Original Article

The anti-CXCL4 antibody depletes CD4(+)CD25(+)FOXP3(+) regulatory T cells in CD4+ T cells from chronic osteomyelitis patients by the STAT5 pathway

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Background: Chronic osteomyelitis is associated with the immune suppression. CD4(+)CD25(+) FOXP3(+) regulatory T cells (Tregs) play a key role in the peripheral tolerance to prevent immune responses to self-antigens and allergens. Evidence has suggested that the accumulation and activity of Tregs are regulated by chemokine family member CXCL10 and its receptor CXCR3 in human atherosclerotic lesions. This study aimed to investigated the effect of CXCL4, a member of chemokine family, on Tregs, and the underlying mechanisms.

Methods: CD4+ T cells were isolated from peripheral blood of patients with chronic osteomyelitis or healthy controls. Anti-CXCL4 antibody and recombinant CXCL4 protein were used for treatment. The expression of forkhead box P3 (FOXP3), cytotoxic T lymphocyte antigen-4 (CTLA-4) and phosphorylated signal transducer and activator of transcription 5 (STAT5) were measured to assess the mechanism. STAT5 inhibitor (IST5-002) was used to retard STAT5 pathway.

Results: We found that serum concentration of CXCL4 in chronic osteomyelitis was significantly enhanced. Through the prevention of STAT5 activity, CXCL4 antibody could inhibit the protein expression of CXCL4, CXCR3, FOXP3, CTLA-4 and phosphorylated-STAT5, as well as decrease the percentage of Tregs in CD4+ T cells. Conversely, recombinant CXCL4 protein resulted in the opposite in CD4+ T cells from healthy controls, obviously enhancing Tregs percentage and promoting STAT5 activation, which were significantly reversed by an STAT5 inhibitor.

Conclusions: CXCL4 antagonism inhibited Tregs percentage and Tregs-associated proteins within CD4+ T cells from chronic osteomyelitis patients via blocking the STAT5 pathway.

Keywords: Chronic osteomyelitis; CXCL4; regulatory T cells (Tregs); signal transducer and activator of transcription 5 pathway (STAT5 pathway)

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Introduction

Osteomyelitis covers a large pattern of inflammatory bone disorders caused by infectious organisms or auto-inflammatory processes (1). Currently, posttraumatic or postoperative osteomyelitis, the main subtype of osteomyelitis, stands for 80% of a chronic state of osteomyelitis due to sustaining bacterial biofilm from foreign materials (1,2). Immune suppression frequently occurs among chronic osteomyelitis (3). Noteworthy, osteonecrosis and bone resorption regarding infectious

Table 1 Patient demographics

Group	Sex		Age	Lesion site		
	Male	Female		Tibia	Calcaneus	Femur
Healthy	17	13	41.83±12.52	N/A	N/A	N/A
Osteomyelitis	29	8	42.36±16.87	19	12	6

organisms usually disturb antibiotics treatments of this disease, thus subscribe the bacterial with immune evasion (4). Simultaneously, the immune-regulatory treatment might provide a therapeutic possibility of chronic osteomyelitis (5). Hence, the therapeutic immune modulation or exploring the underlying mechanisms has captured an increased clinical attention to block the chronic osteomyelitis.

CD4(+)CD25(+)FOXP3(+) regulatory T cells (Tregs), marked by the expression of forkhead box P3 (FOXP3), are a specialized subset of CD4+ T cells, accounting for 5–10% of peripheral CD4+ T cells. Tregs exert an immune-suppressive property and are usually up-regulated in chronic inflammatory disease (6). Mechanically, Tregs function in inhibiting potentially auto-reactive T cells, maintaining immune tolerance and controlling immune responses during allergy, autoimmunity, inflammation as well as tumors immunity, which render it the core component of peripheral tolerance (7-10). Therefore, dysfunction or downregulation of Tregs might represent an immunotherapy strategy to restrict chronic osteomyelitis (11).

Tregs express significant amounts of C-X-C chemokine family members that mediate the traffic and recruitment of immune cells to the inflammation sites (12). CXC family member CXCL10 and CXCL14 are documented important roles in immune regulation in inflammatory-associated coronary artery disease, atherosclerosis (13,14), or ischemic stroke (15). CXCL4 is involved in atherosclerosis and other inflammatory diseases through forming a CC-type heterodimer with CCL5 (16), drives chemotaxis of CCR1-expressing cells (17) and its neutralizing antibody activates platelets in autoimmune disorders, especially in thrombocytopenia in the absence of heparin (18). Report has demonstrated that CXCL4 augment the secretion of pro-inflammatory cytokines of human CD4+ T cells, and meanwhile favors the stimulation of T1 and T17 cells, the two subsets of CD4+ T cells, in psoriatic arthritis (19). However, whether CXCL4 regulated Tregs and whether CXCL4 would represent an immunomodulator in chronic osteomyelitis remained largely unknown.

