CONCISE REPORT

Addition of interleukin 1 (IL1) and IL17 soluble receptors to a tumour necrosis factor α soluble receptor more effectively reduces the production of IL6 and macrophage inhibitory protein-3 α and increases that of collagen in an in vitro model of rheumatoid synoviocyte activation

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Objectives: To evaluate the usefulness of combination treatment with cytokine inhibitors.

Methods: A simplified model was set up to evaluate the effect of tumour necrosis factor α (TNF α) soluble receptors (sTNFR) used alone and in combination with soluble interleukin 1 receptor (sIL1R) and sIL17R on the production of markers of inflammation (IL6), of migration of dendritic cells (macrophage inhibitory protein-3 α (MIP-3 α)), and of matrix synthesis (C-propeptide of type 1 collagen (P1CP)). Synoviocytes were stimulated with supernatants of activated peripheral blood mononuclear cells (PBMC) from patients with rheumatoid arthritis (RA). Soluble receptors (sR) were preincubated at 1 μ g/ml alone or in combination with the supernatants before addition to RA synoviocytes. IL6, MIP-3 α , and P1CP production was measured by enzyme linked immunosorbent assay (ELISA) in 48 hour synoviocyte supernatants.

Results: IL6 production decreased by 16% with sTNFR alone compared with no sTNFR ($p < 0.001$) and by 41% with the combination of the three sR ($p < 0.001$). MIP-3 α production decreased by 77% with sTNFR alone compared with no sTNFR ($p < 0.001$) and by 98% with the combination of the three sR ($p < 0.001$). In the presence of sTNFR alone, P1CP production increased by 25% compared with no sR ($p < 0.01$). The combination of the three sR increased P1CP production by 48% ($p < 0.01$).

Conclusion: The effect of sTNFR on IL6, MIP-3 α , and P1CP production by RA synoviocytes stimulated by activated PBMC supernatants was further enhanced when combined with sIL1R and sIL17R.

Treatment of rheumatoid arthritis (RA) with tumour necrosis factor α (TNF α) soluble receptors (sR) markedly improves clinical and laboratory parameters.¹ Thus inhibition of other cytokines which play a part in joint inflammation and are produced by monocytes (interleukin (IL)1) or T cells (IL17) may increase the degree and frequency of response.

We have recently shown that a combination of cytokine inhibitors increased the degree of response in an ex vivo model of synovium inflammation and bone destruction.² Using a more simplified model of interactions between cytokines produced by blood derived cells and acting on resident synoviocytes, we studied the effect of blocking sR on targets involved in the pathogenesis of RA. Firstly, we looked at IL6

production as a marker of inflammation. Secondly, as dendritic cells (DC) are critical for the induction and chronicity of the immune reaction, we studied the production of macrophage inflammatory protein-3 α (MIP-3 α), a chemokine involved in DC migration.³ Thirdly, to study an effect on potential repair, we looked at collagen synthesis using type 1 procollagen carboxy-terminal propeptide (P1CP), a marker of type 1 procollagen synthesis.⁴

In this study, TNF α soluble receptors (sTNFR) had a major effect on MIP-3 α production by RA synoviocytes stimulated with supernatants of activated peripheral blood mononuclear cells (PBMC). Conversely, we showed also a selective stimulation of P1CP synthesis. Moreover, these results were more pronounced when sTNFR were combined with sIL1R and sIL17R.

PATIENTS AND METHODS

Patients

The patients (14 women, six men) studied fulfilled the revised criteria of the American College of Rheumatology and had a median (SEM) age of 55 (3) years (range 31–83) and disease duration of 13 (3) years (2–51).⁵ Rheumatoid factor was present in 15 of the patients. Patients were treated with disease modifying antirheumatic drugs, alone or in combination, associated or not with prednisone or non-steroidal anti-inflammatory drugs.

Soluble receptors

Dimeric human TNFR p80/IgG1:Fc fusion protein (rhu TNFR:Fc), human soluble type II IL1 receptor (hu IL1RII), and murine IL17 receptor fusion protein (muIL17R:Fc) were obtained from Immunex, Seattle, WA, USA.^{6,7}

Peripheral blood mononuclear cells

PBMC were isolated from patients' blood by Ficoll-Hypaque density centrifugation. After one hour adhesion, PBMC (1×10^6 cells/ml) were cultured in RPMI medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum

Abbreviations: DC, dendritic cells; ELISA, enzyme linked immunosorbent assay; IL, interleukin; MIP-3 α , macrophage inflammatory protein-3 α ; PBMC, peripheral blood mononuclear cells; P1CP, type 1 procollagen carboxy-terminal propeptide; PHA, phytohaemagglutinin; PMA, phorbol 12-myristate 13-acetate; RA, rheumatoid arthritis; sR, soluble receptors; sTNFR, TNF α soluble receptors; TNF α , tumour necrosis factor α

Figure 1 Dose-response curve of sTNFR, sIL1R, and sIL17R alone or in combination on IL6 production. Supernatants (SN) of activated RA PBMC were incubated with synoviocytes and increasing concentrations of sTNFR, sIL1R, and sIL17R ranging from 0.1 to 100 µg/ml, used alone or in combination. IL6 levels were measured by ELISA in 48 hour supernatants. Results represent the mean of the effect of supernatants from two different patients with RA on the same RA synoviocytes.

and activated with 5 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO, USA) and 1 µg/ml phytohaemagglutinin (PHA; Sigma). Supernatants were harvested eight hours later.

