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ORIGINAL ARTICLE

The effect of various disease-modifying anti-rheumatic drugs on the suppressive function of $CD4^+CD25^+$ regulatory T cells

Ji Seon Oh · Yong-Gil Kim · Seung Geun Lee · Min Wook So · Seung Won Choi · Chang-Keun Lee · Bin Yoo

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Abstract Accumulating evidence suggests that defects in the function of $CD4^+CD25^+$ regulatory T cells (Tregs) are important in immune-mediated diseases such as rheumatoid arthritis. Here, we investigated the effects of various disease-modifying anti-rheumatic drugs (DMARDs) on Treg function. Tregs and $CD4^+CD25^-$ effector T cells (Teffs) were isolated from peripheral blood mononuclear cells obtained from healthy adults. Isolated Tregs were cultured with the DMARDs methotrexate (MTX), sulfasalazine (SSZ), leflunomide (LEF), or infliximab (INF). We found that each DMARD had a different effect on Treg function. SSZ and LEF inhibited the anti-proliferative function of Tregs on cocultured Teffs and reduced Treg expression of Foxp3 mRNA, whereas MTX and INF did not.

Keywords Regulatory T-lymphocytes · Rheumatoid arthritis · Antirheumatic agents · Methotrexate · Sulfasalazine · Leflunomide · Infliximab

Ji Seon Oh and Yong-Gil Kim contributed equally to this research.

J. S. Oh · S. W. Choi
Division of Rheumatology, Department of Internal Medicine, University of Ulsan College of Medicine, Ulsan University Hospital, Ulsan 682-714, Korea

Y.-G. Kim · M. W. So · C.-K. Lee (✉) · B. Yoo
Division of Rheumatology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, Seoul 138-736, Korea
e-mail: cklee@amc.seoul.kr

S. G. Lee
Division of Rheumatology, Department of Internal Medicine, Inje University Haeundae Paik Hospital, Busan, Korea

Introduction

$CD4^+CD25^+$ regulatory T cells (Tregs) play an important role in peripheral immune tolerance, serving to prevent autoimmune diseases such as rheumatoid arthritis (RA), which is characterized by chronic synovial inflammation that results in progressive cartilage and bone destruction. Tregs are a subset of $CD4^+$ T cells that express high levels of CD25 and exhibit immunosuppressive activity. These cells are generated in the thymus and possibly in the periphery as well [1, 2]. It has been shown that Tregs are capable of suppressing $CD4^+CD25^-$ effector T-cell (Teff) proliferation and cytokine production and directly suppress monocytes, macrophages, and dendritic cells, suggesting a role in both innate and adaptive immune responses [3, 4]. Although the exact mechanism of immune suppression by Tregs is controversial, it is thought to be mediated by a variety of cell contact-dependent and contact-independent mechanisms [5]. Antigen recognition and activation of the Tregs via the T-cell receptor is required to suppress immune response [6]. Tregs have been found to produce IL-10, IL-4, TGF-beta. However, there is a lack of evidence that these cytokines play a role in Treg-mediated suppression [5, 7, 8].

Forkhead box P3 (FoxP3), an intracellular marker for functional Tregs in both mice and humans, is critical for the development and function of Tregs [9–12]. In humans, Foxp3 expression is also induced in $CD4^+CD25^-$ Teffs upon stimulation and leads to acquisition of a regulatory phenotype that is able to suppress in vitro proliferation of autologous $CD4^+CD25^-$ T cells [13, 14]. Another marker for human Treg delineation is CD127, the interleukin 7 receptor-α (IL-7R-α) chain. It has been reported that the expression of CD127 inversely correlates with Foxp3 expression and Treg suppressor activity [15, 16].

