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Anti-IL-6 receptor antibody suppressed T cell activation by inhibiting IL-2 production and inducing regulatory T cells

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ABSTRACT

T cell activation is crucial to the pathogenesis and progression of rheumatoid arthritis. Tumour necrosis factor-α (TNFα) and interleukin (IL)-6 inhibitors show marked efficacy in rheumatoid arthritis patients, but their impacts on T cell activation have remained unclear. To shed light on these impacts, we examined the effects of an anti-IL-6 receptor antibody and an anti-TNFα antibody on T cell activation in two experimental systems: spleen cells stimulated by anti-CD3 antibody, and purified splenic CD4 T cells stimulated by both anti-CD3 and anti-CD28 antibodies. Anti-IL-6 receptor antibody significantly (but only partially) suppressed T cell activation (as indicated by [^{3}H]-thymidine uptake and CD25 expression) and IL-2 production in both systems, and increased the frequency of regulatory T cells among spleen cells. Anti-TNFα antibody had no effects in either system. Neither antibody increased the expression of markers of apoptosis in CD4 T cells. In conclusion, our results show that anti-IL-6 receptor antibody significantly (but only partially) suppressed the T cell receptor signalling-induced activation of CD4 T cells and also suggest that it achieved this partial suppression by the partial inhibition of IL-2 production and the induction of regulatory T cells. In stark contrast, anti-TNFα antibody had no impact on T cell activation. Extrapolating these results to the clinical treatment of rheumatoid arthritis, they suggest that IL-6 blockade inhibits T cell activation, whereas TNFα blockade does not.

1. Introduction

Rheumatoid arthritis is an autoimmune inflammatory disease characterized by symmetric polyarthritis and progressive structural joint damage. Activated T cells are abundantly observed in the inflamed joints of rheumatoid arthritis patients (Fox et al., 1982; Hemler et al., 1986). Moreover, anti-rheumatic drugs, such as methotrexate, tacrolimus and abatacept, that potently inhibit T cell activation, are effective in the treatment of rheumatoid arthritis patients (Olsen and Murray, 1989; Yoshimura et al., 1989; Linsley et al., 1991). These lines of evidence strongly suggest that activated T cells play crucial roles in the progression and maintenance of arthritis in rheumatoid arthritis patients.

Drugs that block the signalling pathways of pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and interleukin (IL)-6 also dramatically reduce disease activity and halt the progression of joint destruction in rheumatoid arthritis patients with an inadequate response to conventional disease-modifying anti-rheumatic drug therapy (Maini et al., 1999; Weinblatt et al., 1999; Nishimoto et al., 2004). TNFα augments the inflammatory response by inducing inflammatory mediators such as prostaglandins, cytokines and chemokines, and expression of adhesion molecules (Feldmann et al., 1992). This suggests that the mechanism of action of TNFα blockers includes the suppression of the inflammatory response, but it has not been clear whether TNFα blockade inhibits T cell activation.

IL-6 also induces inflammatory mediators and adhesion molecules (Suzuki et al., 2010). Moreover, IL-6 promotes IL-4-induced T helper 2 (Th2) differentiation and inhibits IL-12-induced Th1 differentiation (Diehl and Rincon, 2002). Furthermore, IL-6 induces Th17 differentiation in the presence of transforming growth factor (TGF)-β and inhibits the induction of TGF-β-induced regulatory T cells (Bettelli et al., 2006). The therapeutic effects of an anti-IL-6 receptor antibody such as tocilizumab may therefore be the result of inhibiting any combination of the above actions of IL-6, but again, the impact of IL-6 blockade on T cell activation has not been fully understood.

We conducted the present study to shed light on the question of whether anti-IL-6 receptor antibody or anti-TNFα antibody suppresses the activation of stimulated T cells in two *in vitro* T cell-activation systems. One system consisted of spleen cells stimulated by anti-CD3 antibody, and the other system consisted of purified splenic CD4 T cells stimulated by both anti-CD3 antibody and anti-CD28 antibody. Using these two systems, we examined and compared the effects of anti-IL-6 receptor antibody, anti-TNFα antibody and

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cytotoxic T lymphocyte antigen 4 fusion protein (CTLA4-Fc) as a positive control on T cell activation.

2. Materials and methods

2.1. Reagents

Anti-mouse IL-6 receptor antibody (MR16-1) was prepared in our laboratories (Okazaki et al., 2002). Anti-mouse TNFα antibody (clone TN3-19.12) and mouse CTLA4-Fc were purchased from BD Pharmingen (San Diego, CA) and R&D systems (Minneapolis, MN), respectively. Anti-CD3 antibody and anti-CD28 antibody were purchased from Cedarlane Laboratories (Ontario, Canada) and BD Pharmingen, respectively.

