Original Contribution Kitasato Med J 2013; 43: 126-132

Differential in vitro effects of biological agents on cytokine production of peripheral blood mononuclear cells

Satoko Tejima, Toshihiro Tono, Tatsuo Nagai, Shunsei Hirohata

Department of Rheumatology and Infectious Diseases, Kitasato University School of Medicine

Objectives: Our objectives were to determine the effects of etanercept, infliximab, and tocilizumab on interleukin-17 (IL-17) production of peripheral blood mononuclear cells (PBMCs).
Methods: PBMCs from healthy donors were cultured in the presence of staphylococcal enterotoxin B (SEB) with pharmacologically attainable concentrations of biological agents or control IgG (immunoglobulin G). The concentrations of IL-17 in the culture supernatants were measured using ELISA (enzyme-linked immunosorbent assay). The expression of HLA-DR on monocytes were measured by flow cytometry.
Results: Etanercept, infliximab, but not tocilizumab, suppressed IL-17 production of PBMCs activated with staphylococcal enterotoxin B (SEB). Etanercept and infliximab did not influence IL-17 production of CD4+ T cells activated with immobilized anti-CD3 in the complete absence of monocytes. Etanercept and infliximab, but not tocilizumab, significantly suppressed the expression of HLA-DR on SEB-stimulated monocytes.
Conclusions: The results indicate that anti-TNFα agents, but not anti-IL-6 receptor antibody, suppress IL-17 production of SEB-stimulated PBMCs by inhibiting the expression of HLA-DR on monocytes.

Key words: etanercept, infliximab, tocilizumab, interleukin-17, peripheral blood mononuclear cells, rheumatoid arthritis

Introduction

Although the etiology of rheumatoid arthritis (RA) is unknown, increasing attention has been paid to the role of proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) in the pathogenesis.[1,2] In fact, the agents inhibiting these cytokines are effective in the treatment of RA.[3-5] Thus, etanercept, TNF-receptor-IgG Fc fusion protein, and infliximab, anti-TNFα monoclonal antibody suppress the inflammation by inhibition of TNFα, whereas tocilizumab is an anti-IL-6 receptor antibody, which modulates the inflammation by inhibition of binding between IL-6 and IL-6 receptor. Although several studies have disclosed the *in vitro* effects of these biological agents on the immune competent cells,[6,7] the precise mechanisms in RA remain unclear.

On the other hand, it has been recently shown that IL-17 plays an important role in the pathogenesis of RA. Therefore, IL-17 levels in synovial fluids are significantly higher in RA patients than those in osteoarthritis patients.[8] Moreover, IL-17 gene expression in peripheral blood mononuclear cells (PBMCs) of RA patients has been found to be upregulated.[9] It has also been demonstrated that IL-17 stimulates the production and expression of proinflammatory cytokines, IL-1β and TNFα, in human macrophages.[10] It should be noted that IL-6 in addition to transforming growth factor-β (TGFβ) is required for the differentiation of Th17 cells.[11,12] Consistently, IL-6 blockade suppresses collagen-induced arthritis by inhibition of inflammatory Th17 responses.[13] It is, therefore, suggested that tocilizumab has a therapeutic effect on RA by inhibition of IL-17 production. However, the effect of tocilizumab on IL-17 production in humans has not been explored. The current study was undertaken to investigate the effects of tocilizumab as well as etanercept and infliximab on IL-17 production by human PBMCs.

Materials and Methods

A variety of monoclonal antibodies (mAbs) were used in

Received 26 December 2012, accepted 8 January 2013
Correspondence to: Satoko Tejima, Department of Rheumatology and Infectious Diseases, Kitasato University School of Medicine
1-15-1 Kitasato, Minamiku, Sagamihara, Kanagawa 252-0374, Japan.
E-mail: saihara@med.kitasato-u.ac.jp

this study, including fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR (Immunotech), FITC-conjugated control mouse IgG1 (Dako, Glostrup, Denmark), and 64.1 (an IgG2a mAb directed at the CD3 molecule on mature T cells), a gift from Dr. Peter E. Lipsky of the National Institute of Health, Bethesda, MD, USA.

