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Induced T Regulatory Cells Suppress Osteoclastogenesis and Bone Erosion in Collagen-induced Arthritis Better than Natural T Regulatory Cells

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Abstract

Objective—Osteoclasts are responsible for bone destruction in rheumatoid arthritis (RA) and natural CD4⁺Foxp3⁺regulatory T cells (nTregs) can inhibit osteoclastogenesis. This study aims to determine whether TGF- β -induced CD4⁺Foxp3⁺regulatory T cells (iTregs) also suppress osteoclastogenesis and bone erosion in collagen induced arthritis (CIA).

Methods—Osteoclasts were induced from bone-marrow CD11b⁺ cells with RANKL and macrophage colony-stimulating factor (M-CSF), and assessed with tartrate-resistant acid phosphatase (TRAP) staining. CD4⁺ iTregs were generated with TGF- β and added to cultures with different ratios with CD11b⁺ cells. Transwell and antibody blockade experiments were performed to define the mechanism of action. NF- κ B activation was determined by western blot. 3 $\times$ 10⁶ CD4⁺ iTregs, nTregs or control cells were adoptively transferred to DBA1/J mice on day 14 after

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AUTHORS CONTRIBUTIONS

Dr. Song Guo Zheng had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Zheng, Zou, Stohl.

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immunization with CII/CFA. CIA onset and severity were monitored and bone erosion was examined by CT scan.

Results—Both CD4⁺ Tregs almost completely suppressed osteoclastogenesis but only iTregs sustained the effect in the presence of IL-6 *in vitro*. CD4⁺ iTregs but not nTregs and control cells injected after immunization and before of onset of CIA significantly suppressed disease development. Of note, CT scan showed that the joints in CD4⁺ iTregs but not nTregs or control cells infused CIA had less bone erosion. Treatment with CD4⁺ iTregs but not other cells dramatically decreased the levels of NF-κB p65/p50 in osteoclasts *in vitro* and P65/50 and RANKL expression by synovial tissues *in vivo*.

Conclusion—Manipulation of CD4⁺ iTregs may have therapeutic effects on rheumatoid arthritis and other bone erosion related diseases.

Rheumatoid arthritis (RA) is a chronic human autoimmune disease characterized by inflammatory synovial joints, progressive destruction of cartilage, bone erosions and ankylosis. Osteoclasts, the multinucleated giant cells, are responsible for bone resorption and their development and function have been viewed as potential therapeutic targets in RA (1).

Osteoclasts are derived from hematopoietic cells of the myeloid lineage with monocytes or macrophages. These osteoclast precursors (OCPs) can differentiate into mature osteoclasts under the influence of receptor activator of NF-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) (1, 2).

Regulatory T cells (Tregs) play a crucial role in the maintenance of peripheral immunologic homeostasis. Natural Tregs (nTregs) are critically involved in prevention of autoimmune arthritis and suppressing osteoclast formation (3, 4). However, adoptive transfer of nTregs to the established collagen-induced arthritis (CIA) had only limited suppressive effects on disease progression (5). One explanation is that the inflammatory milieu can alter the phenotype and function of nTregs. The increased levels of IL-6 in an inflammatory environment not only abolishes the suppressive activity of nTregs but also promotes nTregs to become T effector cells, including Th1, Th2, Th17 and Tfh cells (6, 7)

We and others have found that TGF-β-induced CD4⁺CD25⁺Foxp3⁺ cells (iTregs) generated *ex vivo* have similar phenotypic and functional features to those of nTregs (8, 9). Unlike nTregs, CD4⁺ iTregs are resistant to IL-6-derived Th17 cell conversion and maintain their phenotypic and functional characteristics in the inflammatory milieu (7). In the current study, we show while both Tregs suppressed osteoclast formation *in vitro*, only iTregs sustained the effect in the presence of IL-6. Additionally, adoptive transfer of iTregs but not nTregs dramatically suppressed bone erosions and joint damage. This may provide a new strategy for treating patients with autoimmune arthritis.

MATERIALS AND METHODS

General

All reported studies were approved by the University of Southern California (USC) IACUC.

Mice

Eight-week-old female DBA1/J mice were purchased from The Jackson Laboratory and housed at USC.

iTregs generation

Naïve splenic CD4⁺CD25⁻ T cells isolated from wild type (WT) DBA1/J mice were stimulated by anti-CD3/CD28 coated beads (five cells to one bead, Invitrogen) in the presence of IL-2 (40 units/ml) with (iTregs) or without (control T cells, Tconts) TGF- β (2ng/ml, R&D systems) for five days. These cells were harvested and freed from the beads with magnetic column, and FoxP3 expression was determined by flow cytometry. 3×10^6 cells were intravenously transferred to DBA/1J mouse on day 14 after collagen II (CII)/CFA immunization.

nTregs isolation and expansion

Splenic CD4⁺CD25⁺ cells sorted from WT DBA1/J mice were expanded with anti-CD3/CD28 beads (three cells to one bead) with IL-2 (300 units/ml) for 7 days. IL-2 was renewed every three-day. Cells were harvested and beads were removed. The percentage of FoxP3 was examined by flow cytometry before and after cell expansion and 3×10^6 cells were adoptively transferred to DBA/1J mouse on day 14 after CII/CFA immunization.