2.2. Animals

Female BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). The mice were specific pathogen free and were kept in cages in a room maintained at 20 to 26 °C and 35% to 75% relative humidity. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd.

2.3. Cell culture

The mice were sacrificed at 8–10 weeks of age, and their spleens extirpated. The spleen was dispersed into single cells and the red blood cells were lysed. CD4 T cells were purified from the spleen cells using MACS CD4 MicroBeads (Myltenyi BioTec, Bergisch-Gladbach, Germany). The purity of the resulting CD4 T cells was more than 95%.

Whole spleen cells (3×10^5/well) stimulated with anti-CD3 antibody (0.1 μg/ml), or CD4 T cells (3×10^5/well) stimulated with both anti-CD3 antibody (0.1 μg/ml) and anti-CD28 antibody (1 μg/ml), were cultured in the presence of a test substance for 24 h or 3 days at 37 °C in RPMI-1640 medium containing 10% FBS (GIBCO, Grand Island, NY) in a 96-well plate. All cultures were performed in triplicate.

2.4. Determinations

2.4.1. Thymidine uptake

Cells were cultured for 3 days and exposed to 37 kBq/well of [^{3}H]-thymidine (Amersham, Buckinghamshire, UK) during the last 5 h of culture. Thymidine uptake by the cells was then measured using a liquid scintillation counter.

2.4.2. Cytokine production

Culture supernatants were collected at 24 h after culture initiation. The concentrations of IL-2 and IL-6 in culture supernatants were measured by Bioplex Cytokine assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Data were analyzed using the BioPlex software Manager 5.0 (Bio-Rad).

2.4.3. Flow cytometry analysis

Cultured cells were collected at the end of culture (3 days after culture initiation). Phenotypic analysis of cultured cells was performed by FACSCalibur and FACSCanto II (BD Bioscience, Franklin Lakes, NJ), using CellQuest software and FACSDiva software (BD Bioscience) for data acquisition and FlowJo software (Tree Star, Ashland, OR) for data analysis.

For cell-surface phenotyping, the cultured cells were stained with anti-CD4 antibody (clone RM4-5; BD Pharmingen), anti-CD25 antibody (clone PC61; BD Pharmingen). For intracellular staining, the cells were fixed and permeabilised using a Cytofix/Cytoperm kit (BD Pharmingen), according to the manufacturer's instructions, and then stained with anti-Foxp3 (clone FJK-16s; eBioscience, San Diego, CA, USA).

Regulatory T cells were identified as $CD4^+$ $CD25^{high}$ $Foxp3^+$. Apoptotic cells were identified by staining with annexin V (BD Pharmingen) and 7-aminoactinomycin D (7-AAD) (BD Pharmingen).

