IL-17A Stimulates the Progression of Giant Cell Tumors of Bone

Meng Xu1, Zhi-Gang Song2, Cheng-Xiong Xu3, Guang-Hua Rong4, Ke-Xing Fan5, Ji-Ying Chen1, Wei Zhang1, Jin-Peng Jia1, Gang Han1, Wei Wang1, Wei Chai1, Wen-Tao Liang1, Wen-Zhi Bi1, and Yan Wang1

1Department of Orthopaedics, and 2Department of Pathology, The General Hospital of Chinese People’s Liberation Army, Beijing, China; 3Departments of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA; 4Center of Therapeutic Research for Hepatocellular Carcinoma, Beijing 302 Hospital, Beijing, China; 5International Joint Cancer Institute, The Second Military Medical University, Shanghai, China.

* First three authors are contributed equally to this work.

Correspondance to:
Cheng-Xiong Xu, Ph.D., Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Institution, Tampa, FL 33625; Tel: 813-966-2161; E-mail: Cheng-xiong.Xu@moffitt.org and
Wen-Zhi Bi, M.D., The General Hospital of Chinese People’s Liberation Army, 28 Fuxing Road, Beijing, 100853, China; Tel: +86-13501175839; Fax: 86-10-6816-1218; E-mail: biwz301@gmail.com and
Yan Wang, M.D., Department of Orthopaedics, The General Hospital of Chinese People’s Liberation Army, 28 Fuxing Road, Beijing, 100853, China; Tel: +86-13501175839; Fax: 86-10-6816-1218; E-mail: leptin1@gmail.com

Running title: IL-17A stimulates osteolysis in GCTB

Key words: IL-17A; Giant cell tumor of bone; progression of GCTBs; osteolysis

Disclosure of Potential Conflicts of Interest: The authors have no conflicting financial interests.

Words: 3348; Figures: 4; Tables: 2
**Translational relevance**

GCTB is a locally osteolytic primary bone tumor and IL-17A is involved in pathological bone reabsorption. Thus, we investigated the effects of IL-17A in GCTBs progression. Here, we found the IL-17A was increased in GCTBs patients and predominantly produced by MNGCs. In GCTBs, IL-17A strongly promotes bone reabsorption by stimulating the expression of pro-osteolytic factors including RANKL. Recent studies have shown that RANKL is one of the important therapeutic targets for GCTBs. In addition, IL-17A significantly promoted cellular proliferation of SCs and angiogenesis in GCTBs. Our results also show that IL-17A significantly increased in high grade GCTBs. These results suggest that IL-17A might represent a useful candidate marker for progression and therapeutic target for GCTBs.
Abstract

Purpose: Giant cell tumors of bone (GCTBs) exhibit aggressive bone lytic behavior. Studies have shown that interleukin 17A (IL-17A) is involved in pathological bone resorption in various skeletal disorders. Thus, we have investigated the role of IL-17A in GCTBs.

Experimental Design: We evaluated the progression of GCTBs using Campanacci grading and Enneking staging systems in 74 GCTB patients. The expression of IL-17A and the IL-17A receptor A (IL-17RA) was assessed in GCTB tissues and in both multinucleated giant cells (MNGCs) and stromal cells (SCs) cultured in vitro using immunostaining and RT-PCR. The effects of IL-17A on the osteolytic activity of the MNGCs and the proliferation of the SCs were investigated using the "pit" formation and MTT assays, respectively. The effects of IL-17A on the expression of pro-osteolytic factors were examined in primary cultured MNGCs and SCs using RT-PCR, Western blotting, and gene expression microarrays.

Results: In GCTBs, we detected abundant levels of IL-17A, which were associated with tumor extension and grade. IL-17A is predominantly produced by MNGCs, whereas IL-17RA is expressed by both MNGCs and SCs in GCTBs. In the MNGCs, the IL-17A increased the mRNA expression of IL-17A and pro-osteolytic enzymes, also enhanced osteolytic ability. In the SCs, the IL-17A stimulated cellular proliferation and the expression of pro-osteolytic factors, including RANKL through myc and STAT3, respectively. In addition, IL-17A stimulated in vivo tumor growth and the extent of angiogenesis in GCTBs.