PBMCs were obtained from healthy adult volunteers with informed consent by centrifugation of heparinized venous blood over sodium diatrizorate-Ficoll gradients. Purified CD4+ T cells were further prepared from PBMCs, which had been treated with L-leucine methyl ester to remove monocytes, by positive selection with anti-CD4 microbeads and MACS (Miltenyi Biotec, Auburn, CA, USA). The CD4+ T cell populations obtained in this manner contained <0.1% CD14+ cells, <0.1% CD19+ cells, and >95% CD4+ T cells. The monocytes were prepared from PBMCs using the Monocyte Isolation Kit II (Miltenyi Biotec). The monocyte populations obtained in this manner contained <0.1% CD3+ cells, <0.1% CD19+ cells, and >93% CD14+ cells.

The reagents infliximab, etanercept, and tocilizumab were purchaced from Mitsubishi Tanabe Pharma (Tokyo), Takeda Pharmaceutical (Tokyo), and Chugai Pharmaceutical (Tokyo), respectively. Control human IgG1 was purified from human IgG1 myeloma serum using DEAE (diethylaminoethanol)-Sepharose column.

Cell cultures

Roswell Park Memorial Institute (RPMI) 1640 medium (Nikken, Kyoto) supplemented with penicillin G (100 U/ml) (Life technologies, Grand Island, NY, USA), streptomycin (100 μg/ml) (Life technologies), L-glutamine (0.3 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (JRH Bio-Sciences, Lenexa, KS, USA) were used for all the cultures.

PBMCs (2.5 $\times$ 10^5/well) were cultured in the presence of staphylococcal enterotoxin B (SEB) (Serva, Heidelberg, Germany) (100 pg/ml) in 96-well U-bottomed microtiter plates (Nunc, Roskilde, Denmark) with control human IgG (10 μg/ml), tocilizmab (10 μg/ml), etanercept (10 μg/ml) or infliximab (10 μg/ml) for 5 days. Recombinant TNFα (10 ng/ml) (PeproTech, Rocky Hill, NY, USA), recombinant IL-6 (10 ng/ml) (a gift from Drs. Toshio Hirano and Tadamitsu Kishimoto, Osaka University Medical School, Osaka) and TGFβ (10 ng/ml) (PeproTech) were added in some experiments.

Anti-CD3 mAb 64.1 was diluted in RPMI 1640 medium (2 μg/ml), and 50 μl were placed in each well of 96-well flat-bottomed microtiter plates (Nunc) and incubated at room temperature for 2 hours, as previously described.[14,15] The wells were then washed once with culture medium to remove non-adherent mAb before the cells were added. Purified CD4+ T cells (1 $\times$ 10^5/well) were cultured in wells with immobilized anti-CD3 with control human IgG (10 μg /ml), tocilizumab (10 μg/ml), etanercept (10 μg/ml) or infliximab (10 μg/ml) for 5 days.

Purified monocytes (1 $\times$ 10^6/well) were cultured in the presence of SEB (100 pg/ml) in each well of the 24-well flat-bottomed microtiter plates (Nunc) with control IgG (10 μg/ml), tocilizumab (10 μg/ml), etanercept (10 μg/ml) or infliximab (10 μg/ml) for 2 days.

After PBMCs and purified CD4+ T cells were cultured for 5 days, cell proliferation was analyzed using Cell Quanti-MTT™ Cell Viability Assay Kits (BioAssay Systems, Hayward, CA, USA), according to the manufacturer's instructions. The optical density value of each wells was measured at 570 nm by a 2 wave-length microplate reader, MTP-450 (Corona Electric, Ibaraki).

