Anti-CD3 Antibody Induces IL-10-producing CD4⁺CD25⁺ Regulatory T Cells, Which Suppress T Cell Response in Rheumatoid Arthritis Patients

Bo-Young Yoon¹*, Mi-La Cho*, Yeon-Sik Hong, Joo-Yeon Jhun, Mi-Kyung Park, Kyung-Su Park, Sung-Hwan Park and Ho-Youn Kim

Division of Rheumatology, Department of Internal Medicine, The Center for Rheumatic Diseases, and The Rheumatism Research Center (RhRC), Catholic Research Institutes of Medical Sciences, Catholic University of Korea, Department of Internal Medicine, Inje University Ilsan Paik Hospital, Seoul, Korea

ABSTRACT

Background: Regulatory T cells (Tregs) have been investigated intensively for some decades. These cells regulate the immune system, prevent overactivated immune responses and can be used therapeutically. For rheumatoid arthritis (RA), understanding the functions and status of Tregs is an important step for understanding immune regulation in this autoimmune disease. Methods: We investigated the percentages, phenotypes and suppressive functions of CD4⁺CD25⁺ Tregs in peripheral blood (PB) of patients with RA. Results: The percentages were higher in the patients (n=12) than in healthy controls (n=10), and the cells expressed the CD45RBlow, CTLA-4 and CCR7 phenotypes. We also investigated the expression of Foxp3 and secretion of interleukin (IL)-10 induced CD4⁺CD25⁺ Tcells by anti-CD3 antibody treatment. A suppressive function of the patients’ cells was shown through coculture with CD4⁺CD25⁺ T cells in vitro. Conclusion: We suggest that, despite their increased numbers and suppressive function, they manage the ongoing inflammation ineffectively. It might be possible to apply IL-10 to induce the proliferation of IL-10-producing Tregs as therapy for RA. (Immune Network 2007;7(3):124-132)

Key Words: Regulatory T cells, CD4⁺CD25⁺ cell, rheumatoid arthritis, IL-10, peripheral blood

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of autoimmune origin affecting the peripheral joints and systemic organs (1). It is characterized by synovial destruction involving T cells, B cells, macrophages and proinflammatory cytokines (2). An essential role in RA has been ascribed to T cells, but the causes of chronic inflammation and T cell autorreactivation remain unclear. However, disturbed immune regulation may be a cause of ongoing inflammatory responses and the production of proinflammatory cytokines in patients with RA.

The CD4⁺CD25⁺ regulatory T cell (Treg) subset is currently a focus for the study of autoimmune diseases, allergic disorders and transplantation immunology. It is evident that these cells play a pivotal role in immune regulation; they can be classified into two groups: naturally occurring CD4⁺CD25⁺ Tregs and interleukin (IL)-10-producing Tregs (3). The naturally occurring CD4⁺CD25⁺ Treg subset has surface markers of CD25⁺, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), glucocorticoid-induced tumor ne-
crosis factor receptor (GITR) and Foxp3 (4-9). In contrast with naturally occurring CD4+CD25+ Tregs, the IL-10 producing Tregs are secondarily immunosuppressive and develop from conventional CD4+CD25+ T cells in the peripheral circulation (3).

In mice, CD4+CD25+ Treg depleted animals develop various autoimmune diseases, such as gastritis and thyroiditis (10). In turn, the cotransfer of CD4+CD25+ T cells is able to control various manifestations of autoimmune diseases, secondary to neonatal thymectomy. The suppressive function of Tregs may act via several mechanisms: from cell-to-cell contact, by the secretion of cytokines and through negative signals from costimulatory molecules (3,11).

In humans, naturally occurring Tregs make up about 5–10% of peripheral CD4+ T cells, and these are able to suppress CD4+ T cell proliferation and modulate monocyte functions (10). These Tregs are expected to be downregulated or defective in several autoimmune diseases. Kukreja et al. reported low numbers of resting CD4+CD25+ T cells in patients with immune mediated diabetes (12). Other investigators showed that although the levels of CD4+CD25+ Tregs are normal in patients with type 1 diabetes, the ability of these cells is markedly lower than in control subjects (12,13).