Signal transducer and activator of transcription 5 (STAT5) plays a key role in the development of Tregs (20). CXCL4 signaling experiments show involvement of mitogen-activated protein (MAP) kinases, Src and p70S6 kinase in cells expressing CXCR3A and CXCR3B (21). Evidence suggests that chemokine CXCL12 activates STAT5 pathway (22). In this work, to study the effect of chemokine CXCL4 on Tregs induction, and whether STAT5 pathway was the underlying mechanism, CD4+ T cells, isolated from patients with chronic osteomyelitis and corresponding healthy donor (HD), were treated with anti-CXCL4 antibody or recombinant CXCL4 protein *in vitro*. STAT5 inhibitor (IST5-002) was used to block STAT5 pathway (23). Our data suggested that CXCL4 regulated the percentage of Tregs in CD4+ T cells via JAK3/STAT5 pathway.

Methods

Enzyme-linked immunosorbent assay (ELISA) assay

To study the involvement of CXCL4 in chronic osteomyelitis, serum sample of 37 patients with chronic osteomyelitis and 30 corresponding healthy people were recruited from Tongde Hospital of Zhejiang Province without significant difference in age and gender between those two groups. The inclusion criteria were as follows: (I) diagnosis with chronic osteomyelitis; (II) age: 18–70; (III) with the patient's informed consent. And the exclusion criteria were as follows: (I) with diseases of heart, liver, kidney and other organs; (II) with diabetes; (III) with other infectious diseases; (IV) with bone tuberculosis and tumor. And the patient demographic was listed in *Table 1*. This study was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province (No. 2016-016), and conducted in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent from the participants was obtained. Human platelet factor 4 (PF4/CXCL4) ELISA assay kit (ab189573, Abcam, USA) was used to determine serum CXCL4 concentration (ng/mL) accordance with the manufacture's procedure.

Preparation of peripheral blood mononuclear cells (PBMCs)

Peripheral bloods (30 mL) from 37 chronic osteomyelitis cases and 30 HDs were collected into heparinized tubes. Then, peripheral blood lymphocytes (PBLs) was acquired, using human PBL isolation kit (P8900, Solarbio, China), and immediately used for the next step.

CD4+ T cells isolation and treatment

CD4+ T cells were freshly isolated from PBLs by via a negative selection principle using MagCellect^{TM} human CD4+ T cell isolation kit (MAGH102, R&D Systems, USA) according to the provided instructions. The purity of CD4+ was above 92%. Freshly isolated CD4+ T were cultured in a complete RPMI media (Hyclone, USA) with 10% fetal calf serum (GIBCO, USA) and 100 U/mL penicillin (Solarbio) being added.

To assess the roles of CXCL4 in regulating Tregs percentage, freshly purified CD4+ T cells from patients were co-cultured with 200 and 500 μg/L of anti-CXCL4 antibody (Abcam Cat# ab9561, RRID:AB_308720) for 24 h. To study the involvement of STAT5 pathway in the promoted effect of CXCL4 protein on Tregs content, CD4+ T cells from healthy people were treated with 100 ng/mL of recombinant CXCL4 protein (795-P4-025, R&D), or 100 ng/mL of recombinant CXCL4 protein plus 10 μM of STAT5 inhibitor (Fox Chase Chemical Diversity, USA) (23).

Flow cytometric analysis of Tregs

CD4+ T cells were diluted with phosphate-buffered saline (10^6 cells/mL), and then stained with FITC-conjugated anti-human CD4 antibody (Thermo Fisher Scientific Cat# 11-0048-42, RRID:AB_1633390), APC-conjugated anti-human CD25 antibody (Thermo Fisher Scientific Cat# 17-0257-42, RRID:AB_11218671), or appropriate isotype control Abs (Mouse IgG1, kappa) for 30 min at 4 ℃ in darkness. For FOXP3 intracellular staining, after cell surface staining, cells were fixed by 2% formaldehyde and permeabilized using human FOXP3 Fix buffer (eBioscience) for 30 min, and then stained with PE-conjugated anti-human FOXP3 antibody (Thermo Fisher Scientific Cat# 12-4777-42, RRID:AB_1944444) or respective isotype control for 30 min. Tregs was defined using flow cytometry (BD Biosciences, San Jose, CA, USA). Live cell population of lymphocyte gated on the basis of FSC/SSC (gate P1) and P2 gating for CD4+ T cells positive cell population was set. The percentage of Tregs in CD4+ cells was calculated using FlowJo 7.6.1 software (Tree Star Inc., Ashland, OR, USA).