Synoviocyte cultures

To isolate synoviocytes, RA synovium pieces were finely minced and digested with 4 mg/ml collagenase (Worthington, Freehold, NJ) in phosphate buffered saline (Life Technologies). Adherent cells were cultured in complete medium. Synoviocytes (10^4 cell/well) were incubated in 96 well plates in a final volume of 200 µl of complete medium. After adhesion, medium was replaced with 200 µl/well of PBMC supernatant and incubated for 48 hours. Direct stimulation of synoviocytes with PHA and PMA had no effect on the production of the factors studied here.

Measurement of IL6, MIP-3 α , and human type 1 collagen production

IL6, MIP-3 α , and P1CP production were measured in 48 hour culture media by enzyme linked immunosorbent assay (ELISA) as described.^{4,8,9}

Statistical analysis

Results were expressed as mean (SEM). Difference between the sR treated supernatants and the non-treated were analysed with the non-parametric Wilcoxon paired *t* test.

RESULTS

Effect of sTNFR, sIL1R, and sIL17R alone or in combination on IL6 production

We first investigated whether the same sTNFR used for the current treatment of RA, would modulate IL6 production. sTNFR was preincubated with RA PBMC supernatants for 30 minutes before addition to synoviocytes. At concentrations ranging from 0.1 to 100 µg/ml, IL6 production by synoviocytes decreased in a dose dependent manner (fig 1). At 1 µg/ml used in previous studies,^{2,9} IL6 production was decreased by 16% (67.7 (2.5) ng/ml with sTNFR *v* 81.0 (1.8) ng/ml without sR;

n=20; *p*<0.001) (fig 2A). The contribution of the IL6 carried over from the PBMC was negligible when compared with the high levels after synoviocyte incubation. Unstimulated synoviocytes did not produce IL6, and supernatants from unstimulated PBMC had no effect on IL6 production.

We next tested the possibility of increasing the effect of sTNFR on IL6 production by controlling cytokines other than TNF α . At 1 µg/ml, sIL1R decreased IL6 production by 14% in patients with RA (69.6 (2.1) ng/ml with sIL1R *v* 81.0 (1.8) ng/ml without sR; *n*=20; *p*<0.001) (fig 2A). With sIL17R, IL6 production decreased by 16% (68.0 (2.6) ng/ml with sIL17R *v* 81.0 (1.8) ng/ml without sR; *n*=20; *p*<0.001) (fig 2A).

Then, we used the combination of the three sR. At 1 µg/ml, IL6 production decreased by 30% with the combination compared with sTNFR alone (47.7 (2.9) ng/ml with three sR *v* 67.7 (2.5) ng/ml with sTNFR; *n*=20; *p*<0.001) and by 41% compared with no sR (47.7 (2.9) ng/ml with three sR *v* 81.0 (1.8) ng/ml without sR; *n*=20; *p*<0.001) (fig 2A). Thus, addition of sIL1R and sIL17R to sTNFR further reduced IL6 production by synoviocytes.

Effect of sTNFR, sIL1R, and sIL17R used alone or in combination on MIP-3 α production

Using the same system, we looked at MIP-3 α production. With sTNFR, the degree of inhibition of MIP-3 α production was much greater than that of IL6. Indeed, MIP-3 α production decreased by 77% (49 (18) pg/ml with sTNFR *v* 212 (39) pg/ml without sR; *n*=20; *p*<0.001) (fig 2B). This production was also decreased with sIL1R by 39% (129 (21) pg/ml with sIL1R *v* 212 (39) pg/ml without sR; *n*=20; *p*<0.01) (fig 2B). Likewise, sIL17R decreased MIP-3 α production by 38% (131 (32) pg/ml with sIL17R *v* 212 (39) pg/ml without sR; *n*=20; *p*<0.01) (fig 2B).

Finally, the combination of the three sR decreased MIP-3 α production by 90% compared with sTNFR alone (5 (2) pg/ml with three sR *v* 49 (18) pg/ml with sTNFR; *n*=20; *p*<0.001) and by 98% compared with no sTNFR (5 (2) pg/ml with three sR *v* 212 (39) pg/ml without sR; *n*=20; *p*<0.001) (fig 2B).