Tregs may also suppress or regulate immune responses in RA. It is generally assumed that Tregs in patients with RA must be depleted or do not function adequately. Recently, investigators have demonstrated the existence and the function of Tregs in peripheral blood (PB) and synovial fluid (SF) from patients with RA (14-16). Some have reported no differences in the percentages of CD4+CD25+ T cells between the PB of patients with RA and that from healthy controls (15,16). However, others have observed higher percentages of CD4+CD25+ T cells in PB from patients with RA (14). Lawson et al. suggested that early stages of RA might be associated with a deficit in the CD4+CD25high regulatory T cell population in PB (17).

These studies showed that the time of action of Tregs might be an important element in their functions. Thus, in the early phase of RA, the suppressive function of CD4+CD25+ Tregs might be effective therapeutically. Here, we investigated the presence and function of Tregs in patients with RA compared with healthy controls. We also studied the possibility of inducing CD4+CD25+ Treg function by cytokine treatment.

Materials and Methods

Patients. Informed consent was obtained from 12 patients (four men and eight women) with RA who fulfilled the 1987 revised criteria of the American College of Rheumatology (formerly the American Rheumatism Association) (18). The mean age (±SD) of the patients with RA was 51.1±12.3 years, ranging from 28 to 73 years (Table I). All medications were stopped 48 h before entry to the study. Comparisons were made with 10 healthy control subjects (three men and seven women), who had no rheumatic diseases. The mean age of the controls was 34±7 years (range 21–45 years). Informed consent was also obtained from the control subjects, and the protocol was approved by the Catholic University of Korea Human Research Ethics Committee.

Reagents. Anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) were obtained from BD Biosciences (San Diego, CA) and recombinant human (rh) IL-10 was purchased from R&D Systems Inc. (Minneapolis, MN).

Proliferation assay. Aliquots of 2×10⁴ freshly isolated CD4+CD25+ cells and CD4+CD25+ cells from human PB mononuclear cells (PBMC) together with 1×10⁵ irradiated (500 Rads) syngeneic T-depleted splenocytes were plated in triplicate in 96-well round-bottomed plates (Costar). The final volume was 200 µl in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1 µg/ml of anti-CD3 and anti-CD28 mAbs. To analyze proliferation in response to IL-10 activation, con-
trol IgG (10 μg/ml) rhIL-10 (10 ng/ml) and anti-IL-10 mAb (10 μg/ml) were added as indicated. Wells were pulsed with 1 μCi [3H]thymidine (PerkinElmer, Gaithersburg, MD) for the last 18 h of the 72 h culture and harvested onto filter membranes using a Wallac harvester (Tomtec, Hamden, CT). The incorporated [3H]thymidine was then measured using a Wallac Betaplate counter (PerkinElmer).

**Suppression assay.** To test the suppressive function of CD4+CD25+ on anti-CD3 and CD28 stimulation, freshly isolated CD4+CD25+ cells from PBMC were stimulated in triplicate with 1×10^5 irradiated (5000 Rads) CD4 T-depleted PBMC in the presence of 1 μg/ml anti-CD3 and anti-CD28 mAbs. The CD4+CD25− and CD4+CD25+ cells were plated at 2.0×10^5 cells per well alone or in combination with each cell type in triplicate. The cells were cocultured at a 1:1 ratio in a final volume of 200 μl of complete medium in 96-well round-bottomed plates for 72 h. Wells were pulsed with 1 μl Ci [3H]thymidine 18 h before harvesting.

**RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR).** Total cellular RNAs were extracted using the TRIZOL reagent (Invitrogen), and isolated total RNAs were reverse-transcribed using the Qmni-script RT kit (Qiagen, Hilden, Germany). Quantita-

---

Figure 1. Increased percentages of CD4+CD25+ T cells in the PB of patients with RA. (A) Mononuclear cells were isolated by Ficoll density gradient centrifugation. Cells were stimulated with soluble or membrane bound (mb form) anti-CD3 and anti-CD28 mAbs for 48 h. Cells were stained with fluorescent-labeled anti-CD4 and anti-CD25 mAbs and analyzed by flow cytometry. The bold number in each upper right quadrant indicates the percentage of CD4+CD25+ T cells. Results are mean percentage of CD4+CD25+ T cells ± SD (RA-PB; n = 12, Normal PB; n = 10). *p < 0.05, **p < 0.01 vs normal control at the same time point by Student’s t-test.
tive RT-PCR analyses of IL-10, and Foxp3, as well as the control GAPDH mRNA transcripts, were carried out using the assay-on-demand gene-specific fluorescent labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA).