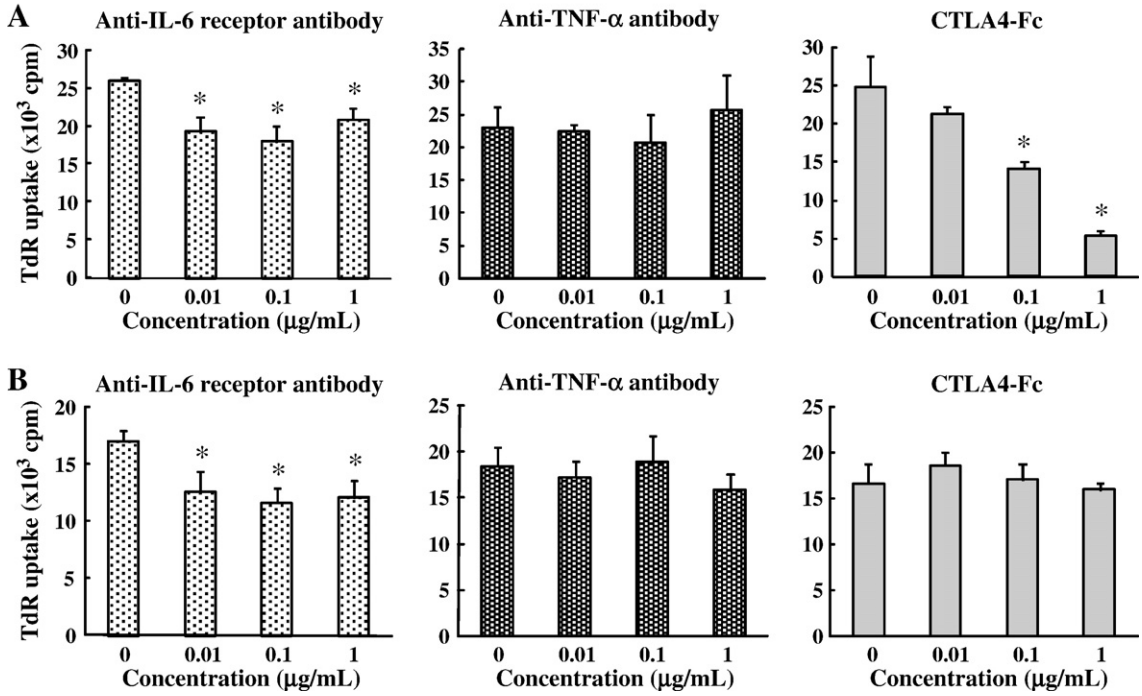

Fig. 1. Effects on thymidine uptake. Whole spleen cells were stimulated with anti-CD3 antibody (A) and purified splenic CD4 T cells were stimulated with both anti-CD3 and anti-CD28 antibodies (B) for 3 days in the presence of test substance. After culture, uptake of [^{3}H]-thymidine was measured. Each column and vertical line indicates the mean and S.D. of triplicate cultures. *P<0.05 vs. control (no test substance added) by Dunnett's multiple comparison test.

2.5. Statistical analysis

Statistically significant differences were analyzed by the unpaired *t*-test and Dunnett's multiple comparison test using a software package (Statistical Analysis System: SAS Institute Japan, Tokyo, Japan), with the significance level set to 5%.

3. Results

3.1. Effects on thymidine uptake

In the study using whole spleen cells, anti-TNFα antibody had no effect on thymidine uptake, whereas CTLA4-Fc inhibited thymidine uptake in a concentration-dependent manner (almost completely at 1 μg/ml). Anti-IL-6 receptor antibody also significantly inhibited thymidine uptake, but the degree of inhibition did not increase with increasing concentration, and never exceeded approx. 30% (Fig. 1A).

Similar results were obtained in CD4 T cells, except that CTLA4-Fc had no effect in these cells (Fig. 1B).

3.2. Induction of apoptosis

To check cell viability, we studied the expression of 7-AAD in CD4 T cells. 7-AAD-positive cells were necrotic. When spleen cells were not stimulated by anti-CD3 antibody, 70.8% of cells became 7-AAD-positive during 3 days in culture. However, when cells were activated by anti-CD3 antibody, 7-AAD-positive cells decreased to 32.3% of the total. The proportions of 7-AAD-positive cells in the anti-IL-6 receptor antibody group, the CTLA4-Fc group and the anti-TNFα antibody group were 51.4%, 57.4% and 27%, respectively (Fig. 2A).

We next evaluated whether cells underwent apoptosis, by staining and by measuring the expression of annexin V in 7-AAD-positive cells. In the control group, annexin V expression was markedly increased compared with the non-stimulated group. The pattern of annexin V expression in the anti-IL-6 receptor antibody and anti-TNFα antibody groups was similar to that in the control group. However, annexin V expression in the CTLA4-Fc group was similar to that in the non-stimulated group (Fig. 2B).

3.3. Cytokine production and CD25 expression

Analysis of the spleen cell culture supernatants (Fig. 3A) showed that IL-2 production was inhibited by anti-IL-6 receptor antibody and more markedly inhibited by CTLA4-Fc, but was not inhibited by anti-TNFα antibody. IL-6 production was also markedly inhibited by CTLA4-Fc, but was not inhibited by either anti-IL-6 receptor antibody or anti-TNFα antibody.

Analysis of the CD4 T cell culture supernatants (Fig. 3B), showed that IL-2 production was inhibited by anti-IL-6 receptor antibody, but not by anti-TNFα antibody or CTLA4-Fc. IL-6 production in CD4 T cells was below the limit of detection.

In addition, we checked the expression of CD25 in CD4 T cells as an activation marker. Expression of CD25 was suppressed by CTLA4-Fc and anti-IL-6 receptor antibody (Fig. 3C). The suppressive effect of anti-IL-6 receptor antibody was weaker than that of CTLA4-Fc. However, anti-TNFα antibody did not suppress CD25 expression.

3.4. Effect of TNFα on thymidine uptake

As TNFα blockade had no effect on thymidine uptake in either system (see Section 3.1), we performed an additional experiment to examine whether exogenous TNFα enhances T cell activation.

