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IL-3 Attenuates Collagen-Induced Arthritis by Modulating the Development of Foxp3+ Regulatory T Cells

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IL-3, a cytokine secreted by Th cells, functions as a link between the immune and the hematopoietic system. We previously demonstrated the potent inhibitory role of IL-3 on osteoclastogenesis, pathological bone resorption, and inflammatory arthritis. In this study, we investigated the novel role of IL-3 in development of regulatory T (Treg) cells. We found that IL-3 in a dose-dependent manner increases the percentage of Foxp3+ Treg cells indirectly through secretion of IL-2 by non-Treg cells. These IL-3-expanded Treg cells are competent in suppressing effector T cell proliferation. Interestingly, IL-3 treatment significantly reduces the severity of arthritis and restores the loss of Foxp3+ Treg cells in thymus, lymph nodes, and spleen in collagen-induced arthritis mice. Most significantly, we show that IL-3 decreases the production of proinflammatory cytokines IL-6, IL-17A, TNF-α, and IL-1 and increases the production of anti-inflammatory cytokines IFN-γ and IL-10 in collagen-induced arthritis mice. Thus, to our knowledge, we provide the first evidence that IL-3 play an important role in modulation of Treg cell development in both in vitro and in vivo conditions, and we suggest its therapeutic potential in the treatment of rheumatoid arthritis and other autoimmune diseases. The Journal of Immunology, 2011, 186: 2262–2272.

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egulatory T (Treg) cells play a crucial role in controlling autoimmunity, and they prevent the development of chronic inflammatory and autoimmune diseases by suppressing autoreactive T cells (1). Treg cells generated in the thymus are called natural Treg cells. Treg cells could also be generated outside the thymus from peripheral naïve CD4+CD25−T cells under both in vitro and in vivo conditions and are referred as adaptive or induced Treg cells (2, 3). The forkhead transcription factor (Foxp3) is the master regulator for the development and function of Treg cells (4, 5). The in vitro conversion of naïve CD4+CD25−Foxp3−T cells to CD4+CD25+Foxp3+ Treg cells depends on the presence of TGF-β1 (2, 6, 7) and of IL-2, which is essential for TGF-β–mediated induction of Foxp3 (8, 9). Both TGF-β1 and IL-2 play an important role in the induction, maintenance of Foxp3 expression, and function of Treg cells under in vitro and in vivo conditions (10). Treg cells in rheumatoid arthritis (RA) patients have a defect in their ability to suppress proinflammatory cytokine production by activated T cells and monocytes (11, 12). Also, deficiency of Foxp3 results in the paucity of CD4+CD25+ Treg cells and leads to severe multiorgan autoimmune diseases in both mice and humans (13, 14). Thus, the development of functional Treg cells holds promise for the treatment of RA and other autoimmune diseases.

IL-3, a cytokine secreted by Th cells, stimulates the proliferation, differentiation, and survival of pluripotent hematopoietic stem cells (15). In previous studies, we have shown that IL-3 is a potent inhibitor of osteoclastogenesis and it inhibits both RANKL- and TNF-α–induced mouse osteoclast formation (16, 17). We have recently shown that IL-3 inhibits RANKL-induced human osteoclasts derived from blood monocytes and bone marrow cells of osteoporotic individuals (18). IL-3 also inhibits TNF-α–induced pathological bone resorption in the presence of other proinflammatory cytokines such as IL-1α, TGF-β1, TGF-β2, IL-6, and PGE2, and pretreatment with IL-3 prevents the development of inflammatory arthritis in mice, and protects cartilage and bone destruction in the joint (19). These results suggest that IL-3 has an anti-inflammatory activity.

In RA patients and in a collagen-induced arthritis (CIA) mouse model, Treg cells are defective and there is increased osteoclastogenesis (11, 20–22). Decreased circulating Treg cell numbers are associated with increased osteoclast formation and bone resorption in humans (23). Also, Treg cells are potent inhibitor of osteoclast differentiation and bone resorption, and they prevent development of CIA (22, 24). Therefore, we hypothesized that the anti-inflammatory activity of IL-3 may be through regulation of Treg cell development. In this study, we investigated the role of IL-3 in regulation of Treg cell differentiation in both in vitro and in vivo conditions. We demonstrate that IL-3 increases the percentage of Foxp3+ Treg cells indirectly through secretion of IL-2 by non-Treg cells. Importantly, IL-3 treatment decreases severity of arthritis and restores the loss of Foxp3+ Treg cells in lymph nodes, spleen, and thymus in CIA mice. Thus, we reveal the novel role of IL-3 in modulation of Treg cell development and suggest its therapeutic potential in treatment of RA and other autoimmune diseases.