The surface antigens on monocytes and the induction of apoptosis were analysed by flow cytometry. Briefly, after incubation, the cells were washed once by phosphate buffered saline (PBS) containing 2% normal human serum and 0.1% sodium azide (staining buffer). The cells were then reacted in suspension by incubating for 30 minutes at 4℃ with saturating concentrations of FITC-conjugated anti-HLA-DR or control mAbs. After the cells were washed once with staining buffer and then twice with PBS, they were counterstained with PE (phycoerythrin)-conjugated Annexin V (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The cells were then analyzed using Cell Lab Quanta SC (Beckman Coulter, Miami, FL, USA). To identify viable cells, the gating for the staining with Annexin V was used.

The concentrations of IL-17 and interferon-γ (IFNγ) in the supernatants were measured using a Human IL-17A ELISA Development Kit, and a human IFNγ ELISA Development Kit, respectively (Peprotech).

Statistical significance was evaluated by the Wilcoxon signed-rank test.

Results

Initial experiments examined the effects of various concentrations of each biological agent on IL-17 production of PBMCs activated with SEB. As shown in Figure 1A, etanercept and infliximab, but not tocilizumab, suppressed IL-17 production of PBMCs in a dose-response manner. The inhibitory effects of etanercept and infliximab were marked at 10 μg/ml, which was found

A

IgG
TCZ
ETN
IFX

IL-17 (pg/ml)
Drug concentration (μg/ml)

B

PBMC (without additional cytokines)

P = 0.0001
P = 0.0001
P = 1.0000
IL-17 (pg/ml)

P = 0.0001
P = 0.0001
P = 0.8552
IFNγ (pg/ml)

IgG TCZ ETN IFX

PBMC (with additional cytokines)

P = 0.0156
P = 0.0156
P = 0.2188
IL-17 (pg/ml)

P = 0.0156
P = 0.0156
P = 0.5781
IFNγ (pg/ml)

IgG TCZ ETN IFX

Figure 1. Effects of biological agents on the production of IL-17 and IFNγ of PBMCs. **A**. PBMCs from a healthy individual were cultured in the presence of SEB (100 pg/ml) with various concentrations of etanercept, infliximab, tocilizumab, or control IgG. After 120 hours of incubation, the supernatants were assayed for IL-17 and IFNγ. The data are representative of 7 different experiments. **B**. PBMCs from 14 healthy individuals were cultured in the presence of SEB (100 pg/ml) with etanercept (10 μg/ml), infliximab (10 μg/ml), tocilizumab (10 μg/ml) or control IgG (10 μg/ml). Recombinant TNFα (10 ng/ml), recombinant IL-6 (10 ng/ml) and recombinant TGF β were added in 7 individuals as indicated. After 120 hours of incubation, the supernatants were assayed for IL-17 and IFNγ. Statistical significance was evaluated by the Wilcoxon signed-rank test.