Osteoclastogenesis

Bone-marrow cells were isolated from WT DBA1/J mice. CD11b⁺ cells were purified with CD11b-PE antibody (eBioscience) and anti-PE microbeads (MACS). The purity was routinely >97%. The cells were stimulated for 4 days with M-CSF (30ng/ml) and RANKL (50ng/ml) (R&D systems). When T cells were present in the cultures, anti-CD3 antibody (1ug/ml, BD Biosciences) was added to cultures. M-CSF and RANKL were renewed every twoday. In other cultures, nTregs, iTregs, or Tconts were added to wells at the ratios indicated in the figures on the first day of the cultures. Anti-TGF- β , anti-IL-4, anti-IFN- γ , anti-IL-10, anti-IL-10R anti-CTLA-4, anti-GITR, or anti-PD1 antibodies (all 10 μ g/ml, R&D systems), or ALK5 (TGF- β RI) inhibitor (Sigma) was added to some cultures. In other experiments, transwell experiments were performed to determine whether cell-contact was required for the suppression. After four-day culture, the cells were stained with tartrate-resistant acid phosphatase (TRAP) (Sigma) according to the manufacturer's instructions, and TRAP⁺ cells were enumerated by microscopy.

Induction and assessment of arthritis

CIA was induced with CII/CFA in eight-week-old female DBA1/J mice. Disease severity was monitored every other day following transfer of iTregs according to previously established scoring system (5). Bone erosions were assessed by CT scan and an index of bone erosion was calculated using established standards.

Western blotting

The levels of NF- κ B P65/P50 were determined by Western blotting. Cultured cells as above or synovial tissues isolated from knee joints were harvested. These cells were depleted of T

cells and remaining non-T cells (2×10^6) or synovial tissues were lysed by lysing buffer. Protein extracts were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with anti-NF- κ B P65 or P50 monoclonal antibody (Cell Signaling).

CT Imaging

Mice were anesthetized with 2% Isoflurane in oxygen. CT imaging was performed using Siemens Inveon CT scanner and Inveon Acquisition Workplace software (Siemens Medical Solutions USA Inc.). The datasets were loaded into Amira 5.2.2 and viewed using the Voltex display and the VolrenRed pseudo-color scale (Visage Imaging Inc.).

Statistical analysis

All statistics were determined using software from GraphPad Software. Parametric testing between two groups was performed by unpaired *t* test. Parametric testing among three or more groups was performed by one-way ANOVA. *P* < 0.05 was considered to be significant.

RESULTS

iTregs but not nTregs suppress osteoclastogenesis in the presence of IL-6 *in vitro*

To determine whether iTregs also can suppress osteoclastogenesis as nTregs (3, 4), the effects of iTregs and nTregs on osteoclastogenesis suppression were directly compared. FoxP3 expression was very similar in nTregs and iTregs (Supplemental Figure 1) and displayed similarly suppressive activities against T cell immune responses as previously reported (7). Addition of iTregs at a 1:2 ratio to cultures of CD11b⁺ cells stimulated with M-CSF and RANKL decreased generation of TRAP⁺ cells to the same degree as did nTregs. In contrast, addition of identical numbers of freshly isolated CD4⁺CD25⁺ cells or Tconts had no effect (Figure 1A and B). The suppressive effects of iTregs are potent, since addition of iTregs to CD11b⁺ cells at a 1:32 ratio still significantly suppressed osteoclast formation that was even superior to nTregs (Figure 1B). These data demonstrate that iTregs at least develop a similarly suppressive ability as nTregs to suppress osteoclastogenesis *in vitro*.