Fluorescence-activated cell sorting (FACS) analysis. PBMC were stained with three-color fluorescence including FITC-labeled anti-CD4, PE-labeled anti-CD25, APC-labeled anti-CD45RB\textsuperscript{low}, and anti-γδ TCR and anti-CCR7 mAbs. Intracellular expression levels of CTLA-4, PD-1 and IL-10 were determined by staining with APC-labeled anti-CTLA-4, anti-PD-1 and anti-IL-10 mAbs using Cytofix/Cytoperm kits (BD PharMingen, San Diego, CA). Data were analyzed using a FACS Calibur flow cytometer with CELLQuest\textsuperscript{TM} software (BD Biosciences, Mountain View, CA).

FACS cell isolation. PBMC were isolated from PB samples of healthy human donors by Ficoll density gradient centrifugation (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The indicator (CD4\textsuperscript{+CD25\textsuperscript{-}}) and suppressor (CD4\textsuperscript{+CD25\textsuperscript{+}}) cell fractions were isolated from 150×10\textsuperscript{6} PBMC. Cells were incubated with 0.4 ml each of FITC-labeled anti-CD4 and anti-CD25PE (BD PharMingen) mAbs for 30 min at 4°C. These cell fractions were then sorted using a FACS Vantage (Becton Dickinson Biosciences, San Jose, CA, USA). On the forward-angle and side-scatter plots, the sort regions were constrained to the lymphocyte population. Sorted cells were collected into serum-containing medium, washed with RPMI medium and assessed for Treg activity.

Cytokine analysis. To determine amounts of IL-10, supernatants after activation with soluble anti-CD3 alone or with anti-CD28 assays were tested for the presence of IL-10 by ELISA according to the manufacturer’s instructions (BD Pharmingen). The color reaction was measured at 405 nm using an ELISA reader (Tecan) and analyzed with Magellan ELISA-software (Tecan).

Statistical analysis. Data are expressed as the mean±SEM. Statistical analysis was performed using Student’s t-test for matched pairs and p<0.05 was considered significant.

Results

Increased percentages of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells in PB of patients with RA compared with healthy controls. The per-

![Figure 2. Activated CD4\textsuperscript{+}CD25\textsuperscript{+} T cells in patients with RA express CD45RB\textsuperscript{low}, CCR7 and CTLA-4. *p<0.05; **p<0.01. PBMC from RA patients (RA, black bars) and normal control (Normal, gray bars) were cultured alone and stimulated with soluble anti-CD3 mAb for 48 hours. Cultured cells were surface-stained with FITC-anti-CD4, PE-anti-CD25, APC-anti-CD45RB and anti-CCR7 Ab and intracellularly stained with APC-anti-CTLA-4 and anti-PD-1 Ab. CTLA-4 and PD-1 expression on CD4\textsuperscript{+}CD25\textsuperscript{+} T cells were analyzed using flow cytometry. Results are the mean ±SD of 5 independent experiments. *p<0.05, **p<0.01 vs normal control at the same time point by Student’s t-test.](image-url)
Activated CD4\(^+\)CD25\(^+\) T cells in patients with RA produce the cytokine IL-10 and express Foxp3 and IL-10.