In spleen cells, the highest concentration of TNFα (100 ng/ml) significantly enhanced thymidine uptake, and this enhancement was inhibited in the presence of anti-IL-6 receptor antibody (Fig. 4A). In CD4 T cells, results showed a significant increase in uptake with 10 ng/ml

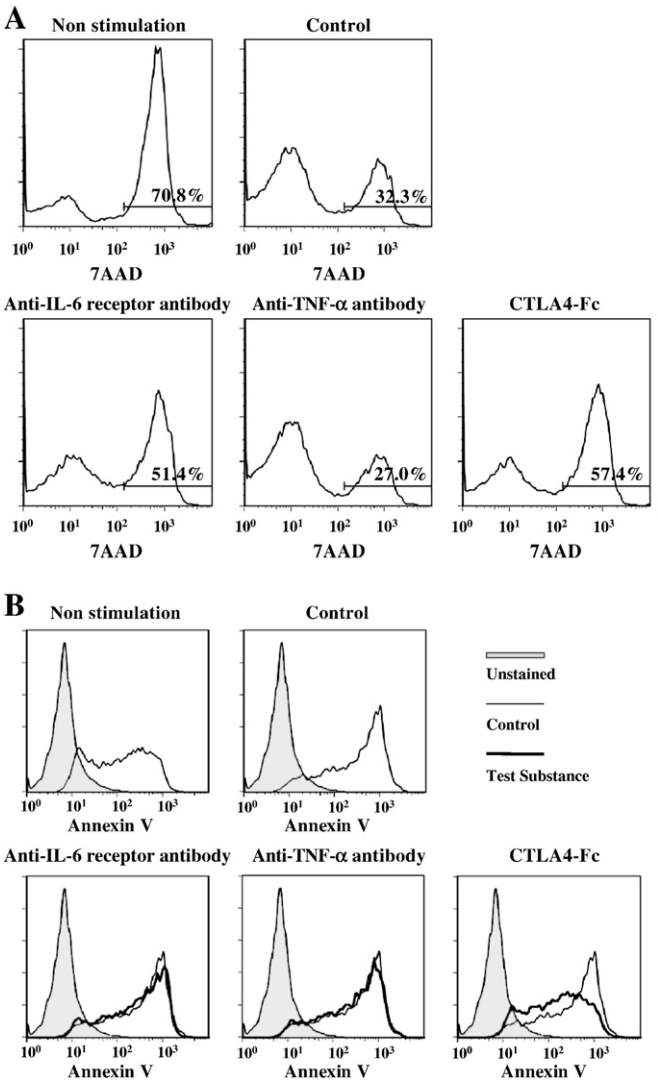

Fig. 2. Analysis of dead cells. Whole spleen cells were stimulated with anti-CD3 antibody for 3 days in the presence of test substance. After culture, flow cytometry analysis of CD4-gated cells was performed. (A) Dead cells ($CD4^+$/7-AAD^+), (B) Apoptotic cells ($CD4^+$/7-AAD^+/annexin V^+).

TNFα, but did not clearly indicate enhancement of thymidine uptake by TNFα, since the highest concentration used (100 ng/ml) had no significant effect (Fig. 4B).

3.5. Induction of IL-2 production by IL-6

To investigate the effect of exogenous IL-6 on IL-2 production, we cultured CD4 T cells in the presence or absence of IL-6 with or without stimulation by both anti-CD3 and anti-CD28 antibodies, and determined IL-2 concentration in the supernatant. Results showed that there was a trend towards an increase in IL-2 production induced by T cell receptor stimulation, and this induction was significant on stimulation in the presence of exogenous IL-6 (Fig. 5).

3.6. Effects on the frequency of regulatory T cells

Anti-IL-6 receptor antibody treatment increased the frequency of regulatory T cells among the cultured spleen cells (from 9.7% to 14.9%), whereas anti-TNFα antibody did not, and CTLA4-Fc actually decreased the frequency of regulatory T cells (from 9.7% to 5.6%). The