Recent studies have revealed an inherent functional instability of nTregs in the presence of IL-6 which is not shared by iTregs (7). To determine whether such dichotomy extends to suppression of osteoclast differentiation, we directly compared the abilities of both Tregs to inhibit generation of TRAP⁺ cells $\pm$ IL-6. In the presence of IL-6, nTregs lost their suppressive ability whereas the ability of iTregs to suppress osteoclastogenesis was maintained (Figure 1C and D).

iTregs suppress bone erosions *in vivo*

Given the potent ability of iTregs to suppress osteoclastogenesis *in vitro* and the important role for osteoclasts in bone erosions in patients with inflammatory arthritis, we tested whether adoptive transfer of iTregs could suppress bone erosions *in vivo* in CIA. 3×10^6 nTregs, Tconts and iTregs were adoptively transferred to DBA/1J mice on day 14 after CII/CFA immunization. CT imaging was performed to determine anatomical differences between arthritic mice and those therapeutic groups. As shown in Figure 2A, the CIA mice

at 56-day after immunization showed the chronic destructive phase of polyarthritis in the feet and decreased spacing between the metatarsals and phalanges compared to normal mice. Interestingly, iTregs-infused CIA showed near normal joint spacing in right feet, slightly narrowed joint spacing in the left feet and no evident bone erosion. Conversely, Tconts- or nTreg-infused CIA exhibited a similar degree of bone erosion in feet as CIA model although vertebral bone erosion is not evident in various groups. Additionally, CD4⁺ cell infusion did not affect the frequencies of OCPs CD11b⁺CD115⁺ cells in BM and spleens (Supplemental Figure 2). This study demonstrates that injection of CD4⁺ iTregs markedly suppress bone erosion in CIA mice that is likely related to their ability to suppress osteoclastogenesis in the inflammation-like environment.

Suppression of bone erosion by CD4⁺ iTregs corrected with the control of CIA disease progression

We next asked whether the suppression of bone erosion in CIA relates to disease development. 3×10^6 nTregs, Tconts and iTregs were adoptively transferred to DBA/1J mice on day 14 after CII/CFA immunization. As shown in Figure 2B, a significant delay in disease onset was observed in iTregs-infused CIA compared to Tconts- or nTreg-infused CIA. We further observed a close relationship between CIA severities and bone erosion in CIA, or CIA receiving Tconts or nTregs. Of note, disease development was dramatically prevented and significantly related to the suppression of bone erosion in iTregs-infused mice ($r=0.79$, $p<0.001$) (Figure 2C). These data reveal that injection of CD4⁺ iTregs can suppress disease development and bone erosion under inflammatory conditions.

CD4⁺ iTregs suppressed the osteoclastogenesis in a cell-contact but not cytokine dependent way

Tregs suppress immune response through cell-contact and/or the production of immunosuppressive cytokines. We also studied the factor(s) responsible for suppressive effects of Treg subsets on osteoclastogenesis. We observed that addition of anti-TGF- β , anti-IL-10, anti-IL-10R, anti-IL-4, anti-IFN- γ , anti-PD1 or anti-GITR did not significantly alter the suppressive effects of both iTregs and nTregs on osteoclast formation compared to addition of control isotype IgG. However, the neutralization of CTLA-4 almost completely blocked the suppressive activity of nTregs and significantly abolished the effect of iTregs, and addition of ALK5 inhibitor partially revised the suppression of iTregs but not nTregs on osteoclastogenesis (Supplemental Figure 3A). In addition, transwell experiments that separate Tregs from CD11b⁺ cells completely abolished the suppression of both Tregs against osteoclast formation (Supplemental Figure 3B), suggesting that cell-cell contact between Tregs and CD11b⁺ cells, CTLA-4 signal pathways play an important role in the inhibitory formation of osteoclast, at least *in vitro* although TGF- β R signaling is only involved in iTreg suppression.

CD4⁺ iTregs blocked the osteoclastogenesis via NF- κ B-P65/P50 and RANKL pathways

It has been well known that the RANK/RANKL-initiated NF- κ B pathway plays a key role in the early stage of osteoclast formation (10), we thus hypothesize that iTregs inhibit osteoclastogenesis through a modulation of these pathways. To address this possibility,

CD11b⁺ cells were co-cultured with or without both Tregs at 2:1 ratio for four days and cells were harvested and depleted of T cells. CD3⁺ cells in the remaining cell population were less than 0.5% after this removal. Protein was prepared and purified from remaining cells and then subject to western blotting analysis. The expression of NF- κ B P65/P50 was markedly decreased in CD11b⁺ that had been primed with iTregs or nTregs compared with control, nonetheless, only iTregs but not nTregs sustained this effect in the presence of IL-6 (Figure 3A). Moreover, NF- κ BP 65/P50 and RANKL expression by synovial tissues isolated from CIA mice infused with iTregs was much lower than that in CIA or CIA infused with nTregs (Figure 3B). These results suggest that iTregs may interfere with RANKL-mediated NF- κ B pathway activation and eventually lead to the suppression of osteoclastogenesis under the inflammation condition *in vitro* and *in vivo*.