In a study of antigen-induced arthritis, depletion of Tregs in immunized animals before arthritis induction was shown to exacerbate disease, whereas transfer of Tregs into immunized mice at the time of induction decreased the severity of disease [17]. It has been shown that early active, antirheumatic drug-naïve RA patients have a smaller proportion of Tregs in the peripheral blood than controls, whereas stable, well-controlled RA patients receiving therapy have Treg numbers similar to controls [18]. In addition, it has been reported that Tregs from RA patients are unable to suppress pro-inflammatory cytokine production, and treatment with anti-tumor necrosis factor (TNF)-α antibodies restores the capacity of Tregs to inhibit cytokine production [19]. TNF has been shown to inhibit suppressive activity and downregulate Foxp3 expression in Tregs [20]. These findings suggest that Treg suppressive function in patients with active RA is decreased significantly and can be restored by appropriate treatments. However, the direct effects of various disease-modifying anti-rheumatic drugs (DMARDs)—the mainstay in the treatment of RA—on Tregs have not yet been systematically addressed. An in vitro study showed that the suppressor function of umbilical cord blood-derived Tregs was modestly reduced by methotrexate (MTX), suggesting that such drugs may adversely affect the suppressive function of Tregs [21]. However, given that the function of Tregs in RA patients could be recovered after appropriate treatment (e.g., anti-TNF agents) and MTX is the first-line drug in the treatment of RA, it is unclear whether the function of Tregs are also reduced by MTX or other DMARDs, including sulfasalazine (SSZ) and leflunomide (LEF). The aim of this study was to evaluate the direct effects of these DMARDs (MTX, SSZ, and LEF) on the function of Tregs in peripheral blood.

Materials and methods

Isolation of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from peripheral blood mononuclear cells

Peripheral blood was obtained from five healthy volunteers after obtaining informed written consent. This study protocol was approved by the Asan Medical Center Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll/Plaque (Pharmacia Biotech, Uppsala, Sweden) at 400*g* for 30 min. $CD4^+$ T cells were isolated from PBMCs by negative selection using an LD column (Miltenyi Biotech, Sunnyvale, CA, USA). Purified $CD4^+$ T cells were incubated with anti-human CD25 magnetic beads (Miltenyi Biotech) and separated into $CD4^+CD25^+$ and $CD4^+CD25^-$ fractions by positive selection using an LS column (Miltenyi Biotech). The selected $CD4^+CD25^+$ cell fractions were separated again over an LS column to achieve higher purities.

The isolated cells were activated by incubation with 100 U/ml recombinant human interleukin (IL)-2 (R&D Systems, Minneapolis, MN, USA), 1 μg/ml anti-CD3 antibody, and 1 μg/ml anti-CD28 antibody (Serotec Ltd., Oxford, UK) for 3 days. Purity was assessed using a fluorescence-activated cell sorter (FACS; Becton–Dickinson, Oxnard, NJ, USA). Isolated cells (2×10^5 cells in 90 μl of phosphate-buffered saline) were incubated with 10 μl RPE-conjugated mouse anti-human CD25 and FITC-conjugated mouse anti-human CD4 antibodies (Serotec Ltd., Kindlington, Oxford, UK) for 30 min.

Cell viability assay

The viability of isolated $CD4^+CD25^+$ T cells before and after culturing with each DMARD was assessed using an XTT-based cell proliferation kit (Roche, Karlsruhe, Germany). Briefly, activated $CD4^+CD25^+$ T cells (10^5 cells/well) were cultured with vehicle (control) or different doses of individual DMARDs (0.1, 1, 10, 100, and 1,000 nM MTX; 0.1, 1, 10, and 100 μg/ml SSZ; 1, 10, 30, and 100 μg/ml LEF; 1, 2.5, 10, and 100 μM INF) for 1 week. XTT labeling mixture (50 μl) and electron-coupling reagent (1 μl) were added to each well and plates were incubated for 18 h at 37 °C. Absorbance of wells was measured using a microtiter plate reader at 450 nm, with a reference wavelength of 650 nm. Cell viability assay results were used to determine a maximum dose of each DMARD at which cell viability was unaffected for use in subsequent experiments: MTX, 100 nM; SSZ, 100 μg/ml; LEF, 30 μM; INF, 10 μg/ml.

