrophages in the HO mice at the times indicated. (C) The percentage of M2 macrophages in the HO mice at the times indicated. (D) M1 and M2 macrophages were separately sorted and isolated, cultured for 24 h and the conditioned mediums were collected. Bone marrow derived mesenchymal stem cells (MSCs) were incubated in the M1 or M2 conditioned medium for 48 h. The mRNA expression levels of Runx2, Ocn, Sox9, and Sp7 in MSCs were examined using PCR. n = 3. Data are presented as mean ± SD. *, $p<0.01$; #, p□0.05.