Conclusion: IL-17A stimulates the progression of GCTBs and might represent a useful candidate marker for progression and as a therapeutic target for GCTBs.
Introduction

Giant cell tumor of bone (GCTB) is a benign, locally aggressive, osteolytic tumor that causes significant bone destruction at the epiphysis of long bones. Although rarely lethal, GCTBs exhibit local recurrence in 8%-62% of patients following primary surgical treatments, and up to 5% of GCTBs develop pulmonary metastases and spontaneous malignant transformation in 2% of patients (1, 2).

GCTBs are comprised of three major cell populations: spindle-shaped mononuclear stromal cells (SCs), osteoclast-like multinucleated giant cells (MNGCs) and the mononuclear cells of monocyte/macrophage origin (3). The SCs are the only proliferating population and represent the neoplastic component of GCTBs. The bone resorption activity of GCTBs is primarily attributed to the MNGCs, the development and activity of which are significantly regulated by SCs via a complex network of cytokines and chemokines (1). For instance, the MNGCs and their precursors express the receptor activator of nuclear factor kappa (RANK), through which the SCs regulate their formation and osteolytic function by constitutively expressing the RANK ligand (RANKL).

Interleukin-17A (IL-17A), a CD4⁺ T cell-derived proinflammatory cytokine, has been implicated in pathological bone resorption in various skeletal disorders (4, 5). Osteoclasts are the predominant cause of bone resorption during both physiological and pathological conditions (6), IL-17A, either derived from the synovial fluids of patients with rheumatoid arthritis (RA) or as a recombinant protein, has been demonstrated to exhibit significant ability to transform monocytes into osteoclasts (7-10). Although GCTB is characterized by the ubiquitous presence of “osteoclast-like” MNGCs and massive bone destruction (1), the role of IL-17A in the pathogenesis of this disease remains poorly understood. Thus in this study, we investigated the role of IL-17A in the progression of GCTBs.

Materials and Methods

Patient samples and the evaluation of GCTB progression in patients

Tumor samples and blood were obtained from 74 patients with GCTB, 8 patients with rheumatoid arthritis (RA) and 10 patients with osteosarcoma (OS). Blood samples were also obtained from 20 healthy individuals. All GCTB patients...
were received extended curettage, with no adjuvant therapy. The clinical characteristics of all of the GCTB patients are summarized in Table 1. The progression of the GCTB was evaluated using the Campanacci grading (11) and Enneking staging systems (12). This research was approved by the Research Ethics Board of the General Hospital of People's Liberation Army.

The isolation and primary culture of osteoclast-like multinucleated giant cells (MNGCs) and stromal cells (SCs)

The GCTB samples were minced (within 4 h of surgical resection) in a Petri dish using ophthalmic scissors and incubated at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) with 0.5 mg/ml collagenase IV (Sigma) and 200 µg/ml DNase I (Sigma) at 37°C for 1 h. The resulting single-cell suspensions were washed three times with complete DMEM (supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin; all from Invitrogen). The cells were resuspended and centrifuged at 700 rpm for 1 min. The large, heavy MNGCs were enriched in the precipitates and were carefully collected. The supernatants, which predominantly contained SCs and mononuclear cells, were also collected for the further culture of the SCs. To separate the MNGC cells, the cells in the precipitates were stained with 5 µg/ml Hoechst 33342 (Sigma) at 37°C for 90 min. Due to their huge size and multiple nuclei, the Hoechst-stained MNGCs could be clearly separated using their increased Hoechst signal and Forward Scattering (FSC) by fluorescence activated cell sorting (FACS). The purified MNGCs were immediately cultured in complete DMEM at 37°C in humidified air with 5% CO₂. To identify the osteoclastic features of the cultured primary MNGCs, the cells were stained using a Tartrate-resistant acid phosphatase (TRAP) staining kit (Cellgarage), and the TRAP-positive cells were observed on day 2 by light microscopy (Supplementary Fig. S1A). The SCs were obtained by continuous culture, as previously described (13, 14) (Supplementary Fig. S1B).

Isolation of peripheral blood mononuclear cells (PBMCs)

For analysis Th17 cells, blood samples were obtained from 3 patients with GCTB, and 5 healthy control individuals. PBMCs were purified from peripheral blood by centrifugation, using a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech).
Cytokine assay and flow cytometric analysis

The IL-17A in the serum and the media of primary cultured cells were measured using the human IL-17A ELISA kit (R&D), according to the manufacturer’s protocols. For detect the IL-17A or IL-17RA-positive cells in MNGCs and SCs, the cells were labeled with phycoerythrin (PE)-conjugated-IL-17 mAbs or PE-conjugated-IL-17RA mAbs (eBioscience), according to the manufacturer’s protocols. Th17 cells in blood were detected according to Shen’s methods (15).