CD4+ T cells

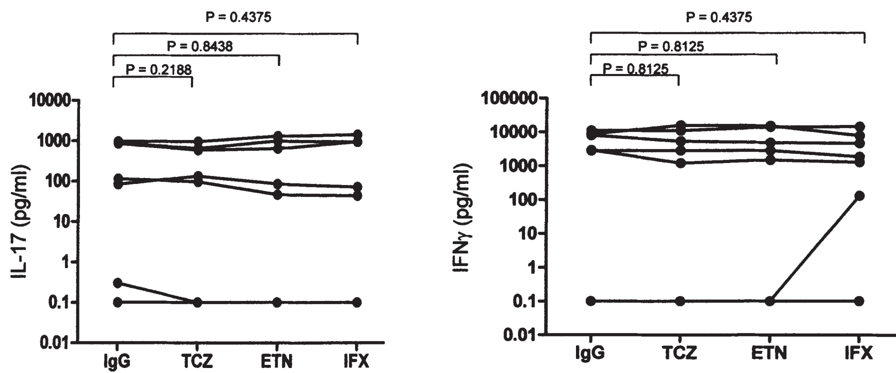

Figure 2. Effects of biological agents on the production of IL-17 and IFNγ production of immobilized anti-CD3-stimulated CD4+ T cells. Purified CD4+ T cells (1 $\times$ 10^5/well) from 7 healthy individuals were cultured in the presence of immobilized anti-CD3 with etanercept (10 μg/ml), infliximab (10 μg/ml), tocilizumab (10 μg/ml), or control IgG (10 μg/ml). After 120 hours of incubation, the supernatants were assayed for IL-17 and IFNγ. Statistical significance was evaluated by the Wilcoxon signed-rank test.

to be around the minimal pharmacologically attainable concentration.[16-18] As shown in Figure 1B, etanercept and infliximab, but not tocilizumab, significantly suppressed the production of IL-17 as well as that of IFNγ by SEB-stimulated PBMCs at the concentration of 10 μg/ml. The results indicate that the suppressive effects of etanercept and infliximab are not specific for Th17 cells. Of note, addition of TNFα, IL-6, and TGFβ, at concentrations that are approximately 10 times higher than those in culture supernatants of PBMCs stimulated with SEB in the absence of TNF inhibitors, did not restore the suppressive effects of etanercept and infliximab, indicating that the suppressive effects cannot be accounted for by the neutralization of these cytokines.

The next experiments examined the effects of each biological agent on the production of IL-17 and IFNγ by CD4+ T cells activated with immobilized anti-CD3. As shown in Figure 2, none of the biological agents influenced the production of IL-17 or IFNγ of immobilized anti-CD3-stimulated CD4+ T cells at 10 μg/ml. The results indicate that the suppression of the production of IL-17 and IFNγ of SEB-stimulated PBMCs by etanercept and infliximab is not a result of their direct effects on T cells. It is possible that the suppression of the production of IL-17 and IFNγ of PBMCs activated with SEB by TNF inhibitors might be due to the inhibition of cell proliferation. Therefore, we examined the effects of 10 μg/ml of each biological agent on the proliferation of SEB-stimulated PBMCs and immobilized anti-CD3-stimulated CD4+ T cells. As shown in Figure 3A, etanercept as well as infliximab significantly suppressed the proliferation of PBMCs compared with control IgG, whereas tocilizmab did not have any significant effects. Again, addition of TNFα, IL-6, and TGFβ did not restore the suppressive effects (Figure 3A). As shown in Figure 3B, etanercept, infliximab, and tocilizumab did not suppress the proliferation of immobilized anti-CD3-stimulated CD4+ T cells. These results indicate that the