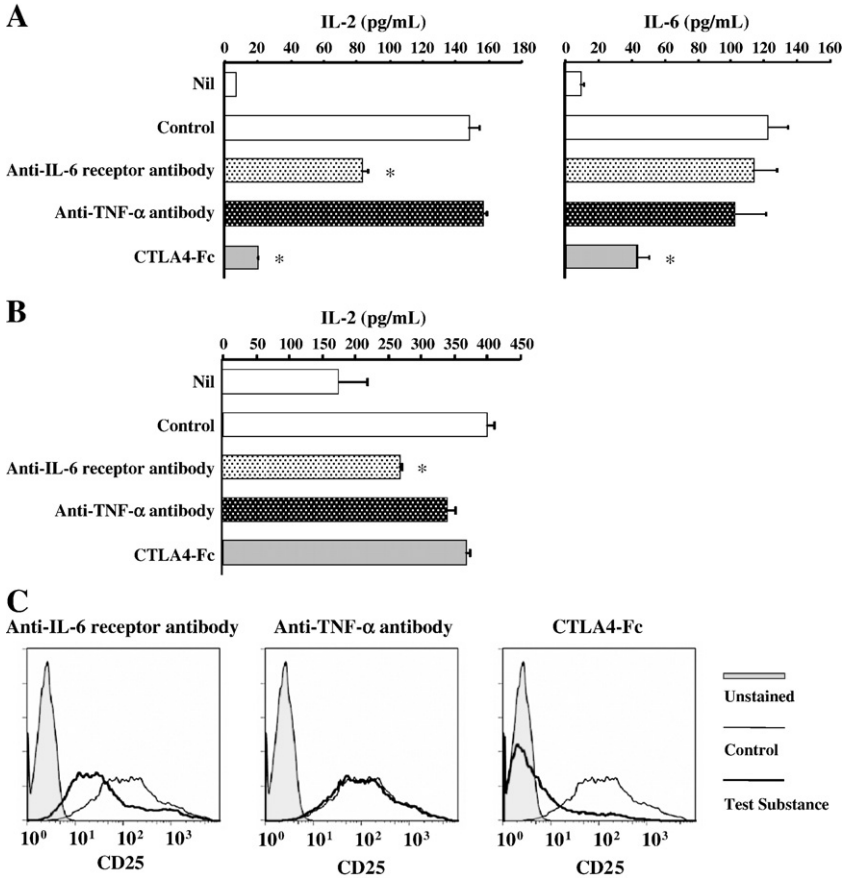

Fig. 3. IL-2 and IL-6 concentrations in supernatants. Whole spleen cells were stimulated with anti-CD3 antibody in the presence of test substance for 24 h. After culture, supernatant was collected and concentrations of IL-2 (left) and IL-6 (right) were measured (A). CD4 T cells were stimulated with both anti-CD3 and anti-CD28 antibodies in the presence of test substance for 24 h. After culture, the concentration of IL-2 in supernatant was measured (B). IL-6 in CD4 T cell supernatant was below the limit of detection. Whole spleen cells were stimulated with anti-CD3 antibody in the presence of test substance for 3 days. After culture, CD25 expression of CD4 T cells was analyzed (C). Nil: Non-stimulated. *$P<0.05$ vs. control by *t*-test.

addition of IL-6 decreased the frequency of regulatory T cells (from 9.7% to 7.2%) (Fig. 6).

Similar results were found among the cultured CD4 T cells (data not shown).

4. Discussion

There is no doubt that activated T cells are involved in the pathogenesis of rheumatoid arthritis. The dramatic therapeutic effects of anti-cytokine therapies such as anti-TNFα antibodies and the anti-IL-6 receptor antibody raise the question as to whether these treatments inhibit the activation of T cells in rheumatoid arthritis. In the present study, we examined the effects of anti-IL-6 receptor antibody, anti-TNFα antibody and CTLA4-Fc on T cell activation in two experimental systems: spleen cells stimulated by anti-CD3 antibody, and purified splenic CD4 T cells stimulated by both anti-CD3 and anti-CD28 antibodies.

We obtained the following results for T cell activation, taking thymidine uptake as an indicator. (1) Anti-IL-6 receptor antibody partially suppressed T cell activation in both systems. (2) Anti-TNFα antibody had no effect in either system. (3) CTLA4-Fc dramatically suppressed T cell activation in the spleen cells, but not in the CD4 T cells. Our data suggest that IL-6 is only partly involved in T cell activation, however, because at all concentrations, anti-IL-6 receptor antibody achieved only about 20%–30% inhibition of T cell activation, in both experimental systems.

To explore the mechanism of decrease in thymidine uptake, we checked cell viability by measuring 7-AAD expression in cells. The data indicated that when T cells were not stimulated by anti-CD3 antibody, almost all cells were necrotic. MR16-1 and CTLA4-Fc increased numbers of 7-AAD-positive cells and the expression of 7-AAD was negatively related to thymidine uptake. In addition, the expression of CD25 (activation marker) was decreased by MR16-1 and CTLA4-Fc and was positively related to thymidine uptake. These results clearly demonstrated that the decrease in thymidine uptake by MR16-1 and CTLA4-Fc resulted from inhibition of activation.