Immunostaining

Western blot and immunohistochemistry (IHC) were performed as previously described (16). For detect the expression of IL-17A and IL17-AR in MNGCs and SCs, cells were seeded into 8-well chamber slides (BD Science). After 24 hrs, the expression of IL-17A and IL17-RA was detected using routine immunofluorescence (IF) methods.

Bone resorption assay

The MNGCs were seeded onto dentine slices (ALPCO Diagnostic) and incubated in the presence or absence of IL-17A (Genescript) at 37 °C in humidified air with 5% CO₂ for 2 days. The slices were rinsed with PBS, incubated overnight in 1 M ammonium hydroxide, and stained with 1% toluidine blue in 0.5% sodium tetraborate solution. The number of resorptive areas or “pits” per low power field on each bone slice was counted using reflective light microscopy.

Gene expression microarray and real-time quantitative RT-PCR analysis

For the microarray assay, the IL-17RA-positive stromal cells (IL-17RA−SCs) were treated with DMSO or 10 ng/ml IL-17A (Genescript) for 48 hrs. The RNA was then amplified using the Illumina TotalPrep RNA amplification kit and hybridized onto Illumina HumanHT-12v4 expression BeadChips, according to the manufacturer’s protocol. The hybridization data were acquired using an iScan Bead Array scanner (Illumina) and pre-processed using quantile normalization (in
compliance with MIAME guidelines). From microarray results, we selected >1.5-fold changed genes. Analysis of the pathways was performed using Ingenuity Pathway Analysis 8.6 software (Ingenuity Systems).

For the qRT-PCR analysis, the total RNA of the cells was isolated using the TRIzol reagent (Invitrogen), according to the manufacturer’s protocols. Gene expression was quantified by real-time quantitative RT-PCR using QuantiTect SYRB Green dye (Qiagen). The primer sequences were as follows: MMP-9 (179 bp) forward 5'-TTGACAGCGACAAGAAGTG-3', MMP-9 reverse 5'-GCCATTACGTCGTCATAT-3'; cathepsin K (127 bp) forward 5'-CCCAGCAGTATGACACCCTT-3', cathepsin K reverse 5'-AAACGCCAACAGGAACCACA-3', IL-17 (154 bp) forward 5'-AACCGATCCACCTACCTTG-3', IL-17 reverse 5'-TCTCTGTCTGGATGGGACA-3', IL-17RA (268 bp) forward 5'-CAGACCTGCGTATTTACCTTG-3', RANKL (211 bp) forward 5'-AGAGAACACCGGTATTTACCTTG-3', RANKL reverse 5'-GCTAGATGACACCTCCTCCA-3', GAPDH (219 bp) forward 5'-GCAGGGGGAGCCAAAAAGGT-3' and GAPDH reverse 5'-TGGGTGGCAGTGATGGCATGGG-3'.

**MTT assay**

The cells were seeded into 96-well cell culture plates at 4 x 10^4/well density. The next day, the cells were treated with the indicated concentrations of IL-17A for the indicated times. For the inhibitor studies, a myc inhibitor (50 µmol/L 10058-F4, Santa Cruz Biotech.) or a STAT3 inhibitor (20 µmol/L AG490, Selleckchem) were used as pretreatments at 30 min prior to the IL-17A treatment, and the cell proliferation was measured after 48 hrs of IL-17A treatment. The cellular proliferation was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (Sigma), according to the manufacturer's protocol.

**Animal models**

IL-17RA^+^SCs were isolated from 3 GCTB patients and transfected with indicated plasmid (IL-17A and shRNA of IL-17RA plasmid from Origene). Then, 2 x 10^6 of IL-17RA^+^SCs were injected s.c. into the intrascapular region of the neck (8 nude mice/group). Nodules were excised 4 weeks after inoculation.