Figure 2 Effect of sTNFR, sIL1R, and sIL17R alone or in combination on IL6, MIP-3α, and P1CP production by RA synoviocytes stimulated with supernatants of activated PBMC from patients with RA. (A) IL6 production; (B) MIP-3α production; (C) P1CP production. Synoviocytes were cultured with supernatants (SN) of activated PBMC from patients with RA (n=20: IL6; n=14: MIP-3α and P1CP) and 1 μg/ml of sTNFR, sIL1R, sIL17R, alone or in combination. IL6 (ng/ml), MIP-3α (pg/ml), and P1CP production (ng/ml) were measured by ELISA in 48 hour synoviocyte supernatants. sR-treated SN versus non-treated SN: *p<0.001, **p<0.01.

Thus, addition of sIL1R and sIL17R to sTNFR had a massive effect on MIP-3α production.

Effect of sTNFR, sIL1R, and sIL17R used alone or in combination on P1CP production

The final consequence of RA inflammation results in matrix destruction and defective repair. We investigated the production of P1CP, a marker of type I procollagen synthesis. In the presence of sTNFR alone, such production increased by 25% (141.4 (12.5) ng/ml with sTNFR v 113.0 (9.1) ng/ml without sR; n=14; p<0.01) (fig 2C). Unlike sTNFR, no effect was seen with sIL1R and sIL17R alone.

The combination of the three sR increased P1CP production by 19% compared with sTNFR alone (167.8 (15.4) ng/ml with three sR v 141.4 (12.5) ng/ml with sTNFR; n=14; p<0.01) and by 48% compared with no sR (167.8 (15.4) ng/ml with three sR v 113.0 (9.1) ng/ml without sR; n=14; p<0.01) (fig 2C).

In conclusion, addition of sIL1R and sIL17R to sTNFR increased collagen synthesis. Combined together, these results indicate that the inhibition of the proinflammatory cytokines IL1 and IL17 further increased the inhibitory effect seen with TNFα alone.

DISCUSSION

To study the role of cytokines in the complex interactions found in RA synovium, we set up a simplified model with supernatants of activated RA PBMC producing proinflammatory cytokines and looked at their effect on RA synoviocytes. In this model, cytokines derived from activated PBMC induce the secondary production of soluble factors by synoviocytes. In particular, the cytokines studied here act on, but are not produced by, synoviocytes. TNFα and IL1 are mainly from monocytes and IL17 is produced by T cells. In addition, these results are in line with those obtained with synovium samples, which are obviously more difficult to obtain.^{2,9}

Having defined these conditions, we performed experiments to inhibit the action of the cytokines present in these supernatants. Because of additive or synergistic effects between cytokines, interpretation of a specific inhibition is rather complex. In early studies on IL17 in RA, when supernatants from RA synovium were added to synoviocytes, IL6 production was inhibited by two thirds with a blocking anti-IL17 antibody.¹⁰ Levels of IL17 could not be high enough to explain the inhibition of IL6 production, indicating an interaction between TNFα, IL1, and other factors, and IL17.

Clinical studies with soluble IL1 receptors have been performed thus far with the type I receptor, which has poor affinity for IL1β and high affinity for interleukin 1 receptor antagonist.¹¹ Here, we used the type II sIL1R, with the highest affinity for IL1β. In addition to the role of IL1 and TNFα produced by monocytes, the role of T cell derived cytokines has been debated. IL17 is exclusively produced by activated T cells. High concentrations of IL17 are produced by the RA synovium.¹⁰ IL1 in combination with IL17 increased IL6 secretion by synoviocytes synergistically.¹² Moreover, IL17 had a direct effect on the production of matrix metalloproteinase-1 and collagenase activity as well as on osteoclast resorption.^{13,14} Thus, IL17 may affect bone metabolism in RA and represents a potential target for RA treatment. The addition of sIL17R increased the effect of sTNFR and of sIL1R in part by reducing the enhancing effect of IL17 on IL1 and TNFα production.

DC are the major antigen presenting cells found in RA synovium, and are likely to have an important role in the pathogenesis of RA by presenting arthritogenic antigen to T cells.¹⁵ MIP-3α may also favour the recruitment of DC to synovium, as a role for MIP-3α was shown in the recruitment of immature DC at sites of inflammation.³ Here, we show that the three proinflammatory cytokines increased MIP-3α production by synoviocytes. In our model, this production was strongly

decreased with sTNFR, and almost completely inhibited with the combination of the three sR. Thus, inhibition of MIP-3 α production may contribute to a reduced immune activity inside the synovium.

Induction of repair of joint destruction is the major challenge of RA treatment. Here, PICP production was significantly increased only with sTNFR and in combination with sIL1R and sIL17R. A role for IL1 in cartilage destruction has been suggested in animal models, and its role in bone and cartilage repair is not clear. Our results suggest that the control of TNF α , either alone or together with the control of IL1 and IL17, is critical for the production of repair factors.

In conclusion, the effect of sTNFR in current treatment on these markers of inflammation, migration of DC, and matrix synthesis, was further enhanced by the addition of IL1 and IL17. Combination treatment may potentially increase the proportion of responding patients and the rate, degree, and duration of their respective response.

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