Infliximab Reduces the Frequency of Interleukin 17–Producing Cells and the Amounts of Interleukin 17 in Patients With Rheumatoid Arthritis

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Background: To detect frequency changes in interleukin 17 (IL-17)$^+$ CD4$^+$ T cells and the amounts of IL-17 in supernatants between baseline and 30 weeks after Infliximab combined with methotrexate (MTX) or MTX-alone therapy.

Methods: Flow cytometry was used to analyze the frequency of IL-17$^+$ CD4$^+$ T cells in rheumatoid arthritis (RA) patients and control subjects at baseline and 30 weeks after therapy. Secretion of IL-17 by peripheral blood mononuclear cells was measured by enzyme-linked immunosorbent assay.

Results: The percentages of IL-17$^+$ CD4$^+$T cells were increased in the peripheral blood mononuclear cells of patients with RA compared with healthy subjects. The percentages of IL-17$^+$ CD4$^+$T cells were correlated with the number of swelling joints and C-reactive protein of RA patients. Likewise, concentrations of IL-17 in supernatants from patients with RA were significantly higher compared with those from control subjects. After infliximab combined with MTX or MTX-alone therapy, the number of swelling joints, erythrocyte sedimentation rate, C-reactive protein, rheumatoid factor, and Disease Activity Score 28 decreased significantly compared with baseline. Only in the infliximab + MTX group that the frequency of T_H17 cells and concentration of IL-17 decreased.

Conclusions: These data support the hypothesis that infliximab therapy can have an effect on T_H17 cells and decrease disease activity.

Key Words: infliximab, rheumatoid arthritis, interleukin 17

(*J Investig Med* 2010;58: 905–908)

CD4$^+$ effector T cells were first subdivided into 2 groups, T_H1 and T_H2 cells, and autoimmune diseases, particularly arthritis, were believed to be mainly driven by T_H1 cells.[1] However, there were always some inconsistencies with this notion, and in recent years, additional effector T-cell subsets have been described, including interleukin 17 (IL-17)–producing cells (T_H17 cells).[2,3] The latter differentiate from naive cells in response to transforming growth factor β, acting with proinflammatory cytokines, particularly IL-6, but with additional roles for IL-21 and IL-1.[4] In addition, IL-23 promotes the expansion and survival of T_H17 cells.[5]

These ideas are well established in murine studies, and T_H17 cells play critical roles in a number of animal models of autoimmunity, including murine arthritis models.[6] Rheumatoid arthritis (RA) is one of the forms of arthritis in which elevated serum levels of IL-17 have been reported,[7] and there was a relationship between IL-17 and IL-23 p19.[8] We previously demonstrated that peripheral blood mononuclear cells (PBMCs) from patients with RA showed increased numbers of IL-17$^+$ CD4$^+$T cells compared with control subjects.[9] These data suggest a role of IL-17–secreting CD4$^+$T cells in RA.

Anti–tumor necrosis factor α (anti–TNF-α) antibody, infliximab, showed a highly successful effect on RA treatment. It can decrease levels of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), Disease Activity Scores of 28 joints (DAS28), and so on.[10] Although the effect of infliximab is well known, the biological mechanism by which the clinical effect is obtained is not very clear. Previous study indicated that infliximab reduced the serum levels of cytokines, chemokines, and adhesion molecules, such as IL-6, IL-8, monocyte chemotactic protein 1, IL-18, and matrix metalloproteinases 1, 3, and 9 in RA patients.[11] Kageyama et al.[10] reported that the serum levels of IL-23 in RA patients at posttreatment with infliximab decreased.

Interleukin 17 has recently been considered to be a cytokine that plays an essential role in the disease process of RA and related with IL-23.[12] Therefore, in this study, we investigated whether infliximab and methotrexate (MTX), respectively, affect the T_H17 cells in RA patients.

MATERIALS AND METHODS

Patients

Twenty-five RA patients received infliximab combined with MTX (infliximab + MTX group; 23 women and 2 men; mean age, 46.4 [SD, 8.3] years; mean disease duration, 8.6 [SD, 6.3] years). Infliximab was injected intravenously at a dose of 3 mg/kg at baseline, then at 2 and 6 weeks, and every 8 weeks thereafter. At the same time, MTX was also administered at a constant dosage (10–15 mg/wk). Blood analyses were performed just before infusion of infliximab at baseline and at 30 weeks after the initial treatment with infliximab.