---

Downloaded from clincancerres.aacrjournals.org on May 29, 2017. © 2013 American Association for Cancer Research.
Statistical analysis

Kaplan-Meier survival analysis and chi-square test were used for calculating the recurrence rate of GCTB and the association of IL-17A expression with clinical parameters, respectively. All of the other results are expressed as the mean values ± standard deviation (SD), and statistical significance was analyzed by unpaired Student's t-test or one-way analysis of variance (ANOVA) and Duncan multiple range tests using SAS statistical software package version 6.12 (SAS Institute, Cary, NC). \( p \leq 0.05 \) was considered to be statistically significant.

Results

IL-17A is significantly increased in GCTB and correlates with progression

Recent studies have shown that IL-17A is abundantly increased in several tumor types and such increased IL-17A levels stimulate tumor progression (17-19). Consistent with other tumors, the serum levels of IL-17A were significantly increased in GCTB patients, compared to healthy individuals, and the results similar to RA patients (Fig. 1A). Because the Th17 cells release IL-17A, we measured the proportion of Th17 cells in the blood of GCTB patients and healthy individuals, respectively. But there was no difference between GCTB patients and healthy individuals (Fig. 1B). We also measured the IL-17A mRNA expression levels in GCTB, RA and OS tissues by qRT-PCR. Our results show that the IL-17A mRNA expression levels found in using GCTB tissues are similar to those in the RA tissues but higher than those in the OS tissues (Fig. 1C). Next, we investigated the association of IL-17A expression with clinical parameters. In all 74 cases, we found that IL-17A is more frequently overexpressed in aggressive lesions (Campanacci grading system) and extracompartmental extension lesions (Enneking staging system) (Table 2), suggesting that IL-17A might be involved in GCTB progression. Although no statistical significance was observed in the recurrence rate between IL-17A-high and IL-17A-low patient groups, the recurrence rate tended to be higher in the IL-17A-overexpressing patients (Fig. 1D).

IL-17A is predominantly produced by MNGCs, whereas IL-17RA is expressed
by both MNGCs and SCs in GCTBs

As mentioned above, GCTBs are composed of three different cell types, and further, IL-17A induces signal transduction through its receptor. Thus, we next investigated which cell types expressed IL-17RA and secreted IL-17A in GCTB. As shown in Fig. 2A, IL-17A was abundantly and almost exclusively produced by the MNGCs. In contrast, IL-17RA was expressed by both the MNGCs and the SCs. These results were confirmed using cultured primary MNGCs and SCs by IF staining and flow cytometric methods (Figs. 2B and 2C). The IL-17A mRNA was more significantly expressed in the MNGCs (Fig. 2D). We also detected high concentration of IL-17A in the conditioned media from the MNGCs compared to the SCs (Fig. 2D). Taken together, these data suggest that the main producer of IL-17A in GCTBs is the MNGCs, whereas both of the MNGCs and SCs express the receptor, are potential targets of IL-17A.

IL-17A enhances the osteolytic ability of MNGCs and increases the expressions of IL-17A, MMP-9 and cathepsin K in MNGCs

MNGC was reported to act as osteoclast cells, directly causing the massive bone resorption observed in the GCTBs (2, 6, 20). Based on the simultaneous presence of IL-17A and IL-17RA on the MNGCs, we investigated the effects of IL-17A on the capacity of the MNGCs to resorb a mineralized matrix, measured with a “pit” formation assay using dentine slices. As shown in Fig. 3A, IL-17A treatment significantly increased the numbers and areas of the MNGC-mediated bone “pits”, compared to the control group. Moreover, we demonstrated that IL-17A could increase the mRNA expression of MMP-9 and cathepsin K in the MNGCs (Fig. 3B). Interestingly, we further found that the expression of IL-17A in the MNGCs was also upregulated by IL-17A treatment (Fig. 3B). Collectively, these results suggest that IL-17A enhances the osteolytic ability of MNGCs, not only by increasing the expression of pro-osteolytic factors but also via an autocrine feedback loop by upregulating its own expression.

