Induction of Regulatory T Cells by Infliximab in Behçet’s Disease

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PURPOSE. To determine whether infliximab induces the development of Foxp3⁺ T regulatory (Treg) cells in patients with Behçet’s disease.

METHODS. The subjects were patients with refractory uveitis caused by Behçet’s disease, Vogt-Koyanagi-Harada disease, or ocular toxoplasmosis and healthy volunteers. Purified CD4⁺ T cells were obtained from patients with uveitis who were treated with colchicine, cyclosporine, or infliximab. Flow cytometry was used to analyze the expression of Foxp3 on CD4⁺ T cells.

RESULTS. Foxp3 was expressed in a small percentage of CD4⁺ T cells (<5%) from healthy subjects and from patients with active uveitis caused by Behçet’s disease, Vogt-Koyanagi-Harada disease, or ocular toxoplasmosis. The percentage of Foxp3⁺ cells among CD4⁺ T cells was significantly decreased in patients with active uveitis compared with patients with inactive uveitis in the remission stage. Foxp3 was expressed at a similar level in CD4⁺ T cells from healthy donors, colchicine-treated patients, and cyclosporine-treated patients, whereas infliximab-treated patients expressed much higher percentages of Foxp3⁺ cells. Patients who had a high population of Foxp3⁺ cells during infliximab treatment did not experience any subsequent episodes of acute uveitis. On the other hand, patients with a low population of Foxp3⁺ cells during infliximab treatment did experience ocular inflammatory episodes. T cells exposed to infliximab in vitro greatly expressed Foxp3 and produced TGFβ, and the Treg cells significantly suppressed the activation of bystander T cells in vitro.

CONCLUSIONS. Anti–TNF-α therapy may be useful for patients with ocular complications of Behçet’s disease who have a decreased percentage of peripheral Treg cells. (Invest Ophthalmol Vis Sci. 2011;52:476–484) DOI:10.1167/iovs.10-5916

Behçet’s disease can cause uveoretinitis characterized by retinal vasculitis, and acute episodes of refractory uveitis can cause blindness. Uveitis in Behçet’s disease is often refractory to treatment with immunosuppressants such as cyclosporine. A new anti-tumor necrosis factor-alpha (TNF-α) monoclonal antibody, infliximab, greatly suppresses ocular inflammation in patients with Behçet’s disease.¹⁻¹⁰ TNF-α is a proinflammatory cytokine that plays a significant role in immune responses. Patients with Behçet’s disease with active uveitis have high serum levels of TNF-α.¹¹,¹² Recently, we reported that infliximab significantly suppressed the number of acute episodes of intraocular inflammation in Behçet’s disease and that the efficacy of infliximab was much greater than the efficacy of cyclosporine.⁸

Infliximab neutralizes membrane-bound TNF-α and soluble TNF-α and suppresses TNF-α production by macrophages and lymphocytes. An alternative inhibition mechanism of infliximab is the promotion of regulatory T cells (Treg cells) that acquire suppressive functions in the periphery. Clinically, infliximab has been used to treat rheumatoid arthritis, and the treated patients had significantly increased populations of Treg cells that expressed Forkhead box P3 (Foxp3).¹³ Foxp3 is constitutively expressed on peripherally induced Treg cells and naturally occurring Treg cells, and it is essential for Treg cell development and function.¹⁴,¹⁵ In a rheumatoid arthritis study by Nadkarni et al.,¹³ the authors suspected that infliximab-induced Treg cells inducibly express Foxp3 molecules through a transforming growth factor-beta (TGFβ) signal and that Treg cells may provide protection from inflammatory conditions.

In the present study, our goal was to determine whether infliximab induces Foxp3⁺ Treg cells in patients with Behçet’s disease. We found that infliximab can induce Treg cells and that this capacity is related to the effects of infliximab on uveitis in Behçet’s disease. We also demonstrate that infliximab-treated T cells display a regulatory phenotype in vitro.