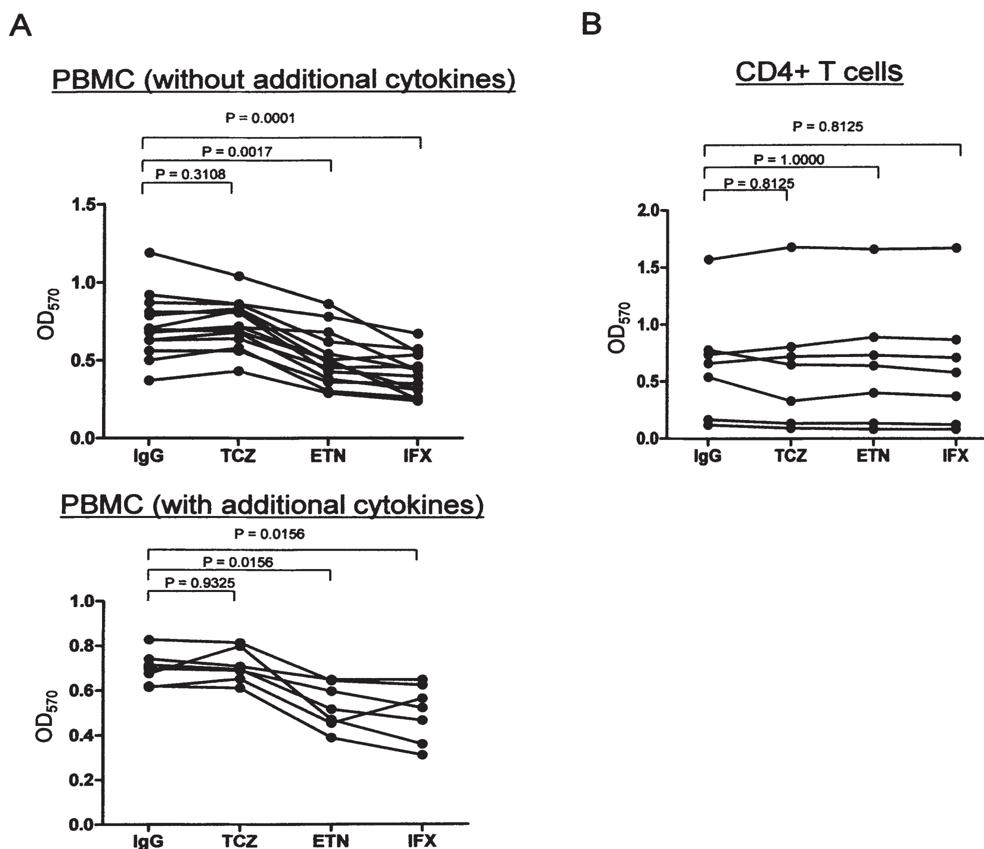

Figure 3. Effects of biological agents on the cell proliferation. **A**. PBMCs from 14 healthy individuals were cultured in the presence of SEB (100 pg/ml) with etanercept (10 μg/ml) or infliximab (10 μg/ml), tocilizumab (10 μg/ml), or control IgG (10 μg/ml). Recombinant TNFα (10 ng/ml), recombinant IL-6 (10 ng/ml), and recombinant TGFβ (10 ng/ml) were added in 7 individuals as indicated. **B**. Purified CD4+ T cells (1×10^5/well) from 7 healthy individuals were cultured in the presence of immobilized anti-CD3 with etanercept (10 μg/ml), infliximab (10 μg/ml), tocilizumab (10 μg/ml) or control IgG (10 μg/ml). After 120 hours of incubation, cell viability was determined by the colorimetric MTT assay. Statistical significance was evaluated by the Wilcoxon signed-rank test.

suppressive effects of etanercept and infliximab on the production of IL-17 and IFNγ of PBMCs activated with SEB are accounted for by their inhibitory effects on the proliferation of SEB-stimulated PBMCs. The activation of PBMCs with SEB requires the presence of antigen-presenting cells including monocytes. Thus, SEB stimulates T cells after binding on the major histocompatibility complex (MHC) class II antigens on antigen-presenting cells.[19] Etanercept and infliximab suppressed the proliferation and cytokine production of PBMCs stimulated with SEB without direct effects on immobilized anti-CD3-stimulated CD4+ T cells. Therefore, it is possible that etanercept and infliximab might suppress the activation of PBMCs stimulated with SEB by inhibiting the function of monocytes.

The next experiments, therefore, examined the effects of each biological agent on the expression of MHC class II antigens on monocytes activated with SEB. Figure 4A shows a typical histogram of the expression of HLA-DR on annexin V-negative monocytes from a normal healthy donor. Etanercept and infliximab, but not tocilizumab, markedly suppressed the expression of HLA-DR. Accordingly, both etanercept and infliximab significantly suppressed the expression of HLA-DR on SEB-stimulated monocytes from 9 healthy individuals compared with control IgG, whereas tocilizumab did not have any significant effects on the expression of HLA-DR (Figure 4B). These results indicate that etanercept and infliximab suppress the proliferation and cytokine production of SEB-stimulated PBMCs by inhibiting the expression of MHC class II antigens.