Materials and Methods

Mice

BALB/c mice 6–8 wk old and DBA/1J mice 8–10 wk old were obtained from the Experimental Animal Facility of the National Centre for Cell Science (NCCS, Pune, India). Water and food were provided ad libitum. All pro-

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Abbreviations used in this article: CIA, collagen-induced arthritis; CII, collagen type II; μ-CT, microcomputed tomography; RA, rheumatoid arthritis; rm, recombinant murine; Treg, regulatory T.
cедерings involved animals were conducted according to the requirements and with the approval of the Institutional Animal Ethics Committee of NCCS.

Abs and reagents

The following reagents were purchased from BD Biosciences: purified anti-CD3ε (145-2C11, NA/LE), purified anti-CD28 (37.51, NA/LE), PerCP-Cy5.5-anti-CD4 (RM4-5), PE-Cy7-anti-CD25 (PC61), biotinylated anti-CD123 (5B11), purified anti-IL-2 mAb (S4B6), PE-anti–pSTAT5 (pY694), streptavidin-PE, biotin-rat IgG2a-κ, mouse IgG1-κ, GolgiStop, and recombinant murine (rml)-IL-3. Allophycocyanin-anti-Foxp3 (FJK-16s), PE-anti-IL-2 (JES6-5H4), allophycocyanin-rat IgG2a-κ, PE- rat IgG2b-κ, Foxp3 staining buffer, and RBC lysis buffer were obtained from eBioscience. rml-IL-2 and recombinant human TGF-β1 were obtained from R&D Systems. CFSE was obtained from Molecular Probes. PMA and ionomycin were purchased from Sigma-Aldrich. Serum-free medium X-Vivo 15 was obtained from Lonza.

Isolation of T lymphocytes

For isolation of CD4+CD25+ and CD4+CD25− T cells, lymphocytes harvested from spleens of 6- to 8-wk-old mice were incubated with CD4 T cell enrichment mixture (Miltenyi Biotec), and untouched CD4+ T cells were isolated using autoMACS (Miltenyi Biotec). The purified CD4+ cells were further incubated with PE-anti-CD25 followed by incubation with anti-PE beads (Miltenyi Biotec) to isolate CD4+CD25+ and CD4+CD25− T cells, according to the manufacturer’s instructions. The purity of sorted CD4+CD25+ and CD4+CD25− T cells was typically >96%.

In vitro Treg cell development

For in vitro Treg cell differentiation, CD4+ T cells (10^6 cells/well of 48-well plate) from spleenocytes were stimulated for 4 d with plate-bound anti-CD3ε (10 μg/ml) plus soluble anti-CD28 (2 μg/ml) Abs in serum-free X-Vivo 15 medium. Where indicated, the medium was supplemented with recombinant human TGF-β1 (5 ng/ml) and rml-IL-2 (20 ng/ml) with or without different concentrations of IL-3. Cells were then harvested and the percentage of Foxp3+ Treg cells was analyzed by FACS.

In vitro suppression assay

The induced and natural Treg cells generated as described above were harvested and various cell ratios of CD4+CD25+ Treg cells treated with or without IL-3 were added to freshly isolated CFSE (2.5 × 10^5/well) to give a “crowding control”. Proliferation of effector T cells was determined using intracellular Foxp3 staining performed using allophycocyanin-conjugated anti-Foxp3 Ab in permeabilization buffer for 45 min on ice. Cells were washed thoroughly and analyzed by flow cytometry.

Intracellular staining of IL-2 production was performed by stimulating cells for 5–6 h with PMA (50 ng/ml) and ionomycin (500 ng/ml). After 1 h, GolgiStop (1 μg/ml) was added to block cytokine secretion. Cells were surface stained with PerCP-Cy5.5-anti-CD4 and PE-Cy7-anti-CD25 Abs for 30 min on ice. Cells were washed thoroughly with wash buffer, fixed-permeabilized with Foxp3 permeabilization buffer for 30 min on ice, and stained intracellularly with allophycocyanin-conjugated anti-Foxp3 and PE-conjugated anti-IL-2 Abs in permeabilization buffer for 30 min on ice. Cells were washed thoroughly and analyzed by flow cytometry.

For pSTAT5 labeling, T cells were stimulated and fixed with Lyse/Fix buffer (BD Biosciences) for 30 min at 37°C, washed and permeabilized with Perm Buffer III (BD Biosciences) for 30 min at 4°C, washed with FACS buffer, and stained with PE-pSTAT5 for 30 min at room temperature. Cells were washed and analyzed by FACS.

Cytometric bead array flex

Levels of IL-1, IL-2, IL-5, IL-6, IL-10, IL-17A, TNF-α, and IFN-γ in culture supernatants were assessed by fluorescent bead-based technology using cytometric bead array (CBA) Flex sets according to the manufacturer’s instruction (BD Biosciences). Fluorescent signals were read and analyzed on a FACSCanto II flow cytometer (BD Biosciences) with the help of BD FCAP Array software (BD Biosciences).