Proliferation and suppression assays

The suppressive function of isolated $CD4^+CD25^+$ Tregs on the proliferation of $CD4^+CD25^-$ Teffs was assessed by coculturing-activated $CD4^+CD25^+$ Treg from healthy individuals with $CD4^+CD25^-$ Teffs (10^5 cells/well) in 96-well plates at different ratios of Tregs and Teffs (1:1, 0.5:1, 0.25:1, 0:1). The cells were cultured with 10 μg/ml phytohemagglutinin (PHA; Sigma, St. Louis, MO, USA) for 7 days; 1 μCi [^{3}H]thymidine (Amersham Pharmacia Biotech, Uppsala, Sweden) was added for the last 16 h. Cell proliferation was measured on the last day of culture using a β-counter (Perkin-Elmer LAS, Wellesley, MA, USA).

The effect of DMARDs on the suppressive function of $CD4^+CD25^+$ Tregs was determined by culturing activated $CD4^+CD25^+$ Treg (10^4 cells/well) with vehicle control or individual DMARDs (MTX, 100 nM; SSZ, 100 μg/ml; LEF, 30 μM; INF, 10 μg/ml) for 7 days. Cultures were performed in α-MEM (Gibco-BRL, Grand Island, NY)

supplemented with 10 % FBS, 2 mM L-glutamine 1 %, 100 U/ml penicillin, and 100 μg/ml streptomycin with the medium replaced every 3 days. After removal of media to the extent possible (to minimize the possible effect of DMARDs on $CD4^+CD25^-$ Teffs), $CD4^+CD25^+$ Tregs were cocultured with $CD4^+CD25^-$ Teffs (10^4 cells/well) in 96-well plates. Cell proliferation assays were then performed as described above.

RNA extraction and reverse transcription-polymerase chain reaction for Foxp3 mRNA expression

Activated $CD4^+CD25^+$ Tregs (2×10^5 cells/well) were cultured with vehicle control or each DMARD (MTX, 100 nM; SSZ, 100 μg/ml; LEF, 30 μM; INF, 10 μg/ml) for 7 days and then lysed by addition of 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated, and first-strand cDNA was synthesized by incubating for 1 h at 37 °C with 1 μl M-MLV reverse transcriptase (200 U), 5 μl 5 × RT buffer (25 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 5 μl of a 2.5-mM dNTP mixture, and 0.5 μl RNase inhibitor, in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). Foxp3 mRNA was estimated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using the primers 5′-ACA CCA CCC ACC ACC GCC ACT-3′ (sense) and 5′-TCG GAT GAT GCC ACA GAT GAA GC-3′ (antisense). The amplification protocol consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 64 °C for 10 s, and extension at 72 °C for 50 s, followed by a final extension at 70 °C for 5 min. The PCR products were electrophoresed on 1.5 % agarose gels (Amersham Pharmacia Biotech, Uppsala, Sweden) and stained with ethidium bromide (0.5 μl/ml) for 30 min.

Statistical analysis

The Mann–Whitney test was used to compare the effects of each DMARD with that of vehicle control. Comparison between 3 or more experimental groups was performed by Kruskall–Wallis test or Jonckheere-Terpstra test where appropriate. p-Values <0.05 were considered statistically significant. All data were analyzed using SPSS v14.0 software (SPSS, Chicago, IL, USA).

Results

Isolation of $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teffs from PBMCs

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from PBMCs by negative and repeated positive selection, and their purity was assessed by FACS. The purity of $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teffs was 95.1 and 98.1 %, respectively (Fig. 1a). $CD25^+$ T cells constituted about 3 % of the $CD4^+$ T-cell population.

Suppressive function of $CD4^+CD25^+$ Tregs on $CD4^+CD25^-$ Teffs

Activated $CD4^+CD25^+$ Tregs from healthy individuals were cocultured with $CD4^+CD25^-$ Teffs (10^5 cells/well) in 96-well plates at different ratios of Tregs and Teffs (1:1, 0.5:1, 0.25:1, 0:1) and incubated in the presence of PHA for 7 days. Cell proliferation assays using [^{3}H]thymidine uptake showed that the suppressive effect of $CD4^+CD25^+$ Tregs on $CD4^+CD25^-$ Teff proliferation was proportional to the ratio of $CD4^+CD25^+$ Tregs to $CD4^+CD25^-$ Teffs (Fig. 1b).