IL-17A stimulates the proliferation and the expression of pro-osteolytic factors in SCs and promotes angiogenesis in GCTBs

Recent studies have shown that IL-17A can affect cellular proliferation (21, 22). As expected, the proliferation rate of the IL-17RA⁺SCs was increased in an IL-17A
dose-dependent manner (Fig. 4A). Further, such results were confirmed in xenograft models. Result show that tumor growth is stimulated by IL-17A overexpression, however knock down of IL-17RA suppressed IL-17A stimulated tumor growth in IL-17RA-SCs (Fig. 4B). In contrast, the IL-17A does not affect the proliferation of IL-17RA-negative SCs (IL-17RA-SCs) (Fig. 4A). In addition, our gene expression microarray results revealed that IL-17A treatment significantly increased the expression of several pro-osteolytic factors in the IL-17RA+SCs, including RANKL, MMP-2 and MMP-9 (Supplementary Fig. S2A). Because RANKL plays a key role in osteolysis, we further confirmed the effects of IL-17A on RANKL expression at both the mRNA and protein levels, and we observed the significantly increased expression of RANKL in the IL-17RA+SCs (Fig. 4C). Notably, the IL-17RA-negative SCs (IL-17RA-SCs), which were collected from post-sorting cultures and subjected to the same treatment and analysis as the IL-17RA+SCs, showed no statistically significant changes in any of the experiments (Fig. 4C). In addition to the pro-osteolytic factors, we also demonstrated that the pro-angiogenic factor VEGF was increased by IL-17A treatment in IL-17RA+SCs (Supplementary Fig. S2A). Such results were also confirmed by the IHC analysis of the GCTB tissues. Our IHC results show that the VEGF expression and angiogenesis rates were significantly increased in the IL-17A high-expressing GCTB tissues compared to the IL-17A low-expressing GCTB tissues (Fig. 4D).

**IL-17A stimulates cellular proliferation and RANKL expression in IL-17RA+SCs through myc and STAT3 signaling, respectively**

We further investigated how IL-17A enhances RANKL expression and SC proliferation. Previous studies have shown that IL-17A directly increases RANKL expression through IL-6 and STAT3 signaling (23). Consistent with those findings, our gene expression microarray results also showed that IL-17A increased IL-6 and STAT3 expression (Supplementary Fig. S2A). Further, we using qRT-PCR and Western blotting confirmed that IL-17A induced STAT3 expression in primary cultured MNGCs (Fig. 4C). In addition, our results show that STAT3 inhibitor treatment can suppress IL-17A-induced RANKL expression in IL-17RA+SCs (Supplementary Fig. S3A). The gene array pathway analysis suggested that myc might be responsible for the IL-17A effects on SC proliferation (supplementary Fig. S2B). Indeed, the addition of a myc inhibitor to the culture of IL-17A-treated SCs in
vitro effectively reduced cellular proliferation (Supplementary Fig. S3B). These data suggest that IL-17A stimulates RANKL expression and cellular proliferation through STAT3 and myc, respectively.

**Discussion**

In this study, we found that IL-17A was significantly increased in patients with GCTBs compared to healthy individuals (Figs. 1A and 1C). In addition, we demonstrated that increased IL-17A in the GCTBs was from the abnormal secretion of IL-17A in the MNGCs (Fig. 2). In this study, we demonstrated that increased IL-17A is closely correlated with the grade and extension of the GCTBs (Table 2). This result suggesting the IL-17A can be a prognostic marker for GCTB progression.

To identify which cell populations are the targets of IL-17A in the GCTBs, we investigated the expression of IL-17RA in the GCTBs. In the IL-17A receptor family, receptor A (IL-17RA) is main receptor of IL-17A and plays an important regulatory role in expression of genes, including cytokines, chemokines, and tissue remodeling effectors (24). In this study, we identified that IL-17RA is expressed by both the MNGCs and the SCs (Figs. 2A-2C), suggesting that IL-17A might play an important role in GCTB by regulating the function of both MNGCs and SCs.