Injection and assessment of CIA

Native chicken collagen type II (CII; Sigma-Aldrich) was dissolved in 50 mM acetic acid at 4°C overnight and then emulsified with an equal volume of CFA (Chondrex, containing 4 mg/ml Mycobacterium tuberculosis; strain H37Rv). Male DBA/1J mice 6–8 wk old were injected s.c. at the base of the tail with 100 μl emulsion containing 200 μg CII. At day 21 after the primary immunization, mice were boosted s.c. with 200 μg CII emulsified with an equal volume of IFA (Sigma-Aldrich). Treatment with rml-IL-3 began with the secondary immunization, and cytokine was administered i.p. daily (1.5 μg/mouse/d in two divided doses) until day 35 after primary immunization.

Mice were observed by two independent blinded examiners every fifth day and monitored for signs of arthritis onset using two clinical parameters: paw swelling and arthritic score. Paw swelling was assessed by measuring thickness of the affected hind paws with 0–10 mm calipers. Clinical arthritis was assessed using the following system: grade 0, no swelling; grade 1, slight swelling and erythema; grade 2, pronounced edema; and grade 3, joint rigidity. Each limb was graded, giving a maximum possible score of 12 per animal. Inflammatory swellings of soft tissues were radiologically evaluated by taking soft x-ray photographs (Siemens).

Histology

For histological analysis, mice were killed by cervical dislocation on day 36. Whole knee joints were removed and fixed for 4 d in 10% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding. Next, 5-μm sections were prepared and stained for H&E.

Microcomputed tomography

Microcomputed tomography (μ-CT) of the tibiae was performed using a SkyScan 1176 scanner (SkyScan). Scanning was done at 100 kV, 100 μA using a 1-mm aluminum filter and exposure set to 590 ms. In total, 1800 projections were collected at a resolution of 6.93 μm/pixel. Reconstruction of sections was achieved using a modified Feldkamp cone-beam algorithm with beam hardening correction set to 50%. CTAalyzer software (version 1.02, SkyScan) was employed for morphometric quantification of trabecular bone indices such as bone volume fraction (bone volume/tissue volume), trabecular number, trabecular thickness, structure model index, and connectivity density.

Statistical analysis

The results were evaluated by using ANOVA with subsequent comparisons by Student t test for paired or nonpaired data, as appropriate. Statistical significance was defined as p ≤ 0.05. Values are reported as mean ± SEM.

Results

IL-3Rα is expressed by Treg cells

To evaluate the role of IL-3 in Treg cell development, we first determined the expression of IL-3Rα on both natural and induced...
Treg cells. We isolated CD4+CD25+ natural Treg cells from splenocytes of 6- to 8-wk-old BALB/c mice, and induced Treg cells were generated from splenic CD4+CD25− T cells by stimulating with plate-bound anti-CD3e (10 μg/ml) and soluble anti-CD28 (2 μg/ml) mAbs in the presence of TGF-β1 (5 ng/ml) and IL-2 (20 ng/ml). Cells were incubated for 4 d and the expression of IL-3Rα (CD123) was checked at mRNA level by RT-PCR. We observed that both natural and induced Treg cells show strong expression of IL-3Rα (Fig. 1A). Expression of IL-3Rα was also analyzed by FACS on both natural and induced Treg cells by surface labeling for CD4, CD25, and IL-3Rα, and cells were further stained intracellularly for Foxp3. Gating was done on CD4+CD25+ T cells for expression of Foxp3 and IL-3Rα. When the IL-3Rα+ cell population was plotted against Foxp3, we observed that almost all the Foxp3+ Treg cells expressed IL-3Rα (Fig. 1B). These results demonstrate that both natural and induced Treg cells express IL-3Rα at both the gene and protein levels.

**FIGURE 1.** IL-3Rα is expressed by both natural and induced Treg cells. A, CD4+CD25+ natural Treg cells were isolated from splenocytes of 6- to 8-wk-old BALB/c mice. Induced Treg cells were generated from splenic CD4+CD25− T cells stimulated with plate-bound anti-CD3e (10 μg/ml) and soluble anti-CD28 (2 μg/ml) mAbs and incubated for 4 d in the presence of TGF-β1 (5 ng/ml) and IL-2 (20 ng/ml). Both cell populations were analyzed for mRNA expression of IL-3Rα (CD123) by RT-PCR. B, Expression of IL-3Rα was also analyzed on both natural and induced Treg cells by FACS by surface labeling for CD4, CD25, and IL-3Rα, and cells were further stained intracellularly for Foxp3. Gating was done on CD4+CD25+ T cells for expression of Foxp3 and IL-3Rα. Results in A and B are representative of three independent experiments. i, induced; n, natural.