We next examined whether apoptosis was induced in necrotic cells, since a number of reports have indicated that IL-6 is an anti-apoptotic factor in many cell types (Chauhan et al., 1997; Chen et al., 1999; Jee et al., 2001). However, our results show that anti-IL-6 receptor antibody had no effect on the expression of markers of apoptosis (annexin V in 7-AAD$^+$ cells), so it is unlikely that anti-IL-6 receptor antibody induced apoptosis of CD4 T cells. Moreover, the annexin V expression pattern in the CTLA4-Fc group was similar to that in the non-stimulated group. This result also strongly supports the idea that CTLA4-Fc completely inhibited activation by TCR stimulation.

Since IL-2 is known to be an essential cytokine for T cell activation, we measured IL-2 levels in the cell supernatants. The results mirrored those for thymidine uptake/T cell activation ((1)–(3) above). Again, we found that anti-IL-6 receptor antibody significantly, but not completely, suppressed IL-2 production in both systems. In an additional experiment, we found that exogenous IL-6 augmented IL-2 production from T cell receptor-stimulated CD4 T cells, but not from non-stimulated CD4 T cells. Taken together, these results suggest that anti-IL-6 receptor antibody may inhibit CD4 T cell activation by suppressing IL-6-induced IL-2 production. It also suggests that the reason anti-TNFα antibody did

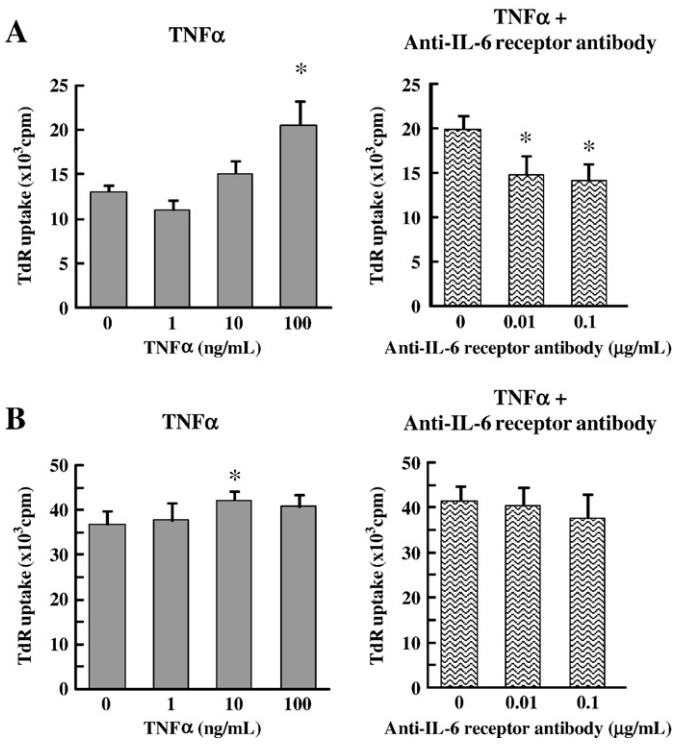

Fig. 4. Effect of TNFα on the activation of T cells. Whole spleen cells were stimulated with anti-CD3 antibody (A) and splenic CD4 T cells were stimulated with both anti-CD3 and anti-CD28 antibody (B) for 3 days in the presence of TNFα (left; control = without TNFα) or 100 ng/ml TNFα + anti-IL-6 receptor antibody (right; control = without anti-IL-6 receptor antibody). After culture, uptake of [^{3}H]-thymidine was measured. Each column and vertical line indicates mean and S.D. of triplicate cultures. *P<0.05 vs. control by Dunnett's multiple comparison test.

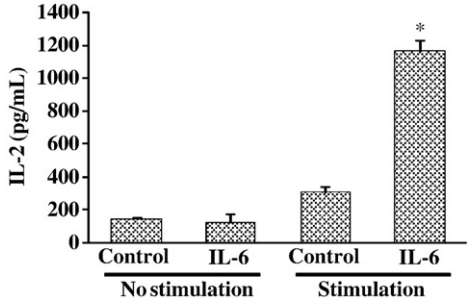

Fig. 5. Enhancement of IL-2 production by exogenous IL-6. Whole spleen cells were stimulated with anti-CD3 antibody for 24 h in the presence of IL-6 (100 ng/ml). After culture, IL-2 concentration in the supernatant was measured. Control: no IL-6 added. *P<0.05 vs. control by t-test.

not inhibit CD4 T cell activation may be that it did not inhibit IL-2 production.