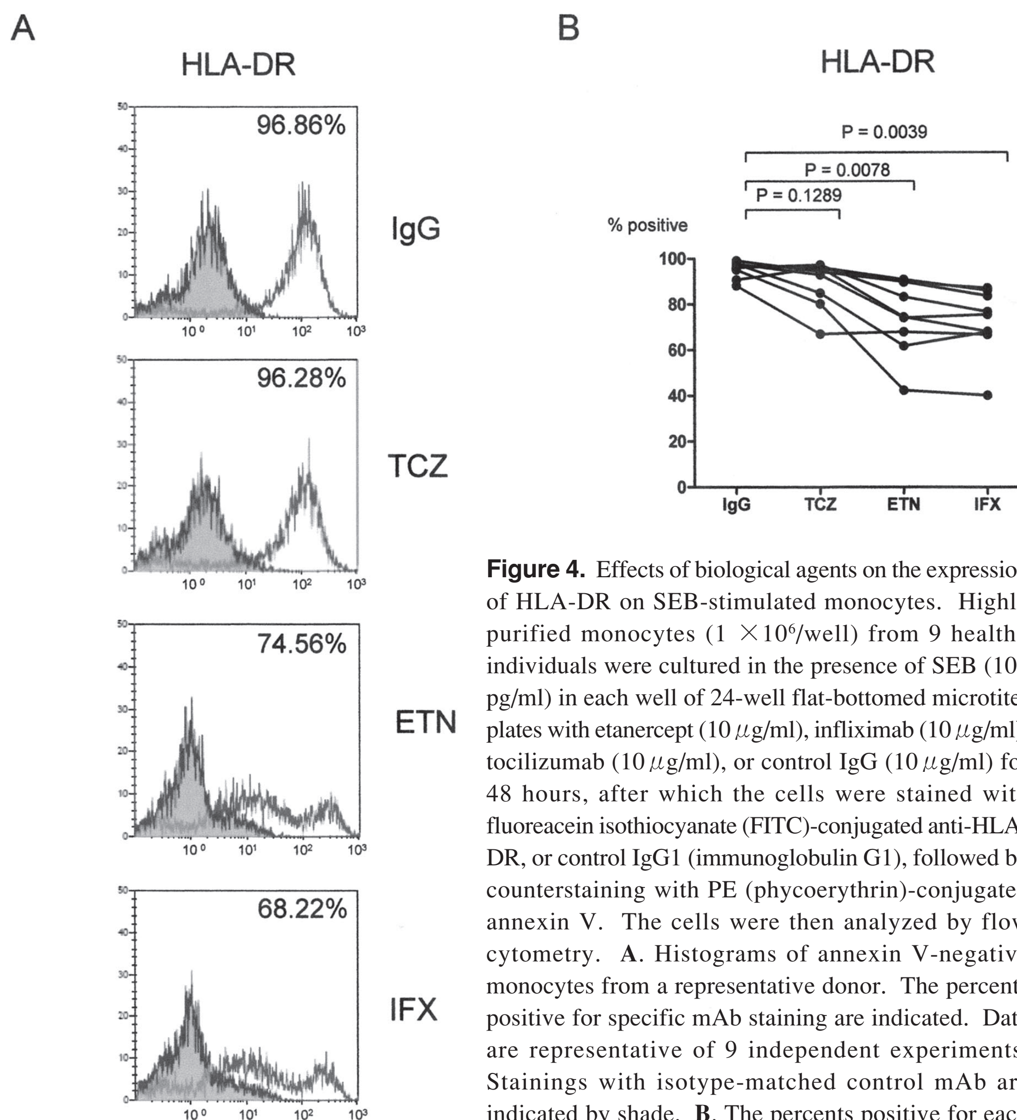

Figure 4. Effects of biological agents on the expression of HLA-DR on SEB-stimulated monocytes. Highly purified monocytes (1 $\times 10^6$/well) from 9 healthy individuals were cultured in the presence of SEB (100 pg/ml) in each well of 24-well flat-bottomed microtiter plates with etanercept (10 μg/ml), infliximab (10 μg/ml), tocilizumab (10 μg/ml), or control IgG (10 μg/ml) for 48 hours, after which the cells were stained with fluoreacein isothiocyanate (FITC)-conjugated anti-HLA-DR, or control IgG1 (immunoglobulin G1), followed by counterstaining with PE (phycoerythrin)-conjugated annexin V. The cells were then analyzed by flow cytometry. **A**. Histograms of annexin V-negative monocytes from a representative donor. The percents positive for specific mAb staining are indicated. Data are representative of 9 independent experiments. Stainings with isotype-matched control mAb are indicated by shade. **B**. The percents positive for each specific mAb staining of monocytes from 9 independent experiments are summarized. Statistical significance was evaluated by the Wilcoxon signed-rank test.

Discussion

We investigated the effects of anti-TNFα agents, etanercept and infliximab, and anti-IL-6 receptor antibody, tocilizumab, on the production of cytokines of SEB-stimulated PBMCs from healthy individuals. The results clearly demonstrate that etanercept and infliximab, but not tocilizumab, suppress the production of IL-17 and IFNγ. Because addition of TNFα, IL-6, and TGFβ, at much higher concentrations than those in the culture supernatants of SEB-stimulated PBMCs in the absence of TNF inhibitors, did not abrogate the suppressive influences of the TNF inhibitors on PBMCs, it is possible that these anti-TNFα agents might have direct action on PBMCs. It is remarkable that infliximab and etanercept showed comparable inhibitory effects. This might be at least in part due to the comparable structures of these compounds. Both infliximab and etanercept have TNF binding sites as well as Fc portions of IgG1. Therefore, both compounds are considered to bind membrane-associated TNF, followed by cross-linking through Fcγ receptors on monocytes and/or macrophages. Further studies are warranted to delineate the precise mechanisms of these compounds.