IL-3 enhances the percentage of both induced and natural Treg cells in vitro and potently suppresses the proliferation of effector T cells

We next examined whether IL-3 promotes conversion of CD4+ CD25+ T cells into Foxp3-expressing Treg cells. Splenic CD4+ CD25+ T cells stimulated with anti-CD3e and anti-CD28 mAbs were incubated with TGF-β1 with or without IL-2 and in the absence or presence of different concentrations of IL-3. After 4 d, cells were analyzed for Foxp3 expression by FACS. We found that IL-3 in a dose-dependent manner increases the TGF-β1–induced conversion of CD4+CD25+ Foxp3− T cells into CD4+CD25+Foxp3+ T cells (Fig. 2A). Fig. 2B represents the average percentage of CD4+CD25+Foxp3+ cells increased by IL-3. To further check whether IL-3 also enhances the percentage of natural Treg cells, splenic CD4+CD25+ T cells were incubated with TGF-β1 with or without IL-2 and in the absence or presence of IL-3 (50 and 100 ng/ml). After 4 d, cells were analyzed for Foxp3 expression by FACS. We observed that IL-3 also increases the percentage of Foxp3+ natural Treg cells (Fig. 2C). Fig. 2D represents the average percentage of CD4+CD25+Foxp3+ cells in presence of IL-3. These results suggest that IL-3 enhances the expression of Foxp3 in both natural and induced Treg cells.

To check whether IL-3–expanded induced and natural Treg cells are functional in suppressing the proliferation of responder/effector T cells, we performed a CFSE-based suppression assay. Freshly isolated, CFSE-labeled conventional CD4+CD25− T cells were cocultured for 72 h with non-Treg cells (unlabeled CD4+CD25− T cells) with or without IL-2 and in the absence or presence of IL-3 (100 ng/ml). CD4+CD25− T cells treated in the presence or absence of IL-3 at different cell ratios. Proliferation of effector T cells was analyzed by FACS for CFSE dilution. We found that IL-3–expanded induced and natural Treg cells inhibit the proliferation of responder T cells in a cell density-dependent manner (Fig. 2E). These results suggest that IL-3–promoted Treg cells are bona fide Treg cells.

IL-3 enhances percentage of Foxp3+ Treg cells indirectly through secretion of IL-2