Twenty RA patients received MTX alone (MTX group; 17 women and 3 men; mean age, 49.0 [SD, 11.2] years; mean disease duration, 8.9 [SD, 9.6] years). The control group consisted of 16 women and 4 men (mean age, 45.0 [SD, 8.9] years).

To evaluate the disease activity of RA in the infliximab + MTX and MTX groups, we measured the ESR and CRP (Bio-Vendor, Minneapolis, MN) and counted the number of swollen joints and tender joints. The DAS28 was also evaluated. Serum rheumatoid factor (RF) levels were also measured by enzyme-linked immunosorbent assay. Clinical and laboratory examinations were performed at baseline and at 30 weeks after the initial treatment with infliximab combined with MTX or with MTX alone. Peripheral blood samples were obtained from all patients. All the patients met the American College of Rheumatology 1987 revised criteria for the classification of RA. Informed consent was given by all patients.

Preparation and Stimulation of PBMCs

Peripheral blood mononuclear cells were purified from peripheral blood by centrifugation, using a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech, Little Chalfont, UK).

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Received March 22, 2010, and in revised form May 17, 2010.
Accepted for publication June 5, 2010.
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Supported by higher education department of Liaoning province research grant number: L2010604, L2010613, and L20060997.

ISSN: 1081-5589
DOI: 10.231/JIM.0b013e3181eb9895

Peripheral blood mononuclear cells were adjusted to a final concentration of 10^6/mL in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% glutamine/penicillin/streptomycin, and 2% HEPES.

For analysis of surface phenotype and intracellular cytokine staining, PBMCs were seeded into 24-well culture plates (Nunc, Naperville, IL) at 2×10^6 cells per well and stimulated ex vivo with phorbol myristate acetate (50 ng/mL; Sigma, St. Louis, MO) and calcium ionomycin (1 kg/mL; Sigma) for 5 hours. GolgiStop (Becton Dickinson, Mountain View, CA) was added at the beginning of the stimulation.

For the measurement of cytokine secretion, PBMCs were seeded into the wells of 96-well culture plates (Nunc) at 10^5/200 μL per well in triplicate and stimulated with beads coated with antibodies to CD3/CD28 (10^5 beads/well; Miltenyi Biotech, Sunnyvale, CA). After incubation for 4 days, cell-free supernatants were collected, and the concentrations of IL-17 in supernatants were assessed using an enzyme-linked immunosorbent assay kit, according to the manufacturer's instructions (eBioscience, San Diego, CA). The samples were diluted before assaying. The detection limit for IL-17 was 4 pg/mL.

Flow Cytometry

Cytometry was used to analyze the surface CD4 and intracellular IL-17 production by PBMCs. For intracellular staining, cells were first stained with antibodies against surface antigens and then fixed and permeabilized using Perm/Fix solution (Becton Dickinson) at room temperature for 20 minutes. Cells were washed with Perm/Wash buffer (Becton Dickinson) and stained with antibodies directed against intracellular cytokines. Live $CD4^+$ T cells were gated, and the percentages of these cells producing IL-17 were detected.

The antibodies used were as follows: allophycocyanin-labeled anti-CD4 (Becton Dickinson) and fluorescein isothiocyanate–labeled anti–IL-17 (eBioscience). Appropriately conjugated IgG antibodies were used as isotype controls.

Statistical Analysis

All data are presented as the mean (SD). The differences in frequency of IL-17^+ $CD4^+$ T cells between patients with RA and control subjects were analyzed by unpaired Student *t* test. The differences in frequency of IL-17^+ $CD4^+$ T cells between baseline and 30 weeks of treatment and IL-17 release by the in vitro–stimulated PBMCs were analyzed by paired *t* test. Spearman rank correlation coefficient was used to test the correlations between the percentages of IL-17^+ $CD4^+$ T cells and the clinical index. All analyses were performed using SPSS17 software (Chicago, IL) and GraphPad Prism 5 software (San Diego, CA).