DISCUSSION

Our study now shows that while both iTregs and nTregs suppressed osteoclast formation *in vitro*, only iTregs sustained this effect in the presence of IL-6. Additionally, iTregs but not nTregs infusion suppressed bone erosion in CIA. Moreover, the suppression exerted by iTregs on bone erosion is related to CIA severities. This is consistent with previous reports demonstrating that elevated IL-6 can reverse the suppressive activity of nTregs and iTreg cells are less sensitive to IL-6 (7, 11). Since IL-6 and other pro-inflammatory cytokines are elevated and critically involved in the pathogenesis of RA, current result suggests that manipulation of iTregs may have an advantage to control bone erosion in RA patients and other patients under inflammatory conditions.

It is less defined how Tregs suppress osteoclast formation. While murine nTreg cell-contact plays a dominant role in the osteoclast suppression (3), others have reported that soluble cytokines are crucial for the functional role of human nTregs in osteoclast formation (12). Our data demonstrate that cell-contact is required for the suppressive effect of iTregs on osteoclast formation *in vitro*. Using antibody neutralization studies, we found that soluble cytokines such as TGF- β 1, IL-4 and/or IL-10 that are not responsible for osteoclast suppression *in vitro*. Although neutralization of CTLA-4 can completely abolished the suppressive activity of nTregs on osteoclast formation (3), it did not completely account for the effect of iTregs on osteoclast suppression. It is possible that the CTLA-4/B7 signal pathway is essential for the development of iTregs but not for nTreg (13), and once iTregs have been established, other inhibitory signals can replace CTLA-4 for their functional activity. Blockade of TGF- β receptor (T β R) I (ALK5) can partially reverse this effect, indicating that T β R signaling may also be involved in the iTregs suppression.

We also demonstrate that iTregs may function through a modulation of osteoclast differentiation. We observe that iTreg treatment did not alter the frequency of osteoclast precursor frequencies. Conversely, addition of iTregs dramatically decreased the NF- κ B activation in CD11b⁺ cells treated with RANKL and M-CSF, regardless of the presence of IL-6. We provide further evidence that iTregs infusion also suppressed the expression of P65/P50, two subunits of mammalian NF- κ B, on joint synovial tissues in CIA mice. It has been known that the osteoclastogenesis requires the combination of RANKL to its receptor RANK. This procedure activates the NF- κ B signaling pathway. NF- κ B controls RANK-

expressing osteoclast precursors to differentiate into TRAP⁺ osteoclasts in response to RANKL and other osteoclastogenic cytokines (14).

We also observe that iTregs suppress the RANKL expression by joint synovial tissues in CIA. It has been documented that RANKL is expressed by osteoclast precursors and activated T cells (14). iTregs might directly suppress RANKL expression by osteoclast precursors, then decreasing osteoclastogenesis and the resultant bone erosion. It is also likely that iTregs suppress the Teff cell differentiation and function in CIA, then indirectly decreasing RANKL expression and bone erosion. This study implicates that manipulation of CD4⁺ iTregs might represent a novel therapeutic strategy for the treatment of RA and other bone erosion related autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CD4⁺ iTregs inhibit osteoclastogenesis *in vitro* in a dose-dependent manner. OCPs were stimulated with M-CSF (30ng/ml) and RANKL (50ng/ml) and co-cultured with CD4⁺CD25⁻ cells, nTregs, Tconts, or iTregs (2:1 ratio) for four days and stained for TRAP. TRAP⁺ cells were counted in high power of microscopy. **A**. Under 2:1 ratio, and data were mean of TRAP⁺ cells $\pm$ SEM of four independent experiments. **B**. TRAP⁺ cells under different ratios of Tregs to CD11b⁺ cells. **C**. TRAP⁺ cells of Tregs to CD11b⁺ cells (2:1) $\pm$ IL-6. **D**. Data were representative photomicrographs of TRAP⁺ cells from the cultures in panel C.

** $p < 0.01$, *** $p < 0.001$ compared with baseline and Tconts or Tregs treatment.

Figure 2.

CD4⁺ iTregs suppress bone erosion *in vivo*. **A.** CT imaging of feet and vertebral in CIA, Tcont-, nTreg- and iTregs-infused CIA in 56-day after CII/CFA immunization. 3×10^6 various CD4⁺ cells were adoptively transferred to DBA/1J mice at 14-day after immunization. **B.** Incidences of CIA in DBA/1J mice after immunization or other therapeutic groups. **C.** Correlation of the clinical scores of each group and severities of bone destruction was shown. Each group had five mice. The results are from two independent experiments.

Figure 3.

CD4⁺ iTregs blocks the osteoclastogenesis via a RANKL-mediated NF-κB P65/P50 pathway. **A.** OCPs were cultured or cocultured with CD4⁺ Tconts or iTregs as described above. After four days culture, cells were harvested for western blot analysis to determine the expression of P65 and P50 on non-T cells. **B.** Synovial tissues isolated from joints in different CIA mice or treated with Tregs and P65/P50 and RANKL expression was determined and quantified. All data in A and B were representative of three independent experiments.