PATIENTS, MATERIALS, AND METHODS

Patients

Subjects had Behçet’s disease with refractory uveoretinitis and were patients at Tokyo Medical and Dental University Hospital between 2008 and 2009. The subjects did not have severe active systemic inflammation, even though they had active uveitis during the follow-up periods. Behçet’s disease was diagnosed according to the criteria of the Behçet’s Disease Research Committee of the Ministry of Health and Welfare of Japan.¹⁶ We administered infliximab to 16 patients after informed consent was obtained. Infliximab (5 mg/kg) was administered at weeks 0, 2, 6, and 14 and every 8 weeks thereafter.⁶ We used the patients’ clinical charts to determine the number of acute episodes of uveitis (uveitis attacks) during the treatment period. We prepared four control groups: patients with other clinical entities of uveitis (Vogt-Koyanagi-Harada disease, n = 5; ocular toxoplasmosis, n = 1), patients with Behçet’s disease treated with colchicine (n = 12), patients with Behçet’s disease treated with cyclosporine (n = 11), and healthy volunteers (n = 15). The patients were neither randomly assigned nor masked for this study.

This research study was performed according to the tenets of the Declaration of Helsinki and was approved by the Institutional Ethics Committees of Tokyo Medical and Dental University.

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Supported by the Japan Foundation for Applied Enzymology and Grants-in-Aid for Scientific Research (C) 20592073 and (B) 19390440 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Submitted for publication May 19, 2010; revised July 29, 2010; accepted August 23, 2010.

Disclosure: S. Sugita, None; Y. Yamada, None; S. Kaneko, None; S. Horie, None; M. Mochizuki, None

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Isolation of Purified T Cells

Peripheral blood mononuclear cells were obtained from the patients. Freshly purified T cells were enriched for CD4+ T cells using cell isolation kit (MACS; Miltenyi Biotec, Auburn, CA; >92% of cells expressed the relevant surface marker) and were applied to flow cytometric analysis. Before the assay, we confirmed that the systemic treatment, including infliximab therapy, did not affect the number of T cells in the blood of each patient.

Flow Cytometry

Flow cytometric analysis of CD4+ T cells was performed with fluorescein-isothiocyanate (FITC)-labeled anti-human Foxp3 mAbs (clone PCH101; eBioscience, San Diego, CA). Before staining, fresh CD4+ T cells were incubated with Fc-receptor blocking reagent (Miltenyi Biotec) for 15 minutes at 4°C. After permeabilization, the cells were stained with FITC-labeled anti-human Foxp3 mAbs and phycoerythrin (PE)-conjugated anti-human CD4 antibodies for 30 minutes at 4°C. FITC-conjugated rat IgG2a isotype (eBioscience) was used as the control.

To examine the expression of CD25 on T cells obtained from patients with Behçet’s disease treated with infliximab, the CD4+CD25+ cells were isolated from purified T cells using a human CD4+CD25+ regulatory T-cell isolation kit (MACS; Miltenyi Biotec). These fresh T cells were stained with FITC-conjugated anti-human CD25 (IL-2Rα; eBioscience) and PE-conjugated anti-human CD4 antibodies. Flow cytometry was also used to analyze the expression of glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR; R&D Systems, Minneapolis, MN), CD152 (CTLA-4; eBioscience), and Foxp3 by these CD4+CD25+ cells. As the isotype controls, the cells were also stained with FITC-conjugated rat IgG, FITC-conjugated mouse IgG, or PE-conjugated goat IgG for 30 minutes at 4°C.

The purified CD4+ T cells from patients with uveitis or healthy donors were cocultured with recombinant human IL-2, anti-human CD3, and anti-human CD28 antibodies in the presence (or absence) of infliximab (20 μg/mL) for 24 hours. In companion experiments, these T cells were treated with anti–TGFβ blocking antibody (10 μg/mL, anti–TGFβ1, 2, 3 antibodies, clone 1D11; R&D Systems). Flow cytometric analysis of these cultured T cells was performed with FITC-conjugated anti-Foxp3 and PE-conjugated anti-CD4 mAbs for 30 minutes at 4°C.