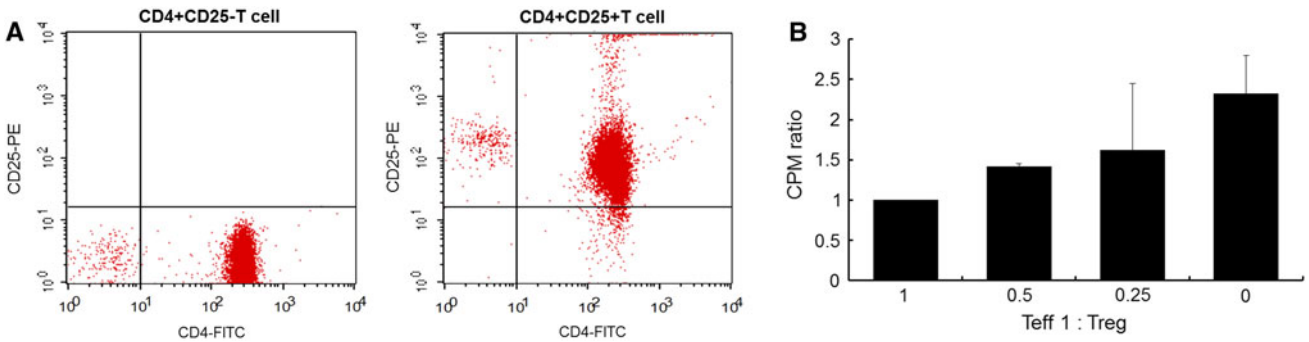

Fig. 1 **a** Purification of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from a healthy individual. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from PBMCs by negative and positive selection, and purity was assessed by FACS. **b** Suppression of $CD4^+CD25^-$ effector T cells (Teffs) proliferation by $CD4^+CD25^+$ regulatory T cells (Tregs). Activated Tregs isolated from healthy individuals were cocultured with Teffs (10^5 cells/well) in 96-well plates at different ratios of Tregs and Teffs (1:1, 0.5:1, 0.25:1, 0:1). Cells were cultured in wells containing PHA for 7 days; proliferation was determined by [^{3}H]thymidine uptake. The suppressive effect of Tregs on Teffs proliferation was proportional to the ratio of Tregs to Teffs ($p < 0.05$). Data represent the mean ± SD of 3 independent experiments

Cell viability after culturing with different doses of various DMARDs

To assess the effects of DMARDs on the survival of $CD4^+CD25^+$ Treg, we cultured activated $CD4^+CD25^+$ Tregs with different doses of each DMARD (MTX, SSZ, LEF, INF) for 1 week, and then performed XTT-based cell proliferation assays. We found that $CD4^+CD25^+$ Treg proliferation was sustained in the presence of all tested doses of each DMARD (Fig. 2).

Effects of DMARDs on the suppressive function of $CD4^+CD25^+$ Tregs

To assess the effect of each DMARD on the anti-proliferative function of $CD4^+CD25^+$ Tregs, we cultured activated $CD4^+CD25^+$ Tregs with each DMARD for 7 days and then plated $CD4^+CD25^-$ Teffs as a responder in 96-well plates at a 1:1 ratio (10^4 cells/well). Cell proliferation assays using [^{3}H]thymidine uptake showed that the anti-proliferative function of $CD4^+CD25^+$ Tregs was not influenced by MTX or INF, but was significantly reduced by SSZ ($p < 0.01$) and LEF ($p < 0.01$) (Fig. 3).

The effects of DMARDs on Foxp3 mRNA expression in $CD4^+CD25^+$ Tregs

To assess the effect of each DMARD on Foxp3 expression in $CD4^+CD25^+$ Tregs, we cultured activated $CD4^+CD25^+$ Tregs with each DMARD for 7 days and then assayed Foxp3 mRNA expression by RT-PCR. Foxp3 mRNA expression in $CD4^+CD25^+$ Tregs was reduced by SSZ and LEF, but not by MTX or INF (Fig. 4).