A hallmark of GCTB is its aggressive bone lytic behavior (25). Both MNGCs and SCs are known to play a critical role in the osteolysis of GCTBs. In the GCTBs, MNGCs exhibit a bone-resorbing function and many osteoclast properties, and they might exceed 50% of the total cell content of the tumor (26). Thus, we conducted a series of experiments to demonstrate whether and how does the IL-17A alters the bone-lytic behavior of MNGCs. IL-17A stimulation significantly enhanced the ability of the MNGCs bone resorption in vitro (Fig. 3A) and increased the production of osteolytic factors such as MMP-9 and cathepsin K (Fig. 3B). In the GCBTs, cathepsin K is the primary proteolytic enzyme, which largely degrades the organic components of bone (27). MMP-9 is an osteoclast bone matrix resorption enzyme that stimulates bone resorption by giant cells (28, 29). According to Zhang et al. report that IL-17A induced the expression of cathepsin K and MMP-9 in osteoclasts and that the inhibition of these enzymes expression reduces the bone resorption efficacy of the osteoclasts (30). These data suggest that IL-17A enhances the osteolytic ability of MNGCs by increasing the expression of MMP-9 and cathepsin K.
For next, we investigated the effects of IL-17A on SC proliferation and the expression of pro-osteolytic factors. Although SCs are not directly responsible for resorptive activity, they can enhance the resorption ability of giant cells by expressing and secreting multiple key components of osteolysis (26, 31, 32). In addition, SCs represent the major neoplastic and proliferative component of GCTBs (31, 33), and the increased proliferation of SCs is closely correlated with the progression of GCTB (34). Our in vitro results revealed that IL-17A can stimulate the proliferation and expression of MMP-2, MMP-9, TGFβ-1 and RANKL in IL-17RA+ SCs (Fig. 4A and B; Fig. S2A). In vivo experiment also have show that IL-17A strongly stimulates tumor growth (Fig. 4B). Previous studies have shown that MMP-2 (35), MMP-9 (36), TGFβ-1 (37) and RANKL (38) are pro-osteolytic factors, these are secreted by SCs and significantly enhance the osteolytic ability of the MNGCs. In particular, RANKL is a key osteolytic regulator because it regulates giant cell formation, osteoclast differentiation and bone resorption (39). In addition, Branstetter et al. reported that the RANKL inhibitor denosumab induces tumor reduction and bone formation in GCTB patients (25). Thus, we using Western blot analysis further confirmed the increased expression of RANKL by IL-17A in SCs and observed the expression of RANKL was significantly increased by IL-17A in the IL-17RA+ SCs (Fig. 4B). Apart from bone resorption, MMPs also play critical roles in tumor angiogenesis by increasing VEGF bioavailability (40, 41). Angiogenesis is well known to contribute to tumor progression, and VEGF signaling is essential for osteoclast formation (42). In this study, we demonstrated that the increased VEGF expression in the SCs (Fig. S2A) and stimulated angiogenesis in the IL-17A-high GCTB patient tissues (Fig. 4D). These results are consistent with other studies. For instance, Ryu et al. reported that IL-17A can increase VEGF production in RA (23) and Numasaki et al. reported that IL-17A promotes tumor angiogenesis (43). Taken together, these data suggest that IL-17A stimulates GCTB progression by promoting angiogenesis and at least partially by promoting cellular proliferation and pro-osteolytic factor expression in SCs.

Finally, to elucidate the mechanism by which the MNGCs derived-IL-17A regulates RANKL expression and enhances proliferation of IL-17RA+ SCs in GCTBs, we performed gene expression profiling and the gene array pathway analysis (Fig. 4B and Fig. S2). Results suggest STAT3 might be involved the RANKL regulation. We using RT-PCR and Western blot analysis further demonstrated that IL-17A could activate the STAT3 pathway and then regulate RANKL expression in the IL-17RA+
SCs (Figs. S3B). This result is consistent with other reports. Hashzume et al. also reported that IL-17A could induce RANKL expression through STAT3 signaling (23). Moreover, through gene expression microarray analysis, we further found the pathway by which IL-17A enhances SC proliferation. Previous studies have demonstrated that myc is involved in cancer growth. However, no information has been available for the IL-17A-mediated regulation of myc. In this study, we report that the IL-17A mediates SC proliferation through myc signaling (Fig. S3A).

The interactions between SC and MNGC are essential for understanding the etiopathogenesis of GCTB (1). The majority of previous studies have focused on and elegantly profiled the mechanisms underlying how SCs regulate MNGCs (1). However, whether and how MNGCs regulate SCs remains poorly understood. Our current study has revealed the important finding that cytokines secreted by MNGCs can regulate the expression of RANKL expression and the proliferation of SCs.

In summary, our study, to the first of our knowledge, has delineated the association of IL-17A with the progression of GCTBs. IL-17A stimulates the progression of GCTBs by promoting angiogenesis, SC proliferation and osteolysis through increasing the expression of pro-osteolytic factors in both the MNGCs and the SCs. Our results suggest that IL-17A is a useful progression marker of GCTB and blocking IL-17A might represent a new therapeutic strategy for GCTB.