IL-2 is essential for TGF-β1–induced conversion of naive CD4+ CD25− Foxp3− T cells into CD4+CD25+Foxp3+ Treg cells (8, 9). To check whether IL-3 enhances TGF-β1–induced Treg cell differentiation in the absence of exogenous IL-2, we stimulated CD4+CD25− T cells with anti-CD3e and anti-CD28 mAbs for 4 d in the presence of TGF-β1 with or without different concentrations of IL-3. We found that IL-3 increased TGF-β1–induced Treg cell differentiation even in the absence of exogenous IL-2 (Fig. 3A). Next, to determine whether the stimulatory effect of IL-3 on induced Treg cells is IL-2–dependent, CD4+ CD25− T cells were stimulated with anti-CD3e and anti-CD28 mAbs for 4 d in TGF-β1 with or without IL-3 and in the absence or presence of anti–IL-2 mAb (10 μg/ml). Anti–IL-2 mAb completely neutralized the stimulatory effect of IL-3 on TGF-β1–induced Treg cells (Fig. 3B). Also, IL-2 secretion induced by IL-3 was significantly inhibited by anti–IL-2 mAb (Fig. 3C). These results suggest that IL-3 induces development of Treg cells indirectly through secretion of IL-2 by non–Treg cells. This was further confirmed by a dose-dependent effect of IL-3 on secretion of IL-2 by CBA. We observed that IL-3 induces secretion of IL-2 in a dose-dependent manner. There was a drastic increase in IL-2 secretion at 100 and 300 ng/ml concentrations of IL-3 (Fig. 3D). Also, the enhanced secretion of IL-2 was directly correlated with a simultaneous increase in expression of IL-2Rα (CD25) on these cells (Fig. 3E).
FIGURE 2. IL-3 enhances the percentage of both induced and natural Treg cells in vitro and suppresses the proliferation of effector T cells. A, Splenic CD4+CD25− T cells stimulated with anti-CD3ε and anti-CD28 mAbs were incubated with TGF-β1 (5 ng/ml) with or without IL-2 (20 ng/ml) in the absence or presence of different concentrations of IL-3. After 4 d, cells were analyzed for Foxp3 expression by FACS. B, Average percentage of CD4+CD25+Foxp3+ cells from three independent experiments of A. Values are expressed as mean ± SEM. *p < 0.05 versus control. C, CD4+CD25− T cells isolated from splenocytes were incubated with TGF-β1 (5 ng/ml) with or without IL-2 (20 ng/ml) in the absence or presence of IL-3 (50 and 100 ng/ml). After 4 d, cells were analyzed for Foxp3 expression by FACS. D, Average percentage of CD4+CD25+Foxp3+ cells from two independent experiments of C. Values are expressed as mean ± SEM. *p < 0.05 versus control. E, Freshly isolated CD4+CD25− T cells (2.5 × 10^5/well) labeled with CFSE were seeded in 96-well plates and cocultured for 72 h with non-Treg cells (unlabeled CD4+CD25− serving as crowding control), with induced Treg cells generated in the presence or absence of IL-3 (100 ng/ml), or with natural Treg cells treated in the presence or absence of IL-3 at different cell ratios. Proliferation of effector T cells was analyzed by FACS for CFSE dilution. Similar results were obtained in three independent experiments. n, natural; i, induced.
FIGURE 3. IL-3 enhances the percentage of Foxp3+ Treg cells indirectly through secretion of IL-2 by non-Treg cells. A, Splenic CD4+CD25+ T cells stimulated with anti-CD3e and anti-CD28 mAbs were incubated with TGF-β1 (5 ng/ml) in the absence or presence of different concentrations of IL-3. After 4 d, cells were analyzed for Foxp3 expression by FACS. B, Splenic CD4+CD25+ T cells stimulated with anti-CD3e and anti-CD28 mAbs were incubated with TGF-β1 with or without IL-3 (100 ng/ml) or TGF-β1, IL-3, and anti–IL-2 (10 µg/ml) mAb. After 4 d, cells were analyzed for Foxp3 expression by FACS. C, Culture supernatants collected after 4 d from experiments in B were analyzed for IL-2 secretion by CBA as per the manufacturer’s instructions. Values are expressed as mean ± SEM. *p < 0.05 versus TGF-β1. **p < 0.001 versus TGF-β1 and IL-3. D, Dose-dependent effect of IL-3 on secretion of IL-2 in Treg cells culture supernatants was analyzed by CBA. Values are expressed as mean ± SEM. *p < 0.001 versus TGF-β1. E, Dose-dependent effect of IL-3 on expression of IL-2Rα (CD25) in induced Treg cells. Values are expressed as mean ± SEM. *p < 0.05 versus TGF-β1. Data in
The induced Treg cell population contains a significant proportion of Foxp3$^+$ cells, which are likely to produce IL-2. Therefore, to further check the source of IL-2 secretion we stimulated splenic CD4$^+CD25^-$ T cells with anti-CD3 and anti-CD28 mAbs for 4 d in the presence of TGF-β1 with or without different concentrations of IL-3. After 4 d, cells were washed, fixed-permeabilized, and stained with PE-labeled p-STAT5 mAb as per the manufacturer’s instruction and analyzed by FACS. Data are averages of three independent experiments. Values are expressed as mean ± SEM. *$p < 0.05$ versus control. B. Splenic CD4$^+CD25^-$ T cells were stimulated for 20 min with IL-2 (5 ng/ml) or IL-3 (30 ng/ml) alone or in the presence of both. Cells were stained with PE-labeled p-STAT5 mAb and analyzed by FACS. C. Average data of three independent experiments of B showing percentage of p-STAT5. Values are expressed as mean ± SEM. *$p < 0.05$ versus control, **$p < 0.05$ versus IL-2 or IL-3.

FIGURE 4. IL-3 synergies with IL-2 for activation of STAT5. A, Splenic CD4$^+CD25^-$ T cells were stimulated for 20 min independently with or without different concentrations of IL-2 or IL-3. Cells were washed, fixed-permeabilized, and stained with PE-labeled p-STAT5 mAb as per the manufacturer’s instruction and analyzed by FACS. Data are averages of three independent experiments. Values are expressed as mean ± SEM. *$p < 0.05$ versus control. B, Splenic CD4$^+CD25^-$ T cells were stimulated for 20 min with IL-2 (5 ng/ml) or IL-3 (30 ng/ml) alone or in the presence of both. Cells were stained with PE-labeled p-STAT5 mAb and analyzed by FACS. C, Average data of three independent experiments of B showing percentage of p-STAT5. Values are expressed as mean ± SEM. *$p < 0.05$ versus control, **$p < 0.05$ versus IL-2 or IL-3.

The induced Treg cell population contains a significant proportion of Foxp3$^+$ cells, which are likely to produce IL-2. Therefore, to further check the source of IL-2 secretion we stimulated splenic CD4$^+CD25^-$ T cells with anti-CD3 and anti-CD28 mAbs for 4 d in the presence of TGF-β1 with or without different concentrations of IL-3. After 4 d, cells were activated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 5–6 h before being analyzed for the secretion of IL-2 by intracellular staining by FACS. Gating was done on two cell populations, CD4$^+CD25^{high}$ and CD4$^+CD25^{low}$, and cells were analyzed for expression of Foxp3 and IL-2. We found that IL-3 in a dose-dependent manner increased the percentage of IL-2-producing CD4$^+CD25^{low}$Foxp3$^+$ cells from 2.3 to 9.1% (Fig. 3F). However, no IL-2$^+$ was produced by CD4$^+CD25^{high}$Foxp3$^+$ cells in the absence or presence of IL-3, even at high concentrations (Fig. 3F). Fig. 3G represents the average data of three independent experiments of the CD4$^+CD25^{low}$Foxp3$^+$ population. Collectively, these results suggest that IL-3 induces development of Treg cells indirectly through secretion of IL-2 by non-Treg cells.