(A) PBMC from RA patients cultured alone and stimulated with anti-CD28 Ab in the absence or presence of soluble anti-CD3 mAb for 48 hours. Cultured cells were surface-stained with FITC-anti-CD4 and PE-anti-CD25 Ab, and intracellularly stained with APC-anti-IL-10 Ab. IL-10 expression on CD4\(^+\)CD25\(^+\) T cells were analyzed using flow cytometry. (B) In parallel experiments, culture supernatants were collected after 48 hours and analyzed by ELISA to determine amounts of IL-10. All samples were run in duplicates and bars indicate means±SD of 5 independent experiments. (C, D) PBMC from RA patients cultured alone and stimulated with soluble anti-CD3 mAb in the absence or presence of IL-10 blocking Ab, recombinant IL-10 for 48 hours. Cultured cells were analyzed by RT-PCR (C) or real time-PCR (D) with specific primer of FoxP3, IL-10 and GAPDH. GAPDH was used as an internal control. *p<0.05, **p<0.01 vs normal control at the same time point by Student's t-test. Results are the mean±SD of 5 independent experiments.

centage of isolated CD4\(^+\)CD25\(^+\) T cells was 16.7% of CD4\(^+\) T cells in PB from patients with RA and 6.1% in healthy controls. After the cells had been stimulated with anti-CD3 and anti-CD28 mAbs for 48 h, they were analyzed by flow cytometry. The percentage of CD4\(^+\)CD25\(^+\) T cells was higher in patients with RA than the controls (Fig. 1A). Mononuclear cells were isolated and stimulated with various conditions. Before stimulation, the mean percentage of CD4\(^+\)CD25\(^+\) T cells from patients with RA (16.1±4.2%) was higher than the controls (6.13±3.5%; p<0.01). After stimulation with anti-CD3 mAb, and anti-CD3 mAb plus anti-CD28 mAb, the mean percentages of CD4\(^+\)CD25\(^+\) T cells in PB from patients with RA were significantly higher than the controls (Fig. 1B).

Activated CD4\(^+\)CD25\(^+\) T cells in patients with RA have the characteristic phenotype of Tregs. CD25 is a marker for Tregs, but is also expressed on activated T cells. Other costimulatory molecule markers such as GITR, OX40 and CTLA-4 are well known. The expression levels of CD45RB\(^{low}\), CTLA-4 and \(\gamma\delta\) TCR in
Figure 4. Suppression of CD4⁺CD25⁺ T cell response by CD4⁺CD25⁺ T cells in PB samples from patients with RA. Purified CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from RA patients were plated either alone or mixed at a ratio in the presence of irradiated (5000 Rads) CD4 T-depleted PBMC and stimulated with soluble (1 and 10 μg/ml) or membrane bound form anti-CD3 mAb for 72 hours. The proliferative response was assessed by [3H] thymidine incorporation after a pulse during the last 16 hours of 72 hours culture. Results are the mean±SD of 5 independent experiments. *p<0.05, **p<0.01 vs CD4⁺CD25⁺ T cell at the same time point by Student’s t-test.

Figure 5. Effects of the addition of exogenous IL-10 on the activation of CD4⁺CD25⁺ T cells in PB samples from patients with RA. PBMC from RA patients (RA, black bars) and normal control (Normal, gray bars) were stimulated with anti-CD28 Ab, recombinant IL-10, IL-10 blocking Ab or recombinant IL-10 plus IL-10 blocking Ab in the absence or presence of soluble anti-CD3 mAb for 72 hours. Cultured cells were stained with FITC-anti-CD4 Ab and PE-anti-CD25 Ab. Stained cells were analyzed using flow cytometry. Results are mean percentage of CD4⁺CD25⁺ T cells ±SD (RA-PB; n=10, Normal PB; n=10).

CD4⁺CD25⁺ T cells from patients with RA were higher than the controls (Fig. 2). The results were emphasized by stimulation with the anti-CD3 mAb and IL-10. Increased IL-10 expression and IL-10 production were observed in the CD4⁺CD25⁺ T cells from patients with RA. Higher CCR7 expression was shown by stimulation with the anti-CD3 mAb (Fig. 2, 3). Expression of Foxp3 is a reliable marker for CD4⁺CD25⁺ Tregs. We observed increased Foxp3 expression in CD4⁺CD25⁺ T cells from patients with RA and this was increased further by stimulation with the anti-CD3 mAb (Fig. 3C, D).