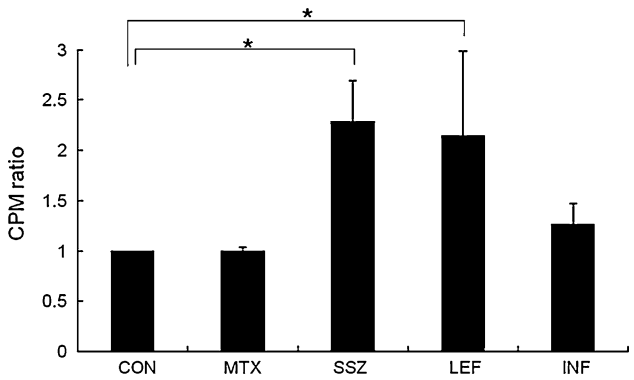

Fig. 3 Effect of various disease-modifying anti-rheumatic drugs (DMARDs) on the anti-proliferative function of $CD4^+CD25^+$ regulatory T cells (Tregs) from healthy individuals. $CD4^+CD25^-$ effector T cells (Teffs) were used as responder cells. After activation and culture with various DMARDs for 7 days, Tregs (10^4 cells/well) were cocultured with Teffs (10^4 cells/well) in 96-well plates. Cells were cultured in wells containing PHA for 7 days; proliferation was determined by [^{3}H]thymidine uptake. The anti-proliferative function of Tregs was significantly reduced by sulfasalazine (SSZ) and leflunomide (LEF), but was not influenced by methotrexate (MTX) or infliximab (INF). $^*p < 0.05$. Data represent the mean ± SD of 3 independent experiments

Discussion

$CD4^+CD25^+$ Tregs are known to play a crucial role in preventing the development of autoimmune disease. Given that RA, an autoimmune disease, develops in the presence of Tregs in peripheral blood and synovial fluid, it is possible that Teffs are less susceptible to Treg-mediated suppression [22], or that Tregs in RA lose their regulatory function.

There are some controversies on the role of Treg in pathogenesis of RA. Some studies reported that the proportion of $CD4^+CD25^+$ Tregs in the peripheral blood of

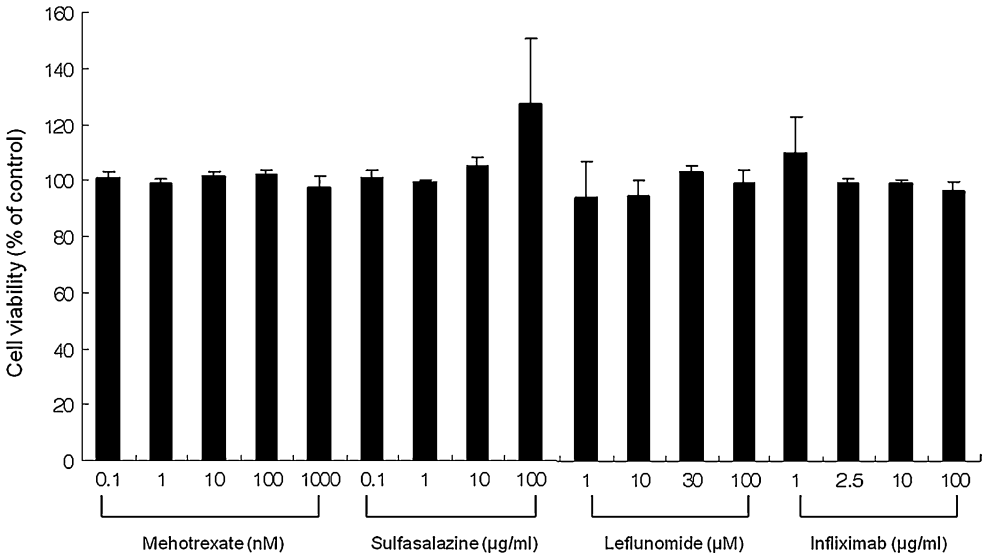

Fig. 2 Viability of $CD4^+CD25^+$ regulatory T cells (Tregs) after culturing with different doses of each DMARD. Activated Tregs from healthy individuals were cultured with various doses of methotrexate (MTX), sulfasalazine (SSZ), leflunomide (LEF), or infliximab (INF) for 7 days, and then cell viability was assessed using an XTT-based cell proliferation assay. Cell viability was not affected by any tested dose of individual DMARDs (p = not significant for each drug). Data represent the mean ± SD of 3 independent experiments