**IL-3 synergies with IL-2 for activation of STAT5**

We next investigated the mechanism of stimulatory effect of IL-3 on Treg cell development. IL-3R signaling is primarily mediated through activation of JAK2 with subsequent phosphorylation and activation of STAT5 (15). Also, STAT5 promotes Treg cell differentiation by regulating expression of Foxp3 (25). Therefore, we examined the effect of IL-3 on STAT5 phosphorylation in T cells in the absence and presence of IL-2. We first performed the titration for both cytokines IL-2 and IL-3 independently for their effects on phosphorylation of STAT5. CD4$^+CD25^-$ T cells were stimulated for 20 min independently with different concentrations of IL-2 or IL-3. Cells were stained for intracellular pSTAT5 and all above experiments are representative of three independent experiments. F, CD4$^+CD25^-$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 4 d in the presence of TGF-β1 with or without different concentrations of IL-3. After 4 d, cells were activated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 5–6 h before being analyzed for the secretion of IL-2 by intracellular staining. Gating was performed for two cell populations, CD4$^+CD25^{high}$ and CD4$^+CD25^{low}$, and cells were analyzed for the expression of Foxp3 and IL-2. G. Average data of three independent experiments of CD4$^+CD25^{low}$Foxp3$^+$ population in experiment F. Values are expressed as mean ± SEM. *$p < 0.05$ versus control.
analyzed by FACS. Both IL-2 and IL-3 independently increased the phosphorylation of STAT5 in a dose-dependent manner (Fig. 4A). The data in Fig. 4A show the average percentage of STAT5 phosphorylation from three independent experiments \((p < 0.05)\). To further check the synergistic effect of IL-2 and IL-3 on phosphorylation of STAT5, we stimulated CD4+CD25+ T cells for 20 min with low concentrations of IL-2 (5 ng/ml) or IL-3 (30 ng/ml) alone or in the presence of both. We found that there was 15.13 and 15.64% phosphorylation of STAT5 by IL-2 and IL-3, respectively. Phosphorylation of STAT5 was increased to 34.67% when both of the cytokines were added together, which was more than that of the additive effect of both cytokines (Fig. 4B). Fig. 4C shows the average percentage of STAT5 phosphorylation from three independent experiments \((p < 0.05)\). Thus, IL-3 activates STAT5 in T cells and synergizes with IL-2 for enhanced activation of STAT5.

**IL-3 reduces severity of arthritis by enhancing the percentage of Foxp3+ Treg cells in vivo in CIA mice**

To investigate the in vivo role of IL-3 in regulation of Treg cells, we used a well-established CIA mouse model of human RA. DBA/1J mice were primed on day 0 with CII and then boosted on day 21. In the treatment group, mice were injected for 15 d with rmlL-3 (1.5 mg/d i.p. in two divided doses at 12-h intervals) from day 21 at the time of booster. Arthritis was manifested by redness and swelling of the paws, including digits. In PBS-injected control mice, no signs of inflammation were seen, whereas CIA mice developed severe inflammation as evidenced by marked swelling and erythema of the hind paws. In contrast, mice treated with IL-3 displayed a significant reduction in paw thickness (Fig. 5A) and mean arthritic score (Fig. 5B). Photographs of hind paws in Fig. 5C show significant reduction of inflammation in mice treated with IL-3. In the presence of IL-3, thickness of the inflammatory soft tissues was also decreased when examined by radiological soft x-rays (Fig. 5D). RA is a chronic inflammatory disorder that ultimately leads to the destruction of joint architecture. By radiological examination we observed that IL-3 treatment prevented damage to articular cartilage (Fig. 5D, enlarged regions). CIA mice had markedly enlarged spleen and inguinal lymph nodes, which were normal in size in IL-3-treated mice (Fig. 5E). By histological examinations of knee joints on day 36, we observed no infiltration of inflammatory cells and no damage to the articular cartilage of control mice. The knee joints in CIA mice showed massive infiltration of polymorphonuclear and other inflammatory cells, and there was multiple superficial cartilage erosion. In contrast, mice treated with IL-3 showed infiltration of few inflammatory cells, and erosion of articular cartilage was not observed (Fig. 5F).

We also assessed trabecular structure of tibiae by μ-CT. In CIA mice there was significant loss of trabecular and cortical bones, which was prevented in IL-3-treated mice (Fig. 6A). Also, a significant increase in trabecular thickness, trabecular number, bone volume fraction (bone volume/tissue volume), connectivity density, and cortical thickness was observed in IL-3-treated mice with respect to CIA mice (Fig. 6B). Bone architecture denoted by the structure model index was not altered. These results suggest that IL-3 maintains normal bone structure in mice. Thus, IL-3 treatment reduces arthritic score and inflammation and prevents damage to bone and cartilage tissues in knee joints in CIA mice.