In Vitro Assays of Treg Cell Activity

To deplete the CD25+ population (including naturally occurring CD25+ regulatory T cells), purified T cells from uveitis patients with Behçet’s disease or healthy donors were enriched for CD4+CD25+ cells by a human CD4+CD25+ regulatory T-cell isolation kit. The CD4+CD25+ T cells in the presence of anti-CD3 and anti-CD28 antibodies (2 μg/mL, respectively) were exposed to infliximab (20 μg/mL) for 24 hours. The T cells were harvested and added (1 x 10⁷ cells/well) as the Treg cells to 96-well plates containing target syngeneic responder pan-T cells (at 10⁵ cells/well) plus anti-CD3 and anti-CD28 antibodies. After incubation for 72 hours, the uptake of [³H]-thymidine was measured to assess cell proliferation (cpm). The level of TGFβ1 in the supernatants of infliximab-treated CD4+ T cells from patients with Behçet’s disease or healthy donors was measured by ELISA (R&D Systems).

Statistical Analysis

Each experiment was repeated at least twice with similar results. Parametric data were analyzed with the Student’s t-test. Nonparametric data were analyzed with the Mann-Whitney U test. Values were considered statistically significant at P < 0.05.
RESULTS

Detection of Foxp3+ T Cells in Uveitis Patients with Behcet's Disease

First, we determined whether healthy donors and patients with active uveitis (caused by Behcet's disease, Vogt-Koyanagi-Harada disease, or ocular toxoplasmosis) had Foxp3+ Treg cells in CD4+ T cells isolated from the peripheral blood. Figure 1 shows representative flow cytometry results from a healthy donor and from uveitis patients before the administration of systemic therapy. The proportion of Foxp3+ Treg cells in CD4+ T cells was low, ranging from 3% to 5% (Fig. 1), in the patients with uveitis and the healthy donor. The proportion of Foxp3+ Treg cells in the patient with Behcet's disease and active uveitis (Fig. 1B) appeared to be lower than the proportion in the uveitis patient with Vogt-Koyanagi-Harada disease (Fig. 1C), the uveitis patient with ocular toxoplasmosis (Fig. 1D), and the healthy donor (Fig. 1A).

We then compared the proportion of Foxp3+ Treg cells in CD4+ T cells between the remission stage and the active uveitis stage of Behcet’s disease (Fig. 2). Patients who had at least one uveitis attack in the past 3 months were considered to be in the active stage, whereas patients who did not have any uveitis attacks for more than 3 months were considered to be in the remission stage. The proportion of Foxp3+ Treg cells in CD4+ T cells during the active uveitis stage (mean ± SD, 2.3% ± 0.7%) was significantly lower than that at the remission stage (5.0% ± 0.4%; P < 0.05). These results suggest that patients with Behcet’s disease in the active uveitis stage have a decreased number of circulating Treg cells.

We then examined the influences of systemic immunosuppressive treatment on the expression of Foxp3+ Treg cells. The proportions of Foxp3+ Treg cells in CD4+ T cells in patients treated with colchicine (3.5%; Fig. 3A) and cyclosporine (4.1%; Fig. 1A) showed an extremely high proportion of Foxp3+ Treg cells in CD4+ T cells (19.2%; Fig. 3C). This observation was confirmed with data from many more patients. The proportion of Foxp3+ Treg cells in CD4+ T cells in patients with Behcet's disease treated with infliximab (11.8% ± 6.2%) was significantly higher than in patients treated with colchicine (3.6% ± 1.4%; P < 0.005) or cyclosporine (4.7 ± 2.8%; P < 0.005) and healthy donors (3.4% ± 1.6%; P < 0.0005; Fig. 4). These data suggest that infliximab induced Foxp3+ Treg cells in treated patients.
whether the proportion of Foxp3 

pressive agents or healthy donors (Fig. 4). We then asked similar to the levels in patients treated with other immunosup-

number of acute episodes of uveitis (uveitis attacks) and the number of uveitis attacks in patients with Behc¸et's disease 

treated with infliximab. Therefore, we examined the relationship between the proportion of Foxp3 

Treg cells in CD4 

T cells and the following clinical data: the number of acute episodes of uveitis (uveitis attacks) and the duration of infliximab treatment. Eleven patients treated with infliximab (Table 1; patients 1–11) had a high population of Foxp3 

Treg cells (>/9%), and none of these patients experienced any episodes of acute uveitis (Table 1). The duration of infliximab treatment in these 11 patients ranged from 4 months to longer than 2 years.