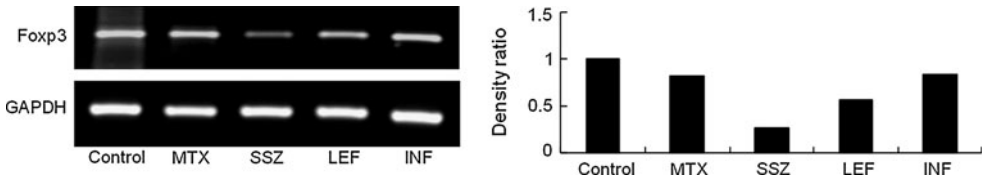

Fig. 4 Foxp3 mRNA expression in $CD4^+CD25^+$ regulatory T cells (Tregs) from a healthy individual. Activated Tregs were cultured with various DMARDs for 7 days. Foxp3 mRNA expression in Tregs was assayed by RT-PCR. Foxp3 mRNA expression in Tregs was reduced by sulfasalazine (SSZ) and leflunomide (LEF), but not by methotrexate (MTX) or infliximab (INF)

RA patients is smaller than controls [23], whereas other studies reported different results [19, 22, 24]. Regarding the function of Tregs, some studies reported that the synovial fluid $CD4^+CD25^+$ Tregs show increased regulatory activity compared with peripheral blood Tregs [22], while Ehrenstein et al. [19] have demonstrated that Tregs derived from peripheral blood of patients with active RA are defective in their ability to suppress pro-inflammatory cytokine production, but not proliferation. This finding differs from other studies using cells from synovial fluid [22–24]. Some of this variability may be explained by differences in the populations of patients, especially in disease stage and therapy, the methods used to purify Treg, or how the suppression assays were performed.

A number of different DMARDs have been used to reduce inflammation and prevent joint destruction in RA. Various actions of each DMARD on RA have been documented, but the exact mechanisms are not fully understood. MTX, as a folate antagonist, inhibits the synthesis of purines and pyrimidines and exerts anti-inflammatory effects by inhibiting proliferation and inducing apoptosis of immune/inflammatory cells as well as inhibiting the production of both monocytic and lymphocytic proinflammatory cytokines involved in RA [25]. MTX, regarded as a cornerstone of RA treatment, is currently recommended as the first DMARD of choice and as the anchor drug to which other DMARDs can be combined and new drugs can be evaluated [26, 27]. LEF inhibits pyrimidine synthesis, resulting in blockade of T-cell proliferation [28]. It also alters the synthesis of cytokines by augmenting the immunosuppressive cytokine TGF-β1 and suppressing the immunostimulatory cytokine IL-2 [29]. SSZ has been shown in vitro to possess multiple anti-inflammatory properties. It inhibits T-cell proliferation, natural killer cell activity, and B-cell activation, resulting in a decrease in immunoglobulin synthesis. Cytokine profiles also are altered by SSZ, resulting in inhibition of the T-cell cytokines IL-2 and interferon-γ, and the monocyte/macrophage cytokines IL-1, TNF-α, and IL-6 [30–32]. The mechanism of these DMARDs in rheumatic disease is still not fully elucidated. Moreover, the effects of these DMARDs on Treg function are not yet known.

The therapeutic effects of anti-TNF agents in RA are thought to be mediated by blocking the TNF-α-mediated inflammatory cytokine cascade. Additionally, anti-TNF agents can induce apoptosis of membrane-bound TNF-α-expressing cells or reduce their numbers through antibody- or complement-dependent cytotoxicity [33–35]. Anti-TNF agents have also been reported to neutralize TNF-α, which binds to its receptor on Tregs and thereby downregulates Treg function [20].