To investigate the in vivo mechanism of IL-3 action in prevention of CIA, we analyzed the effect of IL-3 on Foxp3+ Treg cell development. Mice were sacrificed on day 36 and total lymphocyte populations derived from lymph nodes, spleen, and thymus tissues were analyzed for percentage of CD4+Foxp3+ Treg cells by FACS. As compared with control mice, the percentage of Foxp3+ Treg cells in CIA mice was drastically decreased in thymus, lymph nodes, and spleen. There was a >50% decrease in percentage of Foxp3+ Treg cells in lymph nodes and an ~70% decrease in spleen and thymus. Interestingly, in IL-3-treated mice the percentage of CD4+Foxp3+ Treg cells in all the three tissues was restored to near normal levels (Fig. 7A). Fig. 7B represents the average percentage of Foxp3+ Treg cells in lymph nodes, spleen, and thymus of mice treated with or without IL-3. These results suggest that IL-3 also has a potential to augment the percentage of Foxp3+ Treg cells in vivo.
IL-3 decreases production of proinflammatory cytokines and increases anti-inflammatory cytokines in CIA mice

The pathogenic events that lead to the development of human RA are not fully understood, although the pivotal role of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the induction and maintenance of RA is well documented (26). These cytokines promote the deleterious imbalance in bone metabolism and contribute to enhanced bone destruction. Treg cells in active RA are defective in controlling production of proinflammatory cytokines (11). Treg cells suppress immune response through numerous mechanisms, including the production of anti-inflammatory cytokines, direct cell to cell contact, and by modulating the activation state and function of APCs (27). Therefore, we further examined the effect of IL-3 on the levels of various proinflammatory and anti-inflammatory cytokines in the serum of control, CIA, and IL-3-treated mice. In CIA mice there was a significant increase in production of IL-6, IL-17A, TNF-α, IL-1, and IFN-γ (p < 0.05) and decrease in secretion of IL-5 and IL-10. IL-2 production was also decreased in CIA mice. Surprisingly, we observed that IL-3 significantly increases the levels of IL-10, IL-2, IL-5, and IFN-γ and decreases the levels of IL-6, IL-17A, TNF-α, and IL-1 (Fig. 8). These results suggest that IL-3 has a potential to inhibit production of proinflammatory cytokines and induce anti-inflammatory cytokines.

Discussion

Treg cells represent an important immune mechanism for inducing tolerance to both self and foreign Ags to prevent autoimmunity and unwanted inflammation. Dysfunctional Treg cells are a cause of various autoimmune diseases, allergy, and immunopathology (28). However, it remains to be determined how these cells are involved in regulating the pathophysiology of common immunological disease such as RA. Also, Treg cells potently inhibit osteoclastogenesis and bone resorption. Importantly, the capacity of a single Treg cell to block differentiation of 100 monocytes into osteoclasts underlines the powerful role of Treg cells in regulation of bone resorption (24). Thus, Treg cells appear to be an attractive target cell with substantial therapeutic potential in RA, and they
may act as an important link between the immune and bone system. However, therapeutic applications of Treg cells are limited because of their scarcity. Therefore, development of Treg cells in vitro and in vivo is an essential requirement for their effective clinical intervention. In this study, we investigated the role of IL-3 in regulation of Treg cell development in both in vitro and in vivo conditions.

IL-3 exerts its biological activities by binding to specific high-affinity receptors expressed by hematopoietic stem cells, endothelial cells, and monocytes (29). Previous reports have demon-

FIGURE 7. IL-3 prevents CIA by enhancing the percentage of Foxp3+ Treg cells in vivo. A, Mice were sacrificed on day 36 and total cells derived from lymph nodes, spleen, and thymus tissues were analyzed for percentage of CD4+Foxp3+ Treg cells by FACS. Isotype controls of Foxp3 for each group are shown. B, Average percentage of CD4+Foxp3+ cells from lymph nodes, spleen, and thymus tissues of three mice. Values are expressed as mean ± SEM. *p < 0.05 in all the groups.