Western blot

After fully lysed, total protein within CD4+ T cells was quantified using BCA protein assay kit (Thermo), and 25 μg of which were separated using 15% SDS-PAGE. Proteins of CXCL4, chemokine receptor 3 (CXCR3), FOXP3, cytotoxic T lymphocyte antigen-4 (CTLA-4), STAT5 and p-STAT5 in the electrophoretic pure were transferred onto PVDF membranes (Millipore, USA) and incubated with the primary antibodies: antibody against CXCL4 (Abcam Cat# ab49735, RRID:AB_870744), CXCR3 (Ab154845, Abcam), FOXP3 (Abcam Cat# ab20034, RRID:AB_445284), CTLA-4 (Ab134090, Abcam), STAT5 (Abcam Cat# ab16276, RRID:AB_302363) and phosphorylated (p)-STAT5 (Abcam Cat# ab32364, RRID:AB_778105) and antibody against glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling Technology Cat# 5174, RRID:AB_10622025) at 4°C overnight followed by secondary antibodies (Beyotime, Shanghai, China) for 1 hour at 25 ℃. ECL system (GE Healthcare/Amersham Biosciences) was used for analysis. p-STAT5 was normalized using STAT5 while CXCL4, CXCR3, FOXP3, CTLA-4 and STAT5 were normalized using GAPDH.

Statistical analysis

Data was calculated from three independent experiment, analyzed using GraphPad Prism 7.0 software (La Jolla, CA, USA) and described as mean ± SEM (for serum sample analysis of CXCL4) or mean ± SD (for cell experiments). *T*-test (unpaired, two-tailed) was conducted for comparison between two groups and one-way ANOVA followed by Newman-Keuls post hoc tests was used for more than two groups. P values <0.05 were considered significant.

Results

CXCL4 was up-regulated in chronic osteomyelitis

To study the roles of CXCL4 in chronic osteomyelitis, concentration of CXCL4 in serum samples of patients with chronic osteomyelitis (n=37) and healthy people (n=30) was assessed, using ELISA assay. As shown in *Figure 1*, CXCL4 was significantly enhanced when compared with corresponding healthy control, suggesting the involvement

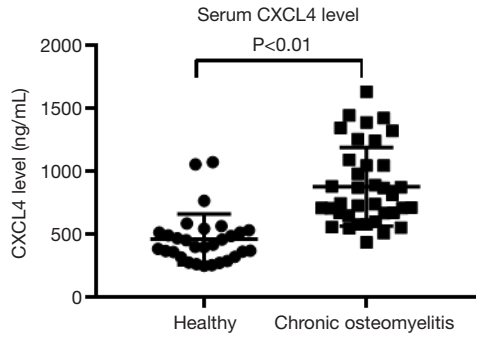

Figure 1 CXCL4 was up-regulated in chronic osteomyelitis. Serum content of CXCL4 form patients with chronic osteomyelitis (n=37) and healthy people (n=30) was assessed, using ELISA assay. ELISA, enzyme-linked immunosorbent assay.

of CXCL4 in chronic osteomyelitis.

Anti-CXCL4 antibody inhibited Tregs percentage and suppressed the activation of STAT5 pathway in CD4+ T cells of patients with chronic osteomyelitis

Figure S1 showed that Tregs percentage, and protein expression of CXCL4, CXCR3, FOXP3 and p-STAT5 was significantly enhanced within CD4+ T cells isolated from patients with chronic osteomyelitis when compared with the healthy control. To study the roles of CXCL4 in regulating Tregs content and the underlying mechanism, isolated CD4+ T cells (*Figure S2*) from patients with chronic osteomyelitis were exposure to IgG control and anti-CXCL4 antibody (200 and 500 μg/L), respectively. As shown in *Figure 2A,B*, anti-CXCL4 antibody decreased the percentage of Tregs in CD4+ T cells. *Figure 2C,D* showed a significant reduction in CXCL4 expression in CD4+ T cells after exposure to anti-CXCL4 antibody (200 and 500 μg/L), demonstrating a successful CXCL4 neutralization using monoclonal antibodies against CXCL4. Besides, anti-CXCL4 antibody decreased protein levels of CXCL4, CXCR3, FOXP3, CTLA-4 and p-STAT5 in a dose-dependent manner (*Figure 2C,D*), suggesting the inhibitory effect of anti-CXCL4 antibody on Tregs content in CD4+ T cells of patients, which was associated with the inactivation of STAT5 pathway.