RESULTS

Increased Frequency of IL-17^+ $CD4^+$ T Cells in PBMCs From Patients With RA

Using flow cytometry, we evaluated the intracellular expression of the cytokines IL-17 by PBMCs from patients with RA and from control subjects, after stimulation with phorbol myristate acetate and ionomycin. We observed a higher proportion of IL-17–producing T cells within the PBMCs of patients with RA (infliximab + MTX group and MTX-alone group) compared with control subjects (1.09% and 0.99% vs 0.56%; $P < 0.01$ for both comparisons) (Fig. 1A). In both groups, there were statistically positive correlations between the frequencies of T_H17 cells and both the CRP level and the swollen joint count ($r = 0.50$, $P < 0.01$; $r = 0.47$, $P < 0.01$, respectively). In contrast, there was no correlation between the frequencies of T_H17 cells and other clinical parameters, including the ESR, RF, and DAS28.

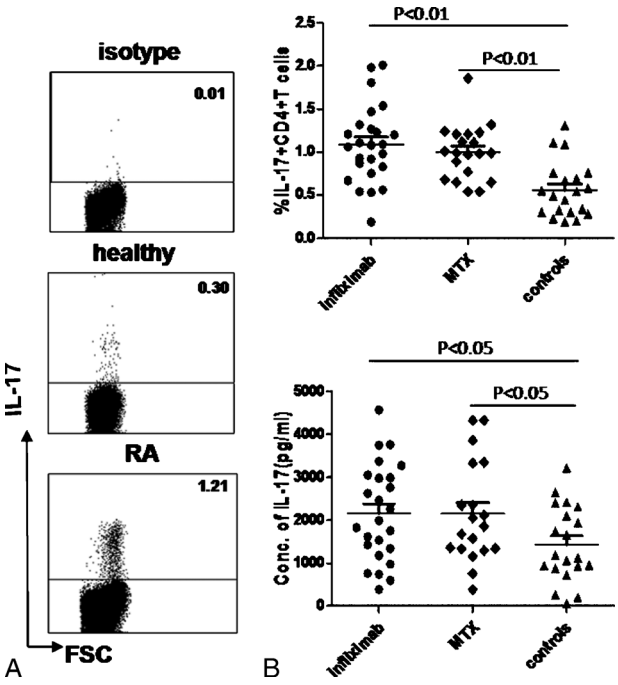

FIGURE 1. A, Frequency of IL-17–positive $CD4^+$T cells in patients with RA and control subjects. B, Concentration of IL-17 in culture supernatants of PBMCs from patients with RA and control subjects at 4 days after stimulation with anti-CD3/CD28.

Increased Secretion of IL-17 by PBMCs From Patients With RA

After stimulation with microbeads coated with anti-CD3 and anti-CD28, the amounts of IL-17 in supernatants from the PBMCs of patients with RA (2160.01 [SD, 1127.42] pg/mL, 2157.84 [SD, 1138.94], respectively) were significantly higher than those in PBMCs from control subjects (1435.94 [SD, 883.71], $P < 0.05$ for both comparisons) (Fig. 1B).

Clinical and Laboratory Disease Activity of RA Patients Decreased After Treatment

In the infliximab + MTX group, CRP levels decreased from 2.69 mg/dL at pretreatment to 1.47 mg/dL ($P < 0.01$) at 30 weeks after the initial infusion. Erythrocyte sedimentation rate levels also decreased, from 53.6 mm/h at pretreatment to 37.8 mm/h ($P < 0.01$) at 30 weeks, after the initial infusion. Serum RF levels, DAS28 score, and the number of swollen joints also significantly decreased at 30 weeks compared with those at pretreatment (Table 1).

In the MTX group, CRP, ESR, DAS28, and the number of swollen joints were significantly decreased at 30 weeks after the initial administration of MTX ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.05$, respectively),whereas RF levels were decreased but have no statistical significance($P > 0.05$).

Frequency of IL-17^+ $CD4^+$T Cells and Concentration of IL-17 in the Supernatants of PBMCs From RA Patients Decreased at Posttreatment in the Infliximab Group But Not in the MTX Group

In the infliximab + MTX group, frequencies of IL-17^+ $CD4^+$T cells were significantly decreased from 1.09% at pretreatment to 0.89% ($P < 0.05$) at 30 weeks after the initial infusion (Fig. 2A). After stimulation with microbeads coated with

TABLE 1. Changes in Clinical and Laboratory Parameters in Patients With RA Treated With Infliximab Combined With MTX and MTX Alone