FIGURE 8. Effects of IL-3 on production of various cytokines in CIA mice. Serum samples of mice were analyzed on day 36 for secretion of various proinflammatory and anti-inflammatory cytokines by CBA. Data are represented as mean ± SEM (n = 5). Similar results were obtained in two independent experiments.
strated that IL-3–sensitive T cell clones activated by CD3 express IL-3Rα, whereas T cell clones that were insensitive to the growth-enhancing effect of IL-3 were negative for IL-3Rα expression (30, 31). These differences in reactivity to IL-3 among different T cell clones might be due to differences in the methods used for establishing such T cell clones (31). To our knowledge, we report in this study for the first time that both natural and induced Treg cells show IL-3Rα expression at both the gene and protein levels. Sustained expression of Foxp3, a key distinguishing feature of Treg cells, is required for the differentiation, suppressor function, maintenance of cell phenotype, and metabolic fitness of Treg cells (32). Mice and humans harboring a loss-of-function mutation in the Foxp3 gene are affected by fatal early onset lymphoproliferative immune-mediated disease affecting a variety of organs and tissues (33). Interestingly, in our system IL-3 significantly increases the percentage of Foxp3+ natural and induced Treg cells, and these cells suppress the proliferation of effector T cells. Thus, IL-3–treated Treg cells are endowed with the classical suppressive function ascribed to naturally occurring Treg cells. We demonstrate that IL-3 promotes development of Treg cells indirectly through secretion of IL-2 by non-Treg cells. IL-2 is an indispensable cytokine for the peripheral maintenance of natural Treg cells. However, natural Treg cells do not produce this cytokine, and the exact source of IL-2 has been a matter of intense research in the last decade (9, 34). Our results are consistent with earlier findings by other groups that the main source of IL-2 is CD4+CD25low non-Treg cells and not CD4+CD25hi Treg cells (35). IL-3 signaling involves activation of STAT5 in hematopoietic stem cells (15). Also, STAT5 activation by IL-3 is critical for Treg cell development and Foxp3 induction (25). Deletion of STAT5 in CD4+ T cells results in marked reduction of Foxp3+ Treg cells in both thymus and periphery, whereas enhanced STAT5 activation promotes Treg cell differentiation (25, 36). We report in this study that IL-3 synergizes with IL-2 for enhanced activation of STAT5 in T cells.

Deficiency of Treg cells leads to breakdown of tolerance in various human autoimmune diseases including type 1 diabetes and RA (11). Also, depletion of Treg cells exacerbates various experimental autoimmune diseases, including CIA (37). Foxp3+ Treg cells constitute 5–15% of peripheral CD4+ T cells (38, 39), and this proportion appears to be reduced in mice genetically prone to autoimmune diseases such as diabetes and CIA (37, 40). In our studies, we found that therapeutic treatment of CIA mice with IL-3 led to a significant rise in the percentage of both natural and peripheral Foxp3+ Treg cells in thymus, spleen, and lymph nodes. An increased percentage of Treg cells improved clinical signs of arthritis and suppressed local and systemic bone destruction. Also, we observed that IL-3–treated Treg cells are more potent in suppressing osteoclast formation in vitro when compared with Treg cells generated with TGF-β and IL-2 (R. Srivastava, unpublished observations). Thus, the increased percentage of Foxp3+ Treg cells in CIA mice by IL-3 may lead to the inhibition of osteoclastogenesis and bone loss, thereby attenuating CIA.

Similar to human RA, various proinflammatory cytokines, including TNF-α and IL-1β, are involved in CIA, a rodent model of human RA. IL-3 significantly suppresses the production of most proinflammatory cytokines (namely, IL-6, IL-17A, TNF-α, and IL-1) and induces the production of various anti-inflammatory cytokines (namely, IL-10, IL-2, IL-5, and IFN-γ) in vivo. Because inflammation and bone loss are two frequently occurring and tightly linked disorders, we next determined the effect of IL-3 on bone loss with the help of μ-CT analysis. Interestingly, μ-CT measurements of tibiae revealed that IL-3 prevents bone loss in CIA mice. Thus, our study clearly demonstrates that IL-3 enhances the percentage of Foxp3+ Treg cells in vitro indirectly through secretion of IL-2, and it also restores the loss of Foxp3+ Treg cells in vivo. Also, IL-3–generated Treg cells have the potential to cure CIA in mice. Furthermore, our data suggest that IL-3 may represent a novel therapeutic strategy to reduce immune responses, inflammation, and bone loss in autoimmune diseases.

Cellular therapy based on ex vivo development of Treg cells and their subsequent transfer to patients is currently the focus of intense research in the treatment of various autoimmune diseases (28). Also, transfer of Treg cells has already been shown to prevent a wide range of experimental autoimmune diseases, including diabetes, experimental encephalomyelitis, and colitis (40–42). Several, preclinical studies have shown that either freshly isolated or ex vivo expanded Treg cells can prevent both local and systemic organ and tissue destruction (43). Two broad therapeutic approaches have been considered: first, to expand Treg cells in vitro with the intention of infusing these cells into patients; and second, to manipulate the immune system in vivo resulting in an increase in Treg cells. Importantly, IL-3 appears to increase the percentage of Treg cells in both in vitro and in vivo conditions. Moreover, inhibition of most proinflammatory cytokines by IL-3 may results in an increase in percentage of Treg cells. Thus, to our knowledge, we reveal for the first time that IL-3 may be useful to expand Treg cells under both in vitro and in vivo conditions, and it may act as an important therapeutic agent to treat RA and other autoimmune diseases.

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Disclosures
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References