Recombinant CXCL4 protein promoted the percentage of Tregs in CD4+ T cells from healthy people via activating STAT5 pathway

To study whether STAT5 pathway was the mechanism, by which CXCL4 regulated Tregs content in CD4+ T cells, CD4+ T cells isolated from healthy people were treated with recombinant CXCL4 (100 ng/mL), or recombinant CXCL4 (100 ng/mL) plus STAT5 inhibitor (10 μM), and then the percentage of Tregs, as well as protein levels of FOXP3, CTLA-4 and p-STAT5 within CD4+ T cells were assessed. As shown in *Figure 3*, with CXCL4 treatment, the proportion of Tregs as well as protein levels of FOXP3, CTLA-4 and p-STAT5 were significantly enhanced when compared with the normal cells. However, recombinant CXCL4-induced the changes of events mentioned above were significantly reversed with additional STAT5 inhibitor treatment, strengthening that recombinant CXCL4 protein increased Tregs percentage and function associated proteins via the activation of STAT5 pathway.

Discussion

Tregs were enhanced in chronic osteomyelitis (11). Active immunosuppression by Tregs induces peripheral tolerance to both self and foreign antigens *in vivo*. Thus, down-regulation of Tregs is a prospect potential to limit immunosuppression during chronic osteomyelitis. Chemokine CXCL10 and its receptor CXCR3 have been demonstrated promoted effect on the accumulation of Tregs (14). Herein, we found serum concentration of chemokine CXCL4 was up-regulated in chronic osteomyelitis (*Figure 1*). To study the effect of CXCL4 on Tregs content of chronic osteomyelitis, CD4+ T cells were isolated from chronic osteomyelitis patients. The *in vitro* data suggested that the percentage of Tregs/CD4+ T cells was remarkably inhibited in the presence of anti-CXCL4 antibody (*Figure 2A*), indicating that neutralization of CXCL4 attenuated the immunosuppression during chronic osteomyelitis by decreasing Tregs number. Conversely, recombinant CXCL4 significantly promoted the percentage of Tregs/CD4+ T cells of healthy people (*Figure 3A*), demonstrating that exogenous increase of CXCL4 contributed to enhanced Tregs numbers.

FOXP3 confers the regulatory activity of Tregs which not only regulates suppressive phenotype of Tregs, but also stabilizes the Tregs lineage (7,24). Loss of FOXP3 impairs immune suppressive activity of Tregs (25,26). Furthermore, Tregs constitutively express CTLA-4, the other marker of Tregs activation, which is not Treg-cell-specific or participates in *in vitro* immunosuppression (8). To elucidate the regulation of CXCL4 on Tregs at molecular levels, protein expression of FOXP3 and CTLA-4 within CD4+ T