It has been reported that IL-6 inhibits TGF-β-induced differentiation of naïve T cells into regulatory T cells and conversely induces Th17 differentiation in the presence of TGF-β (Bettelli et al., 2006). It was therefore considered possible that IL-6 blockade induces regulatory T cells. To test this hypothesis, we analyzed the frequency of regulatory T cells among cultured spleen cells. As hypothesised, the addition of anti-IL-6 receptor antibody increased the frequency of regulatory T cells. We also confirmed that the addition of IL-6 decreased the frequency of regulatory T cells. These results suggest that regulatory T cells induced by IL-6 blockade are involved in the suppression of T cell proliferation, at least in part.

In stark contrast to anti-IL-6 receptor antibody, anti-TNFα antibody had no effect in any of our experiments. Our results therefore strongly suggest that anti-TNFα antibody does not inhibit T cell activation, which

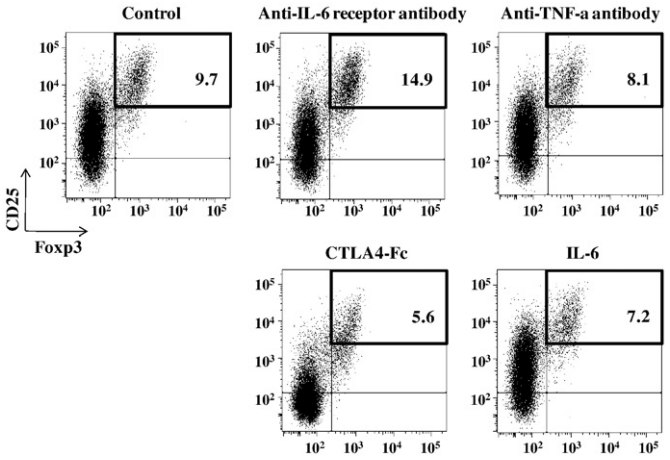

Fig. 6. The frequency of regulatory T cells. Whole spleen cells were stimulated with anti-CD3 antibody for 3 days in the presence of a test substance. After culture, flow cytometry analysis was performed. Regulatory T cells ($CD4^+$ $CD25^{high}$ $Foxp3^+$ cells) are shown, with their frequency, in the upper right rectangle in each plot.

further suggests that its target cytokine, TNFα, is not involved in T cell activation. On testing this hypothesis, we found that although exogenous TNFα did increase thymidine uptake, this increase was inhibited by anti-IL-6 receptor antibody. This makes it clear that it was endogenous IL-6 induced by TNFα, not TNFα itself that caused the increase in thymidine uptake. We previously reported that although TNFα inhibitor suppressed the progression of arthritis in a mouse arthritis model when it was injected after onset of arthritis, it did not suppress the onset of arthritis or the induction of Th17 when it was injected soon after immunization (Fujimoto et al., 2008). Taken together, these results suggest that the anti-rheumatic actions of TNFα inhibitors are mainly based on anti-inflammatory effects.

We showed here that CTLA4-Fc almost completely inhibited thymidine uptake by spleen cells, but not by purified CD4 T cells. CTLA4-Fc binds to CD80 and CD86 expressed on antigen-presenting cells, which were present in the spleen cell system, but which are absent in the purified CD4 T cell system. The presence or absence of antigen-presenting cells can therefore explain the difference in the effects of CTLA4-Fc in the two systems.

In conclusion, anti-IL-6 receptor antibody suppressed the activation of CD4 T cells induced by T cell receptor signalling, by inhibiting IL-2 production and inducing regulatory T cells.

5. Conclusions

In conclusion, our results show that anti-IL-6 receptor antibody significantly (but only partially) suppressed the T cell receptor signalling-induced activation of CD4 T cells. They also suggest that anti-IL-6 receptor antibody achieved this suppression by causing partial inhibition of IL-2 production and the induction of regulatory T cells. In stark contrast, anti-TNFα antibody showed no evidence of any impact on T cell activation. Extrapolating these results to the clinical treatment of rheumatoid arthritis, they suggest that IL-6 blockade inhibits T cell activation, whereas TNFα blockade does not. If that is the case, the mechanism of action of TNFα inhibitors may be based mainly on suppression of the inflammatory response, whereas the mechanism of action of the IL-6 inhibitor, tocilizumab, includes direct immunoregulatory effects in addition to anti-inflammatory effects.