	Infliximab + MTX Group		MTX Group		
	Baseline	**30 Wk**	**Baseline**	**30 Wk**	**Control Group**
Age, y	46.4 (8.3)		49.0 (11.2)		45.0 (8.9)
Sex, no. male/no. female)	2/23		3/17		4/16
Duration of disease, y	8.6 (6.3)		8.9 (9.6)		
Other medications					
NSAID	3/25		4/20		
ESR, mm/h	53.6 (27.53)	37.8 (14.87)*	67.15 (35.76)	45. 75 (23.92)†	ND
CRP, mg/dL	2.69 (2)	1.47 (0.87)*	3.11 (2.23)	1.73 (1.1)†	ND
DAS28	3.76 (2.01)	2.57 (3.26)*	4.21 (3.15)	3.14 (2.98)*	ND
RF	388.68 (471.68)	197.92 (244.38)†	412.65 (596.74)	193 (272.86)	ND
SJC	5.24 (3.83)	2.4 (2.76)*	4.65 (3.6)	2.5 (2.62)†	ND
% T_H17 cells	1.09 (0.45)‡	0.89 (0.34)†	0.99 (0.32)‡	0.92 (0.23)	0.56 (0.32)
Concentration of IL-17, pg/mL	2160.01 (1127.42)§	1541.30 (1148.17)†	2157.84 (1138.94)§	1740.77 (1208.92	1435.94 (883.71)

Values are mean (SD) unless stated otherwise.

*$P < 0.01$ compared with baseline.

†$P < 0.05$ compared with baseline.

‡$P < 0.01$ compared with control subjects.

§$P < 0.05$ compared with control subjects.

SJC indicates swollen joint count; ND, not detected; NSAID, nonsteroidal anti-inflammatory drugs.

anti-CD3 and anti-CD28, the amounts of IL-17 in supernatants from the PBMCs of patients with RA were significantly decreased from 2160.01 to 1541.3 pg/mL ($P < 0.05$) (Fig. 2B). In the MTX group, frequency of IL-17$^+$ CD4$^+$T cell levels and concentration of IL-17 did not show a significant change at 30 weeks after the initial treatment compared with levels at pretreatment (Table 1) ($P > 0.05$, $P > 0.05$, respectively).

DISCUSSION

Many proinflammatory cytokines and chemokines can contribute to the pathogenesis of inflammatory arthritis. In recent years, the idea that arthritis is predominantly driven by the T_H1 subset of T cells, producing interferon γ, has been questioned. In models of autoimmunity such as experimental allergic encephalomyelitis and collagen-induced arthritis, an inability to make or respond to interferon γ resulted in more severe disease rather than abolishing it.[13,14] These findings have led to the discovery of a new T-cell subset, T_H17 cells, making their signature cytokine, IL-17, and it is evident that in many models of autoimmunity, this subset is often the principal driver of inflammation and autoimmune diseases, including arthritis.[15]

Interleukin 17 has previously been demonstrated in the synovial fluid of patients with RA.[16] In addition, IL-17 has been reported to induce human synoviocytes to produce IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor, and it induces osteoclastogenesis and leads to bone resorption.[17]

In this study, we examined the frequencies and characteristics of T_H17 cells in the peripheral blood of patients with RA and compared the results with those of control subjects. We demonstrated that the numbers of T cells that produce IL-17 are significantly increased in the peripheral blood of patients with RA. In accordance with this, increased concentrations of IL-17 were seen in the supernatants of their PBMCs after stimulation with anti-CD3/CD28. These observations are compatible with the idea that IL-17–producing cells contribute to the pathogenesis of inflammatory arthritis and seem to apply to RA. Levels in RA correlated strongly with disease activity as assessed by the swollen joint count or the CRP concentration. This suggests that IL-17 may play some role in the inflammatory processes of RA.

Many studies showed that TNF-blocking treatment gets good results in patients with RA and ankylosing spondylitis.[10,18] In our study, both infliximab + MTX and MTX-alone therapy can suppressed disease activity, including the levels of ESR, CRP, RF, DAS28, and the number of swelling joints. This may be more significant in the infliximab + MTX group. In MTX group, the levels of RF decreased after treatment but had no statistical significance. In the infliximab + MTX group, the changes in RF and DAS28 were much more significant than in the MTX-alone group. More interestingly, in 8 patients who had a good response to treatment with infliximab and MTX, clinical parameters at baseline were significantly higher than in other patients. Those