Grant Support

This work was supported by the National High Technology Research and Development Program of China (2011AA030101).

Authors' Contributions

Conception and design: M.Xu, C.X.Xu, G.H.Rong, K.X.Fang, Y.Wang

Development of methodology: M.Xu, Z.G.Song, C.X.Xu, G.H.Rong, K.X.Fang, Y.Wang

Acquisition of data: M.Xu, Z.G.Song, W.Zhang, J.P.Jia, J.Y.Chen, G.Han, W.Wang, W.Chai, W.T.Liang, W.Z.Bi, Y.Wang
Analysis and interpretation of data: M.Xu, Z.G.Song, C.X.Xu, G.H.Rong, K.X.Fang

Writing, review, and/or revision of the manuscript: M.Xu, C.X.Xu, G.H.Rong, K.X.Fang, W.Z.Bi, Y.Wang.

Administrative, technical, or material support: M.Xu, C.X.Xu, G.H.Rong, K.X.Fang.

Study supervision: C.X.Xu, W.Z.Bi, Y. Wang.

References


arthritides are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production. Arthritis Rheum 2011;63:73-83.


42. Niida S, Kondo T, Hiratsuka S, Hayashi SI, Amizuka N, Noda T, et al. VEGF

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33</td>
<td>45</td>
</tr>
<tr>
<td>Female</td>
<td>41</td>
<td>55</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤30</td>
<td>58</td>
<td>78</td>
</tr>
<tr>
<td>&gt;30</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>Pathological fracture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>No</td>
<td>66</td>
<td>89</td>
</tr>
<tr>
<td>Tumor extension</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (Intracompartmental)</td>
<td>45</td>
<td>61</td>
</tr>
<tr>
<td>T2 (Extracompartmental)</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>Campanacci grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I/II</td>
<td>40</td>
<td>54</td>
</tr>
<tr>
<td>Grade III</td>
<td>34</td>
<td>46</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>No</td>
<td>52</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 2. The association of IL-17A expression with clinical parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL17A</th>
<th></th>
<th></th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>18</td>
<td></td>
<td>0.676</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤30</td>
<td>34</td>
<td>24</td>
<td></td>
<td>0.994</td>
</tr>
<tr>
<td>&gt;30</td>
<td>10</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pathological fracture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>2</td>
<td></td>
<td>0.571</td>
</tr>
<tr>
<td>No</td>
<td>38</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor extension</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (Intracompartmental)</td>
<td>22</td>
<td>23</td>
<td></td>
<td>0.039</td>
</tr>
<tr>
<td>T2 (extracompartmental)</td>
<td>22</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campanacci grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I/II</td>
<td>18</td>
<td>22</td>
<td></td>
<td>0.012</td>
</tr>
<tr>
<td>Grade III</td>
<td>26</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* chi-square test
Figure legends

Figure 1. IL-17A is significantly increased in giant cell tumors of bone (GCTBs) but does not correlate with the recurrence of GCTBs. A, The serum IL-17A levels were examined in 74 GCTB patients, 8 rheumatoid arthritis (RA) patients, 10 osteosarcoma (OS) patients, and 20 healthy individuals by ELISA. B, Th17 cells were detected by flow cytometry using CD4+ and IL-17A antibodies in blood. Blood samples are obtained from 3 GCTB patients, and 5 healthy control individuals. C, IL-17A mRNA expression was determined in tissues of GCTB (n=12), OA (n=8), and RA (n=10) by qRT-PCR. D, The prognosis of the 74 cases of GCTB was followed up for 6 years, and the recurrence was recorded. Kaplan-Meier analysis of 74 patients who received curettage detected no statistically significant correlation between recurrence and IL-17A expression levels. GCTB patients were classified to the "high" and the "low" IL-17A expression group using results from IHC (positive more/less than 25%). a,b,c,d data not sharing the same letter are significantly different from one another in each group (P < 0.05) by ANOVA and Duncan's multiple range test.