References

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., Kuchroo, V.K., 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441, 235–238.

Chauhan, D., Kharbanda, S., Ogata, A., Urashima, M., Teoh, G., Robertson, M., Kufe, D.W., Anderson, K.C., 1997. Interleukin-6 inhibits Fas-induced apoptosis and stress-activated protein kinase activation in multiple myeloma cells. Blood 89, 227–234.

Chen, R.H., Chang, M.C., Su, Y.H., Tsai, Y.T., Kuo, M.L., 1999. Interleukin-6 inhibits transforming growth factor-beta-induced apoptosis through the phosphatidylinositol 3-kinase/Akt and signal transducers and activators of transcription 3 pathways. J. Biol. Chem. 274, 23013–23019.

Diehl, S., Rincon, M., 2002. The two faces of IL-6 on Th1/Th2 differentiation. Mol. Immunol. 39, 531–536.

Feldmann, M., Brennan, F.M., Williams, R.O., Cope, A.P., Gibbons, D.L., Katsikis, P.D., Maini, R.N., 1992. Evaluation of the role of cytokines in autoimmune disease: the importance of TNF alpha in rheumatoid arthritis. Prog. Growth Factor Res. 4, 247–255.

Fox, R.I., Fong, S., Sabharwal, N., Carstens, S.A., Kung, P.C., Vaughan, J.H., 1982. Synovial fluid lymphocytes differ from peripheral blood lymphocytes in patients with rheumatoid arthritis. J. Immunol. 128, 351–354.

Fujimoto, M., Serada, S., Mihara, M., Uchiyama, Y., Yoshida, H., Koike, N., Ohsugi, Y., Nishikawa, T., Ripley, B., Kimura, A., Kishimoto, T., Naka, T., 2008. Interleukin-6 blockade suppresses autoimmune arthritis in mice by the inhibition of inflammatory Th17 responses. Arthritis Rheum. 58, 3710–3719.

Hemler, M.E., Glass, D., Coblyn, J.S., Jacobson, J.G., 1986. Very late activation antigens on rheumatoid synovial fluid T lymphocytes. Association with stages of T cell activation. J. Clin. Invest. 78, 696–702.

Jee, S.H., Shen, S.C., Chiu, H.C., Tsai, W.L., Kuo, M.L., 2001. Overexpression of interleukin-6 in human basal cell carcinoma cell lines increases anti-apoptotic activity and tumorigenic potency. Oncogene 20, 198–208.

Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L.S., Damle, N.K., Ledbetter, J.A., 1991. CTLA-4 is a second receptor for the B cell activation antigen B7. J. Exp. Med. 174, 561–569.

Maini, R., St. Clair, E.W., Breedveld, F., Furst, D., Kalden, J., Weisman, M., Smolen, J., Emery, P., Harriman, G., Feldmann, M., Lipsky, P., 1999. 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 354, 1932–1939.

Nishimoto, N., Yoshizaki, K., Miyasaka, N., Yamamoto, K., Kawai, S., Takeuchi, T., Hashimoto, J., Azuma, J., Kishimoto, T., 2004. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. Arthritis Rheum. 50, 1761–1769.

Okazaki, M., Yamada, Y., Nishimoto, N., Yoshizaki, K., Mihara, M., 2002. Characterization of anti-mouse interleukin-6 receptor antibody. Immunol. Lett. 84, 231–240.

Olsen, N.J., Murray, L.M., 1989. Antiproliferative effects of methotrexate on peripheral blood mononuclear cells. Arthritis Rheum. 32, 378–385.

Suzuki, M., Hashizume, M., Yoshida, H., Mihara, M., 2010. Anti-inflammatory mechanism of tocilizumab, a humanized anti-IL-6R antibody: effect on the expression of chemokine and adhesion molecule. Rheumatol. Int. 30, 309–315.

Weinblatt, M.E., Kremer, J.M., Bankhurst, A.D., Bulpitt, K.J., Fleischmann, R.M., Fox, R.I., Jackson, C.G., Lange, M., Burge, D.J., 1999. A trial of etanercept, a recombinant tumor necrosis factor receptor: Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N. Engl. J. Med. 340, 253–259.

Yoshimura, N., Matsui, S., Hamashima, T., Oka, T., 1989. Effect of a new immunosuppressive agent, FK506, on human lymphocyte responses in vitro. I. Inhibition of expression of alloantigen-activated suppressor cells, as well as induction of alloreactivity. Transplantation 47, 351–356.