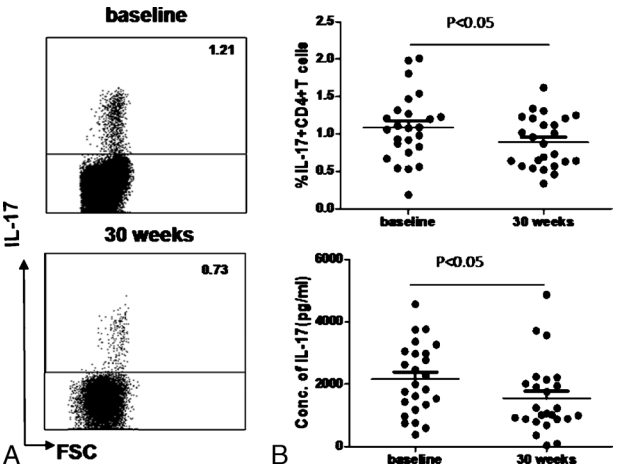

FIGURE 2. Frequencies of IL-17$^+$ CD4$^+$T cells (A) and concentrations of IL-17 in the supernatants ex vivo (B) in RA patients at baseline and 30 weeks after infliximab infusion.

patients who had low disease activities at baseline had mildly improved in symptoms and clinical parameters. This indicated that infliximab may be much more effective in highly active RA patients than in stable patients.

The use of TNF-α inhibitors can affect the production of a variety of cytokines, chemokines, and proteases that may exist as a downstream of a TNF-α cascade in RA patients, such as serum levels of IL-6, IL-23, and IL-15.[10,19] The levels of IL-8, monocyte chemotactic protein 1, IL-18, and matrix metalloproteinases 1, 3, and 9 decreased after infliximab infusion.[18]

Do TNF-α blockings have an effect on T_H17 cells in RA patients? Some studies showed that TNF-α can inhibit phosphate of FoxP3, decrease the function of regulatory T cells, and promote T_H17 cell differentiation. Furthermore, TNF-α can activate IL-6 and promote differentiation of T_H17 cells (reports on the first International RA Forum in Beijing). Blockage of TNF prevents intestinal mucosal inflammation through downregulation of IL-17 and IL-23 p19.[20] All these showed that blocking TNF-α downregulates differentiation of T_H17 cells and the amounts of IL-17.

To analyze the in vivo mechanism of the clinical efficacy of infliximab, we measured the frequency of IL-17$^+$ CD4$^+$T cells and concentration of IL-17 from PBMCs in RA patients at pretreatment and posttreatment of infliximab combined with MTX and of MTX alone. Frequency of IL-17$^+$ CD4$^+$T cells and concentration of IL-17 were significantly decreased by treatment with infliximab combined with MTX but not by that with MTX alone. This indicates the novel function of infliximab and the possibility that the cascade by which anti–TNF-α decreases IL-17 production actually occurs in vivo.

Kageyama et al.[10] showed that infliximab reduces the serum levels of IL-23 but has no effect on IL-17 levels. They detected the serum/plasma levels of IL-17 at baseline and after treatment. What we detected were the changes in frequency of IL-17$^+$ cells and the levels of IL-17 in supernatants ex vivo. First, we also detected the serum levels of IL-17 in patients. We found that most of the serum levels of IL-17 in RA patients and control subjects were undetectable. So it is very difficult to do the statistic analysis.

Kohno et al.[21] compared the gene expression levels of IL-17 in PBMC samples at baseline and 2 weeks after the first infusion of infliximab. They showed that the gene expression levels of IL-17 decreased but not significantly. This may be due to the short-time use of infliximab (only 2 weeks). In our study, we detected the changes in T_H17 cells and IL-17 production after 30 weeks' infusion of infliximab. Thus, we showed the significant decrease in T_H17 cells and IL-17 production ex vivo.

In the study of Yue et al.,[19] they showed that peripheral T_H17 cells in RA patients decreased after adalimumab/MTX treatment, and they got the conclusion that adalimumab and MTX treatment downregulates peripheral T_H17 cells in RA patients. In fact, our findings are very similar with that.

In our study, infliximab can reduce the frequency of IL-17$^+$ CD4$^+$T cells and the amounts of IL-17 in RA patients, and this is considered to be an important action of infliximab in RA treatment. Further study of direct blocking of IL-17 in experimental arthritis or in RA patients is important for the development of new treatments for RA.

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