Next, we determined whether infliximab induces Foxp3 

Treg cells in a patient whose treatment was converted from conventional therapy to infliximab. The proportion of Foxp3 

Treg cells in CD4 

T cells during cyclosporine treatment (3.8%) was increased to 21.2% after the treatment was switched to infliximab (Fig. 5A). The same results were obtained from four other patients for whom the Foxp3 

Treg cells in CD4 

T cells were measured during cyclosporine treatment and then after therapy was converted to infliximab (Fig. 5B).

A few patients with Behc¸et's disease treated with infliximab had a low proportion of Foxp3 

Treg cells in CD4 

T cells, similar to the levels in patients treated with other immunosuppressive agents or healthy donors (Fig. 4). We then asked whether the proportion of Foxp3 

Treg cells in CD4 

T cells might be related to the clinical effectiveness of infliximab on uveitis. Therefore, we examined the relationship between the proportion of Foxp3 

Treg cells in CD4 

T cells and the number of uveitis attacks in patients with Behc¸et's disease treated with infliximab.

Association of Foxp3 Population in CD4 

T Cells with Acute Episodes of Uveitis in Patients with Behc¸et's Disease

For 16 patients with Behc¸et's disease who were treated with infliximab, we examined the correlation between the Foxp3 

Treg cells in CD4 

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All five patients who had a low proportion (<8%) of Foxp3 

Treg cells in CD4 

T cells (Table 1, patients 11–16) experienced acute uveitis attacks during infliximab therapy. The mean proportion of Foxp3 

Treg cells in CD4 

T cells in these five patients was significantly lower than that in the 11 patients who did not experience any uveitis attacks (4.5% ± 2.1% vs. 15.5% ± 4.5%; P < 0.005). Activity scores (uveitis attacks) before infliximab treatment, length of disease, and previous systemic immunosuppressive therapy were not associated with the Foxp3 population in CD4 

T cells (Table 1).

Detection of CD25 

Treg Cells in Uveitis Patients with Behc¸et's Disease after Infliximab Treatment

For the experiments, we used freshly isolated CD4 

CD25 

T cells obtained from uveitis patients with Behc¸et's disease who were treated with infliximab. Then we examined whether these CD4 

CD25 

T cells express Foxp3, GITR, and cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4/CD152). As revealed in Figure 6, fresh CD4 

T cells from patients with infliximab treatment contained high levels of CD25, Foxp3, GITR, and CD152 compared with cells from healthy donors. These results suggest that CD25 

Foxp3 

Treg cells in infliximab-treated patients may circulate to provide protection from severe inflammation in Behc¸et's disease.

Induction of Regulatory T Cells in the Presence of Infliximab In Vitro from Uveitis Patients with Behc¸et's Disease

We next examined whether patients with Behc¸et's disease with active uveitis had Foxp3 

Treg cells and whether the Foxp3 

Treg cells within the CD4 

T population were increased if the T cells were pretreated with infliximab in vitro.
Representative results (Fig. 7A) show that the patient with uveitis had Foxp3$^+$/H11001 T cells, but the proportion of Foxp3$^+$ cells was low (only 1.7% positive in CD4$^+$/H11001 T cells). However, the Foxp3$^+$ population was increased if the T cells were treated with infliximab (14.5% CD4/Foxp3 double positive). Importantly, the Foxp3$^+$ population was decreased when the T cells were treated with infliximab plus anti–TGF$\beta$/H9252 blocking antibodies (4.1% positive; Fig. 7A, upper right histogram). On the other hand, in the case of a healthy donor (Fig. 7A, lower histogram), the positive population was not changed even if the T cells were treated with infliximab (1.4%–3.0% positive).

We also examined whether T cells from patients with Behcet’s disease could produce TGF$\beta$ when treated with infliximab in vitro. The supernatants of T cells in the presence of infliximab contained significantly greater amounts of TGF$\beta$ than the supernatants of T cells without infliximab (Fig. 7B). The supernatants of infliximab-treated T cells from healthy donors contained much greater amounts than those of non-treated T cells, but there was no statistically significant difference between the two groups (Fig. 7B).