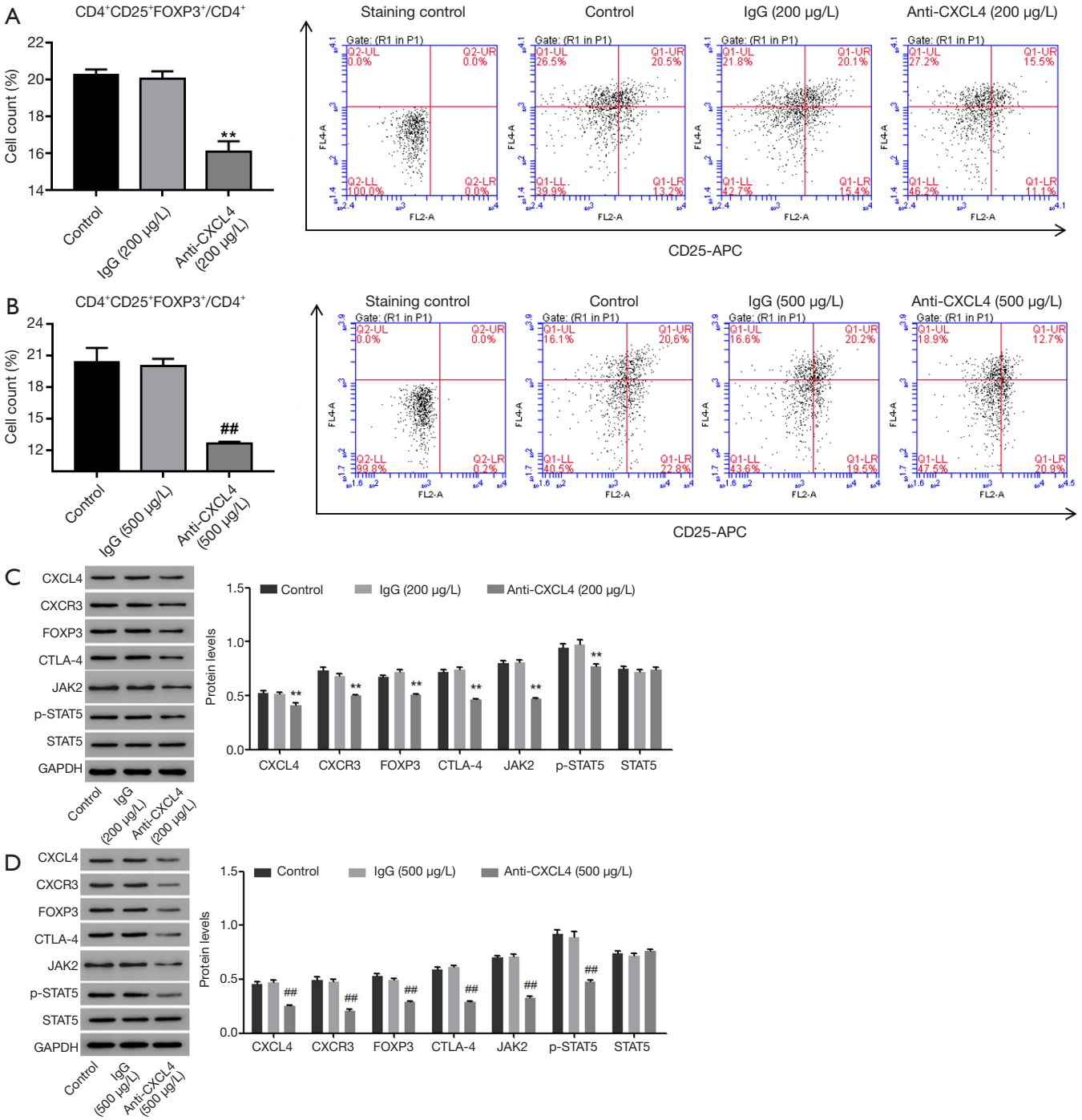

Figure 2 Anti-CXCL4 antibody inhibited Tregs percentage and prevented STAT5 activation in CD4+ T cells of patients. CD4+ T cells isolated from six patients with chronic osteomyelitis were incubated with anti-CXCL4 antibody (200 and 500 μg/L) for 24 h. (A,B) The percentage of Tregs in CD4+ T cells was measured using Flow cytometric analysis, and CD4(+)CD25(+)FOXP3(+) T cells were presented in the upper right quadrant; For staining control in reprehensive Flow cytometric analysis, Mouse IgG2b kappa Isotype Control (eBMG2b), APC, eBioscience™ from Ebioscience with catalog #17-4732-81 was used as an isotype control for anti-CD25 antibody, and Mouse IgG1 kappa Isotype Control (P3.6.2.8.1), PE, eBioscience™ from Ebioscience with catalog #12-4714-82 was used as an isotype control for anti-FOXP3 antibody. Rabbit IgG Isotype Control (Invitrogen, 02-6102) was used as an isotype control for anti-CXCL4; (C,D) protein levels of CXCL4, CXCR3, FOXP3, CTLA-4, STAT5 and p-STAT5 within CD4+ T cells were assessed, by western bolt. GAPDH was used to normalize CXCL4, CXCR3, FOXP3 and CTLA-4 while STAT5 was used to quantified p-STAT5. **, P<0.01 *vs.* IgG (200 μg/L); ##, P<0.01 *vs.* IgG (500 μg/L). Tregs, regulatory T cells.