CD4⁺CD25⁺ T cells are suppressed by CD4⁺CD25⁺ T cells in PB from patients with RA. The CD4⁺CD25⁺ T cells from patients with RA exhibited the typical suppressive function of Tregs (Fig. 4). For stimulation, cells were treated with a soluble anti-CD3 mAb. Activated CD4⁺CD25⁺ T cells maintained their sup-
pressive function. Membrane-bound type anti-CD3 mAb induced stronger proliferation than the soluble type. Thus, both CD4⁺CD25⁺ T cells and CD4⁺CD25⁺ T cells were activated and overcame the suppressive function of CD4⁺CD25⁺ T cells. CD4⁺CD25⁺ T reg produce cytokine IL-10, and exogenous IL-10 induces the expansion of CD4⁺CD25⁺ Treg cells from patients with RA. We investigated cytokine IL-10 levels in the supernatants of activated CD4⁺CD25⁺ T cells in PB from patients with RA. Increasing the anti-CD3 mAb concentrations administered resulted in a gradual increase in the production of IL-10 (Fig. 3B). Exogenous IL-10 also affected the activation of these cells. The effect disappeared after treatment with an anti-IL-10 blocking mAb (Fig. 5). Thus, IL-10 was secreted from these activated CD4⁺CD25⁺ T cells and induced the reactivation of CD4⁺CD25⁺ T cells in an autocrine manner.

Discussion

We found increased percentages and increased suppressive function of CD4⁺CD25⁺ Tregs in PB samples from patients with RA. This result differs from previous studies, which showed reduced expression of the CD4⁺CD25⁺ T cell subset producing IL-10, but not IL-2 or IL-4, in inflamed synovium and in PB samples from such patients (14-16,19). Regarding CD4⁺CD25⁺ Tregs, investigators concur on the increased numbers and suppressive function of such cells in SF from patients with RA (14,15). However, there are differing opinions about Tregs in PB samples from such patients (16,18) and in PB from patients with type 1 diabetes: another T cell mediated autoimmune disease (12,13).

We expected that the Tregs in patients with RA would be depleted or defective. However, the results differed from our expectation. Why do the increased numbers and enhanced suppressive function of Tregs fail to overcome ongoing inflammation in this disease? First, we agree with other investigators' opinions of negative feedback from the immune system (14). An activated immune system induces T cells to cause inflammation and concurrently induces Tregs to suppress it. Despite this negative feedback system, the balance between active CD4⁺ T cells and CD4⁺CD25⁺ Tregs may be more important than absolute cell count and function. Second, the suppressive function of Tregs is decreased in vivo. Here, we tested the function of CD4⁺CD25⁺ Tregs by coculture with CD4⁺ T cells or by cytokine production in vitro. Many variations of the immune system must exist in vivo and the suppressive function of Tregs might be weakened or lost. Reverse activated T cells may not be affected by regulatory factors such as cell-to-cell contact and cytokine production in vivo (5,19,20). One study reported that proinflammatory cytokines - IL-7 and TNF-alpha - lead to diminished suppressive activity of CD4⁺CD25⁺ Tregs (21). Third, this study and those of other investigators showed increased percentages and function of CD4⁺CD25⁺ Tregs in PB samples from patients with RA with relatively low disease activity (low ESR and CRP levels) and long disease duration. In addition, most such patients use corticosteroids, which are known to affect lymphocyte function and CD25 expression (22). Ehrenstein et al. reported compromised functioning of Tregs in PB from patients with RA (19). The study population had a disease activity score > 5.1 and excluded patients taking corticosteroids. Moreover, they found that the compromised function of Tregs could be reversed by TNF-α therapy. The conditions of our study were similar to that report in that patients were sampled after, rather than before, such therapy. Therefore, inflammatory cytokines may affect the function of CD4⁺CD25⁺ Tregs.