It should be noted that anti-TNFα agents did not suppress the proliferation and cytokine production of highly purified CD4+ T cells stimulated with immobilized anti-CD3. Because immobilized anti-CD3 can activate CD4+ T cells in the complete absence of monocytes or other antigen-presenting cells,[14] the lack of suppression by anti-TNFα agents on immobilized anti-CD3 stimulated CD4+ T cells strongly suggested that anti-TNFα agents might suppress the proliferation and cytokine production through interactions with the membrane-bound TNFα on monocytes.

The binding of superantigens such as SEB to HLA-DR on monocytes is mandatory for the stimulation of T cell receptors.[19] Therefore, the efficiency of stimulation with SEB is dependent on the expression of HLA-DR on monocytes. In this regard, etanercept and infliximab markedly suppressed the expression of HLA-DR on monocytes, whereas tocilizumab had no influence at all. It is suggested that the suppression of activation of PBMCs with SEB by these TNF inhibitors is a result of the suppression of the expression of HLA-DR.

It has been shown that IL-6 and TGFβ play important roles in the maturation of Th17 cells.[12,13] Tocilizumab did not exhibit a suppressive effect on IL-17 production in the short culture system using SEB-stimulated PBMCs in the present study. However, a previous study showed that IL-6 blockade suppressed the induction of Th17 cells and the development of autoimmune arthritis in mice,[13] suggesting that tocilizumab might suppress IL-17 production by inhibition of Th17 differentiation *in vivo*. It is known that the transcription factor RORγt is involved in the Th17 differentiation.[20] Therefore, it is possible that the induction of RORγt might precede the differentiation of Th17 as well as the production of IL-17. Therefore, further *in vitro* studies to explore the role of tocilizumab on the expression of RORγt in T cells are warranted to fully delineate the mechanism of tocilizumab.