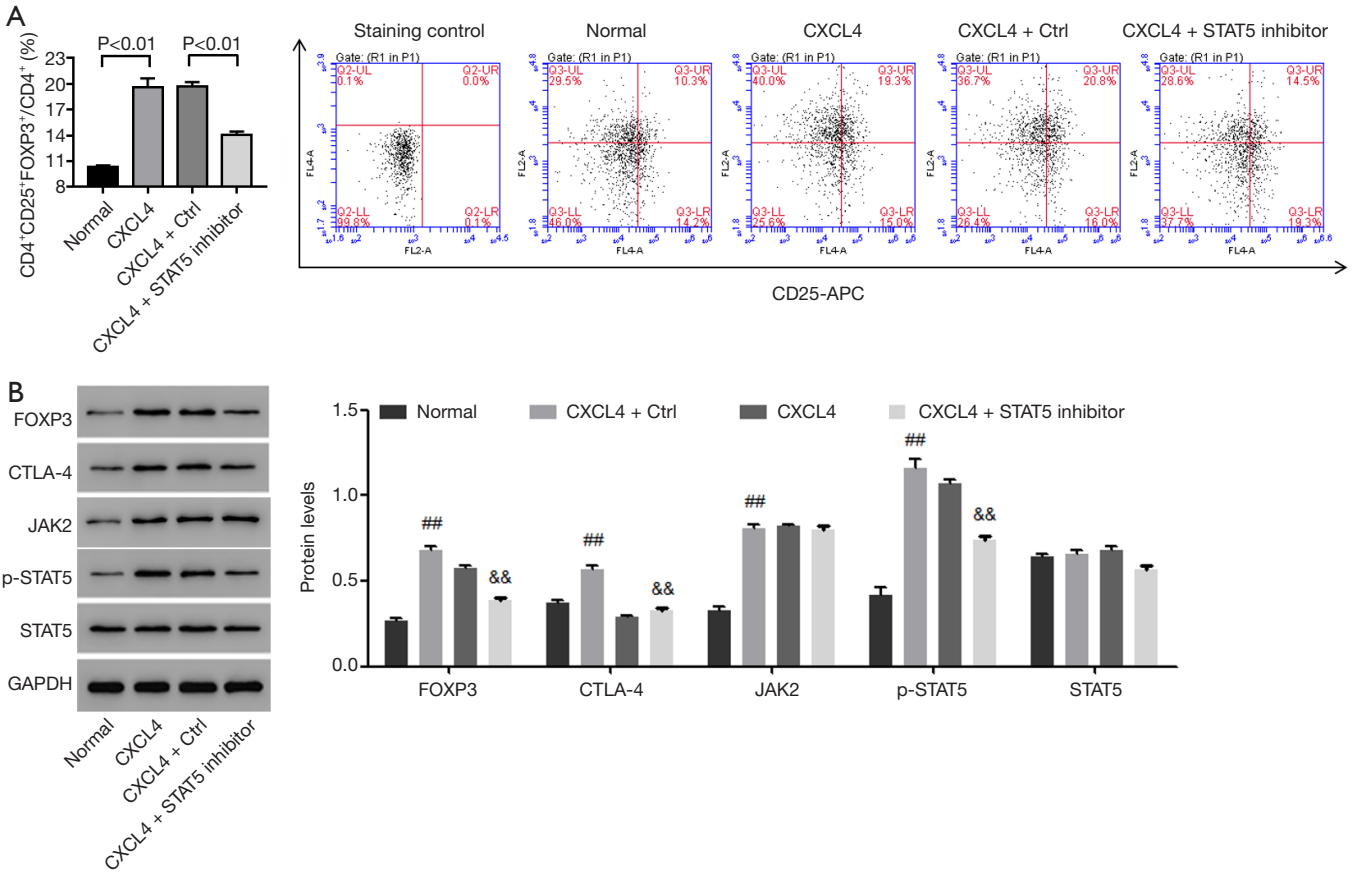

Figure 3 Recombinant CXCL4 protein promoted proliferation of Tregs in CD4+ T cells of healthy people via activating STAT5 pathway. CD4+ T cells isolated from 6 healthy people were treated with 100 μg/L of recombinant CXCL4 [dissolved in a solution of 0.32% Tris HCl, 1.17% Sodium chloride, 50% Glycerol and 0.02% DTT with the final dosage of the solution in culture medium (v/v) being 0.1%] in the presence of 10 μM of STAT5 inhibitor (IST5-002) or vehicle [DMSO, with the final dosage of DMSO in culture medium (v/v) being 0.01%] for 24 h. (A) the proportion of Tregs in CD4+ T cells was measured using Flow cytometric analysis, and CD4(+)CD25(+)FOXP3(+) T cells were presented in the upper right quadrant; For staining control in reprehensive Flow cytometric analysis, Mouse IgG2b kappa Isotype Control (eBMG2b), APC, eBioscience™ from Ebioscience with catalog #17-4732-81 was used as an isotype control for anti-CD25 antibody, and Mouse IgG1 kappa Isotype Control (P3.6.2.8.1), PE, eBioscience™ from Ebioscience with catalog #12-4714-82 was used as an isotype control for anti-FOXP3 antibody; (B) protein levels of FOXP3, CTLA-4, STAT5 and p-STAT5 within CD4+ T cells were assessed by western bolt. ##, P<0.01 *vs.* normal cells; &&, P<0.01 *vs.* CXCL4 + Ctrl. Tregs, regulatory T cells.

cells was assessed in the presence of anti-CXCL4 antibody or recombinant CXCL4 protein. Our results suggested that anti-CXCL4 antibody (200 and 500 μg/L) dose-dependently decreased FOXP3 and CTLA-4 (*Figure 2C,D*). Conversely, recombinant CXCL4 significantly increased FOXP3 and CTLA-4 (*Figure 3B*), indicating the promoted effect of CXCL4 on the activation and function of Tregs at a molecule level. Furthermore, evidence suggested that chemokine receptor CXCR3, secreted on circulating human Tregs, mediates the transportation of Tregs into the peripheral sites of inflammation (27). Inspiringly, our data firstly suggested the reduction of CXCR3 within Tregs in the presence of anti-CXCL4 antibody (*Figure 2C,D*).