Regarding other drugs being used to treat RA, glucocorticoids (GCs) therapy had been reported to increase the frequency of Treg in early clinical studies on patients with different autoimmune diseases [36, 37]. However, other larger studies on patients with autoimmune diseases showed the opposite result [38]. A recent study suggests that short-term GC therapy did not change the relative frequency of circulating Tregs in vivo, neither in immunocompetent human subjects nor in mice. GC treatment in vitro did not have any direct effect on the functional ability of the Treg cells [39]. Calcineurin inhibitors (CNIs), cyclosporin A and tacrolimus, has been shown to inhibit FOXP3 expression and possible suppressor function of Tregs in several in vitro and in vivo studies [40, 41] and to reduce the frequencies of circulating Tregs in renal or liver transplant recipients [42, 43].

In this study, we focused on the possible inhibitory (adverse) effect of DMARDs on the regulatory function of healthy Treg population which are unaffected by such an inflammatory condition. SSZ and LEF significantly attenuated Treg expression of Foxp3 mRNA and suppression of Teffs. In contrast, neither MTX nor INF had any effect on these Treg properties, indicating that these DMARDs do not reduce the suppressive capacity of Tregs. Theoretically, DMARD which do not disturb the suppressive function of Treg might be the ideal choice for RA. For example, in individuals who are in preclinical stage or remission state of RA without any evidence of inflammation, some DMARDs such as SSZ or LEF might disturb autoregulatory function of Treg.

There had been several reports that sulfasalazine induced exacerbation of intestinal and/or extraintestinal manifestations of ulcerative colitis [44, 45]. Although the relationship between the disease flare and Treg function was not known, it deserves much consideration. In a recent study, LEF increased the proportion of $CD4^+CD25^+$ Tregs and FoxP3 mRNA expression in spleen lymphocytes from

collagen-induced arthritis rats both in vivo and in vitro [46]. In contrast, LEF decreases peripheral Treg in a mouse model of allogeneic bone marrow transplantation [47]. Because the environment where the Treg was studied has variable disease states in most studies, it is difficult to know whether the change in Treg function is caused by the drug itself or through environmental changes by the drug. As far as we know, there has been no in vitro human study on the effect of these drugs on Treg function in non-disease condition. Although the intracellular mechanism of SSZ and LEF as well as other DMARDs has not been known exactly, we thought that the negative effect of SSZ and LEF on Treg could be related to downregulation of Foxp3 activation. However, more research is needed to know which intracellular component is affected by these drugs and then makes an influence on Foxp3 activation.

Because this was an in vitro study with a small number of samples, there may be limits to which its results can be extrapolated to explain the effects of DMARDs on Tregs in vivo, where complex mechanisms and interactions among various inflammatory cells and cytokines are likely involved. Although Tregs suppress the activation and/or proliferation and cytokine formation of Teff even in the absence of antigen presenting cells (APCs) in vitro [8, 48], much of the effect of Treg on Teff has been known to depend on the effect on the function of APCs. Our experiment performed in the absence of APCs has a limitation in the evaluation of APCs-mediated effect of Treg. In addition, we defined Treg based on expression of CD4 and CD25 as many previous studies. Although the "classic" $CD4^{+}CD25^{+}$ Tregs are generally $Foxp3^{+}$ and highly immunosuppressive, these population also includes effector T cells [49]. Thus, identification of Treg by low levels of CD127 expression in combination with CD4 and CD25 expression would be a better approach to evaluate the pure $FoxP3^{+}$Treg function [15]. However, a notable finding in this study is that each DMARD may have a different effect on the regulatory function of Tregs. A better understanding of the mechanisms underlying the therapeutic action of each DMARD on Tregs may provide a direction for research into more effective combination regimens of conventional DMARDs or lead to the development of new treatment strategies for RA.

Conclusions

We have shown that each DMARD had a different effect on Treg function. SSZ and LEF inhibited the anti-proliferative function of Tregs on cocultured Teffs and reduced Treg expression of Foxp3 mRNA, whereas MTX and INF did not.

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