In conclusion, these results demonstrated that etanercept and infliximab, but not tocilizumab, suppress proliferation and cytokine production of PBMCs through the inhibition of the expression of MHC class II antigens on monocytes. Further studies to explore the effects on various functions of monocytes other than HLA-DR expression are warranted for a more complete understanding of the mechanism of these biological agents.

Acknowledgments

Financial support: This work was supported by a Parents' Association Grant from Kitasato University School of Medicine and grants from Mitsubishi Tanabe Pharma Co., Tokyo, Takeda Pharmaceutical Co., Tokyo, and Chugai Pharmaceutical Co., Tokyo.
Conflict of interest: none

References

1. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 2007; 7: 429-42.
2. Lipsky PE. Interleukin-6 and rheumatic diseases. *Arthritis Res Ther* 2006; 8 (Suppl 2): S4.
3. Maini R, St Clair EW, Breedveld F, et al. Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. *Lancet* 1999; 354: 1932-9.
4. Fleischmann RM, Schechtman J, Bennett R, et al. Anakinra, a recombinant human interleukin-1 receptor antagonist (r-metHuIL-1ra), in patients with rheumatoid arthritis: a large, international, multicenter, placebo-controlled trial. *Arthritis Rheum* 2003; 48: 927-34.

5. Choy EH, Isenberg DA, Garrood T, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. *Arthritis Rheum* 2002; 46: 3143-50.
6. Mitoma H, Horiuchi T, Tsukamoto H, et al. Mechanisms for cytotoxic effects of anti-tumor necrosis factor agents on transmembrane tumor necrosis factor alpha-expressing cells: comparison among infliximab, etanercept, and adalimumab. *Arthritis Rheum* 2008; 58: 1248-57.
7. Baldwin HM, Ito-Ihara T, Isaacs JD, et al. Tumour necrosis factor alpha blockade impairs dendritic cell survival and function in rheumatoid arthritis. *Ann Rheum Dis* 2010; 69: 1200-7.
8. Kotake S, Udagawa N, Takahashi N, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. *J Clin Invest* 1999; 103: 1345-52.
9. Kohno M, Tsutsumi A, Matsui H, et al. Interleukin-17 gene expression in patients with rheumatoid arthritis. *Mod Rheumatol* 2008; 18: 15-22.
10. Jovanovic DV, Di Battista JA, Martel-Pelletier J, et al. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 1998; 160: 3513-21.
11. Veldhoen M, Hocking RJ, Atkins CJ, et al. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006; 24: 179-89.
12. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, et al. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007; 8: 942-9.
13. Fujimoto M, Serada S, Mihara M, et al. Interleukin-6 blockade suppresses autoimmune arthritis in mice by the inhibition of inflammatory Th17 responses. *Arthritis Rheum* 2008; 58: 3710-9.
14. Geppert TD, Lipsky PE. Accessory cell independent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3. *J Immunol* 1987; 138: 1660-6.
15. Hirohata S, Hashimoto T. Abnormal T cell responses to bacterial superantigens in Behçet's disease (BD). *Clin Exp Immunol* 1998; 112: 317-24.
16. Frey N, Grange S, Woodworth T. Population pharmacokinetic analysis of tocilizumab in patients with rheumatoid arthritis. *J Clin Pharmacol* 2010; 50: 754-66.
17. Tracey D, Klareskog L, Sasso EH, et al. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther* 2008; 117: 244-79.
18. Takeuchi T, Miyasaka N, Tatsuki Y, et al. Baseline tumour necrosis factor alpha levels predict the necessity for dose escalation of infliximab therapy in patients with rheumatoid arthritis. *Ann Rheum Dis* 2011; 70: 1208-15.
19. Kappler J, Kotzin B, Herron L, et al. V beta-specific stimulation of human T cells by staphylococcal toxins. *Science* 1989; 244: 811-3.
20. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor ROR gamma t directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 2006; 126: 1121-33.