Finally, CD4⁺CD25⁺ Tregs may have different functions in different disease stages. Thus, the numbers and function of Tregs were decreased in patients with type 1 diabetes (13). That study involved patients with recent-onset adult type 1 diabetes. CD4⁺CD25⁺ Tregs might be important in the decisive stage when the immune system breaks down or starts to move away from a good balance. We investigated the number and function of CD4⁺CD25⁺ Tregs in the chronic inflammatory stage. The immune system of the patients had shifted to a stage of nonregulation or was unable to reattain normal function. Results of a recent study showed that depletion of CD4⁺CD25⁺ T cells in mice led to an accelerated onset and worsening of collagen-induced arthritis (23). In humans, CD4⁺CD25⁺ Treg levels are decreased in PB samples from patients with early RA (17). It is probable that the importance of CD4⁺CD25⁺ Tregs in autoimmune disease is at the location and stage of action.
We observed some characteristic phenotypes and surface markers of the CD4\(^+\)CD25\(^+\) Tregs. CD45 \(\text{RB}^{\text{low}}\) is an early surface marker of such cells (24). CTLA-4 is also an important surface molecule indicating regulatory mechanisms by negative costimulatory signals (6). Foxp3 expression is the most essential and unique characteristic of CD4\(^+\)CD25\(^+\) Tregs (8,9). Both PD-1 and CTLA-4 are expressed in CD4\(^+\)CD25\(^+\) Tregs and participate in their function. However, it is not an absolute regulatory pathway, and its regulatory functions persist in the absence of PD-1 and PD-L1 (25). Here, we found no difference in the PD-1 expression level of Tregs between patients with RA and healthy controls. CCR7 is one of the lymph node homing receptors, and CD4\(^+\)CD25\(^+\) Tregs expressing CCR7 may enter lymphatic organs in vivo. These characteristics help Tregs to target specific sites in therapeutic applications (26). We noted here an increase in CCR7 expression before and after stimulation by the anti-CD3 mAb.

The secretion and role of cytokines in Tregs have been debated. Investigators consider IL-10 to be an important cytokine that induces the activity of Tregs in vivo (27). We found that CD4\(^+\)CD25\(^+\) Tregs in PB samples from patients with RA secreted IL-10, and that this increased following stimulation by anti-CD3 and anti-CD28 mAbs. We also investigated whether the numbers of Tregs could be increased by IL-10 stimulation and reduced by IL-10 blocking antibodies. We suggest that IL-10 could be used for the expansion of CD4\(^+\)CD25\(^+\) Tregs and treatment of autoimmune diseases. IL-2 and TGF-\(\beta\) are other essential cytokines in the generation and function of CD4\(^+\)CD25\(^+\) Treg subsets (28,29).

CD4\(^+\)CD25\(^+\) Tregs have now been investigated intensively. For therapeutic application, the expansion of such cell types is essential. However, expansion itself is a very delicate process. After expansion, Tregs lose their characteristic phenotypes and suppressive function. TGF-\(\beta\) is central to the conversion of CD4\(^+\)CD25\(^-\) T cells into CD4\(^+\)CD25\(^+\)Foxp3\(^+\) Tregs (30). By increasing the percentages of CD4\(^+\)CD25\(^+\) Tregs in patients with RA, we can expect other problems in that the increased numbers and stable suppressive function of Tregs may not operate well. Therefore, early transfer of Tregs may be important therapeutically before extreme joint inflammation develops in such patients. Tregs have a nonspecific suppressive function (3,11). Antigen-specific expansion of CD4\(^+\)CD25\(^+\) Tregs can establish antigen-specific dominant tolerance to self or nonself antigens. Some have reported the induction of antigen-specific immunologic tolerance by antigen-specific expansion of Foxp3\(^+\)CD25\(^+\)CD4\(^+\) Tregs during tissue transplantation and in patients with autoimmune diabetes (31,32). This approach may also be possible for treating autoimmune diseases as a new therapeutic modality.

In summary, we have reported higher percentages of CD4\(^+\)CD25\(^+\) Tregs in the PB of patients with RA than in healthy controls. The cells exhibited CD45 \(\text{RB}^{\text{low}},\) CTLA-4, CCR7 and Foxp3 phenotypes. IL-10 was secreted by both nonactivated and activated CD4\(^+\)CD25\(^+\) T cells. We suggest that treatment with IL-10 in combination with anti-CD3 antibodies could be used in the expansion of CD4\(^+\)CD25\(^+\) Tregs for the treatment of autoimmune diseases.

References
5. Shevach EM: CD4\(^+\)CD25\(^+\) suppressor T cells: more questions than answers. Nat Rev Immunol 2;389-400, 2002