Activation of STAT5 is sufficient to increase the numbers Tregs, favors FOXP3 expression in Tregs, and facilitates Treg cell function *in vitro* (28,29). STAT5 is a transcription factor, which is involved in cytosolic signaling and in mediating the expression of specific genes. Upon activation of JAKs through cytokine and growth factors stimulation, receptor associated and unphosphorylated

STATs will be phosphorylated, which subsequently results in parallel dimerization and translocation to the nucleus to activate gene transcription (30). STAT5 pathway modulates Tregs function in asthmatics and diabetes (31,32). However, fewer reports have demonstrated the roles of STAT5 in regulating Tregs in chronic osteomyelitis. Our results indicated that the activation of STAT5 within CD4+ T cells was suppressed by anti-CXCL4 antibody while accelerated under recombinant CXCL4 treatment (*Figures 2,3*). Morcinek *et al.* (33) demonstrated that STAT5 could regulate the proliferation and apoptosis of cells, which is probably the cause of number changes in Tregs cell. Furthermore, recombinant CXCL4 induced the promoted effect on Tregs percentage and on the expression of FOXP3 and CTLA-4 within CD4+ T cells were significantly reversed by additional treatment of STAT5 inhibitor, substantiating that activation of STAT5 pathway was the mechanism, by which CXCL4 regulated Tregs content and activation in chronic osteomyelitis.

Conclusions

Anti-CXCL4 antibody inhibited Tregs percentage and reduced the expression of FOXP3 and CTLA-4 in CD4+ T cells, and blocking STAT5 activation was the underlying mechanism. Our data suggested that stimulation of CD4+ T cells with anti-CXCL4 antibody influenced Tregs content and its function. Further effort is needed to explore whether targeting CXCL4 is an immunotherapeutic strategy to block human chronic osteomyelitis.

Acknowledgments

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Footnote

Data Sharing Statement: Available at http://dx.doi.org/10.21037/apm-20-166

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-20-166). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province (No. 2016-016), and conducted in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent from the participants was obtained.