To examine the extent to which infliximab-treated T cells could display a regulatory phenotype, we measured the proliferation of T cells that were activated in vitro in the presence of the induced Treg cells from patients with Behcet’s disease with active uveitis. When CD4$^+$/CD25$^+$ T cells were used as the target cells, infliximab-induced Treg cells from a healthy donor did not suppress the activation of the bystander T cells (Fig. 7C). These results suggest that T cells exposed to infliximab are able to acquire Treg function in Behcet’s disease.

**DISCUSSION**

Here we showed that a low level (3%–5%) of Foxp3$^+$/CD4$^+$ Treg cells was present in the peripheral blood of healthy donors and patients with various clinical entities of uveitis, including Behcet’s disease. Infliximab, but not colchicine or cyclosporine, significantly increased the proportion of Foxp3$^+$ Treg cells in CD4$^+$ T cells in patients with Behcet’s disease. Moreover, the proportion of Foxp3$^+$ Treg cells in CD4$^+$ T cells was significantly associated with the effects of infliximab on uveitis attacks.
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FIGURE 6. Detection of CD25, Foxp3, GITR, and CD152 in CD4<sup>+</sup> T cells from uveitis patients after infliximab treatment. Flow cytometry was used to analyze the expression of CD25, Foxp3, GITR, and CD152 (CTLA-4) on T cells. Fresh CD4<sup>+</sup> T cells from infliximab-treated patients (n = 2) or healthy donors (n = 2) were stained with FITC-labeled anti–CD25 and PE-labeled anti–CD4 antibodies, FITC-labeled anti–Foxp3 and PE-labeled anti–CD4 antibodies, FITC-labeled anti–GITR and PE-labeled anti–CD4 antibodies, and FITC-labeled anti–CD152 antibodies. Before staining, harvested T cells were blocked with human Fc block materials and then permeabilized. FITC-labeled rat IgG, FITC-labeled mouse IgG, or PE-labeled goat IgG was used as an isotype control. The assay was reproducible, and representative results are shown. Gates are based on each isotype control. Numbers in the histograms indicate the percentages of double-positive cells.
acquire the regulatory phenotype depends on the production of TGFβ. TGFβ is a powerful suppressive mediator of activated responder T cells that produce inflammatory cytokines such as TNF-α. Moreover, TGFβ promotes the induction of Foxp3+ Treg cells. Thus, the presence of TGFβ and the blockade of TNF-α contribute to the generation of Treg cells. In fact, CD4+ T cells...
exposed to infliximab in vitro are converted to Treg cells that express Foxp3 and can acquire regulatory functions (Fig. 7). In addition, infliximab-induced Treg cells that express Foxp3 may increase through TGFβ if the T cells are exposed to infliximab. These results indicate that the infliximab-induced Treg cells express Foxp3 through the TGFβ signal. TGFβ is the most important factor for the induction of Treg cells in the periphery.28,29

Monoclonal antibody therapy against TNF-α provides a new approach to the treatment of refractory uveitis, such as Behcet’s disease. Greiner et al.30 recently showed that anti-TNF-α therapy (a recombinant protein generated by fusing the p55 TNF-α receptor with human IgG1 [TNF-Ig]) can modulate the phenotype of peripheral blood CD4+ T cells in patients with posterior segment intraocular inflammation. Neutralizing TNF-α activity in patients with uveitis increased the fraction of IL-10-producing Treg cells. Thus, an alternative function of anti-TNF-α therapy may be the induction of a distinct regulatory T-cell population.

In conclusion, infliximab has the capacity to induce Treg cells in patients with Behcet’s disease, and the induction of Treg cells contributes to the clinical efficacy of infliximab in suppressing refractory uveitis. However, the patient groups in this study underwent phenotyping for nonequivalence in activity scores of the disease, length of the disease, and length of previous immunosuppressive treatment. If we can reproduce our findings in patient groups with equivalent phenotypes or collect data from a new cohort (fresh cases), we can conclude that an increased percentage of Treg cells within the CD4 population after infliximab therapy can act as a biomarker for the therapy.

Acknowledgments

The authors thank Ikuyo Yamamoto and Yuko Kawazoe for providing technical assistance and the doctors of the Uveitis Group at Tokyo Medical and Dental University Hospital for collecting samples.

References