Figure 2. IL-17A is primarily produced in multinuclear giant cells (MNGCs), and the IL-17A receptor A (IL-17RA) is expressed in both MNGCs and stromal cells (SCs). A, Immunohistochemical analysis is show that IL-17A is predominantly located in MNGCs, whereas IL-17RA is expressed in MNGCs and some SCs. A representative photograph of 400×; the white arrows indicate the MNGCs. B, Expression of IL-17A and IL-17RA was assessed in the cultured primary MNGCs and SCs using immunofluorescence. C, IL-17A or IL-17RA-positive cells were detected in MNGCs and SCs by flow cytometry analysis. The MNGCs and SCs were isolated from three OS patients with high IL-17A expression. For IL17 detection the cells were permeabilized. D. The IL-17A mRNA and protein levels were measured in the cultured primary cells and their cultured media. The mean values ± SD are shown. ***p<0.001.
Figure 3. IL-17A stimulates the osteolytic ability and the expression of IL-17A and pro-osteolytic factors in MNGCs. A, The osteolytic ability of MNGCs in the presence of IL-17A was assessed by “pit” formation assay using dentine slices. B, IL-17A significantly increased the mRNA expression levels of IL-17A, cathepsin K and MMP-9 in MNGCs, which were measured by qRT-PCR. The results are expressed as the mean values ± SD. a,b,c,d data not sharing the same letter are significantly different from one another in each group (P < 0.05) by ANOVA and Duncan's multiple range test.

Figure 4. IL-17A stimulates cell proliferation and pro-osteolytic factors expression in IL-17RA-positive stromal cells (IL-17RA⁺SCs) and promotes angiogenesis in GCTB tissues. A, The effects of IL-17A on the proliferation of the IL-17RA⁺SCs and IL-17RA⁻SCs were measured by MTT assay. B, IL-17RA⁺SCs cells were transfected with indicated plasmid and injected to mice. Four weeks after, the mice were sacrificed and measured tumor volume. C, IL-17A increased the expression of RANKL, STAT3, and myc at both the mRNA and protein levels in IL-17RA⁺SCs, but not in IL-17RA⁻SCs. Indicated SCs were treated with 10 ng/ml IL-17A for 48 hrs and then examined at the mRNA and protein levels using qRT-PCR and Western blot, respectively. ***, p<0.001. D, VEGF and microvessels (CD31) were detected in IL-17A-high and IL-17A-low GCTB patient tissues by immunohistochemistry. The magnification is 100x. a,b,c,d data not sharing the same letter are significantly different from one another in each group (P < 0.05) by ANOVA and Duncan's multiple range test.
Figure 1

A. IL-17A (pg/ml) levels in different groups:
- HC
- GCTB
- RA
- OS

B. Percentage of CD+IL17+ cells in blood:
- HC
- GCTB

C. Gene expression levels:
- GCTB
- RA
- OS

D. General Recurrence Rate:
- IL17A high (n=44), recurrence rate = 36.4%
- IL17A low (n=30), recurrence rate = 20.0%

P = 0.086
Figure 2
**Figure 3**

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Control image.</td>
</tr>
<tr>
<td>IL-17A (0.1 ng/ml)</td>
<td>Image 1.</td>
</tr>
<tr>
<td>IL-17A (1 ng/ml)</td>
<td>Image 2.</td>
</tr>
<tr>
<td>IL-17A (10 ng/ml)</td>
<td>Image 3.</td>
</tr>
</tbody>
</table>

B

**Cathepsin K**

- **Relative expression (fold)**
  - **IL-17A (ng/ml)**: 0, 0.1, 1, 10

**MMP 9**

- **Relative expression (fold)**
  - **IL-17A (ng/ml)**: 0, 0.1, 1, 10

**IL-17A**

- **Relative expression (fold)**
  - **IL-17A (ng/ml)**: 0, 0.1, 1, 10
**Figure 4**

**A**

- IL-17RA⁺SCs
- Control
- IL-17A (0.1 ng/mL)
- IL-17A (1 ng/mL)
- IL-17A (10 ng/mL)

**B**

- Control
- IL-17A
- IL-17A/shIL-17RA
- shIL17-RA

**C**

- RANKL
- STAT3
- Myc
- GAPDH

**D**

- High IL-17A
- Low IL-17A
- VEGF
- CD31
Clinical Cancer Research

IL-17A Stimulates the Progression of Giant Cell Tumors of Bone

meng xu, zhi-gang song, Cheng-Xiong Xu, et al.

Clin Cancer Res  Published OnlineFirst July 15, 2013.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-0251

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/07/12/1078-0432.CCR-13-0251.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.