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Supplementary

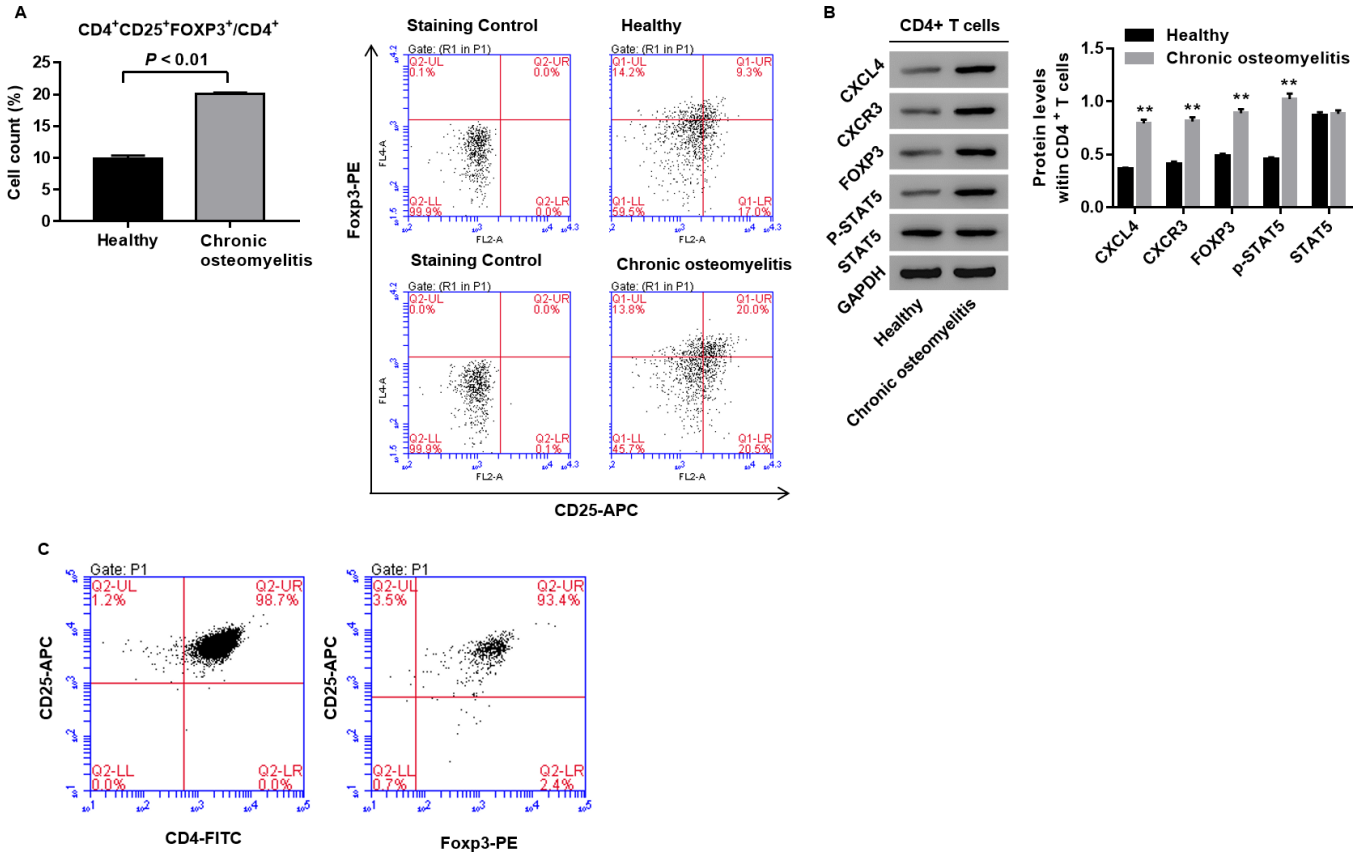

Figure S1 Analysis of Tregs percentage and function associated proteins, expression of CXCL4 and CXCR3, as well as the activation of STAT5 pathway between patients with chronic osteomyelitis and healthy people. (A) Flow cytometric analysis showing the percentage of Tregs in CD4+ T cells, which was presented in the upper right quadrant; for staining control in reprehensive flow cytometric analysis, 5 μL of Mouse IgG2b kappa Isotype Control (eBMG2b), APC, eBioscience™ from Ebioscience with catalog #17-4732-81 was used as an isotype control for anti-CD25 antibody, and 5 μL of Mouse IgG1 kappa Isotype Control (P3.6.2.8.1), PE, eBioscience™ from Ebioscience with catalog #12-4714-82 was used as an isotype control for anti-FOXP3 antibody; (B) Western bolt showing protein levels of CXCL4, CXCR3, FOXP3, STAT5 and p-STAT5 within CD4+ T cells in those two groups. GAPDH was used to normalize CXCL4, CXCR3 and FOXP3 while STAT5 was used to quantified p-STAT5. **, P<0.01 *vs.* healthy people; (C) the purity detection of Treg cells after isolation. Tregs, regulatory T cells.

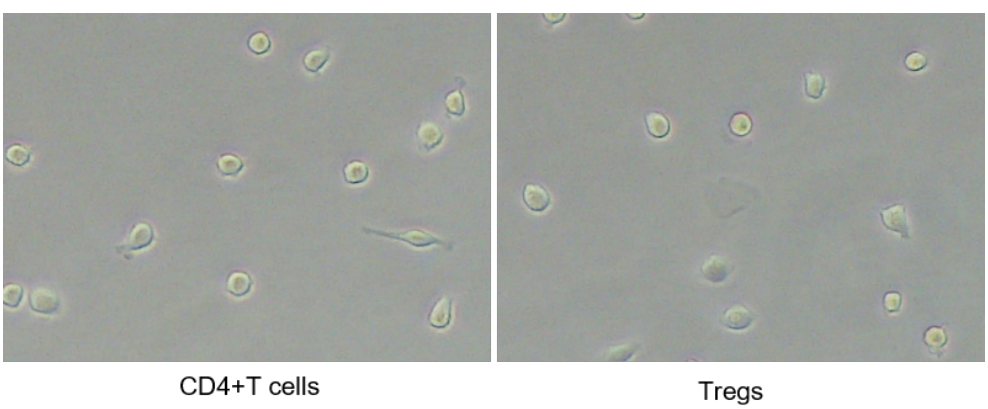

Figure S2 CD4+ T cells and Tregs cells were observed using microscopy with 40× magnification. Tregs, regulatory T cells.