

Tumor Necrosis Factor Blockade Differentially Affects Innate Inflammatory and Th17 Cytokines in Rheumatoid Arthritis

SLADJANA M. ZIVOJINOVIC, NADA N. PEJNOVIC, MIRJANA N. SEFIK-BUKILICA, LJILJANA V. KOVACEVIC, IVAN I. SOLDATOVIC, and NEMANJA S. DAMJANOV

ABSTRACT. *Objective.* To evaluate the effect of a tumor necrosis factor- α (TNF- α) inhibitor (etanercept) on innate inflammatory and Th17 cytokines in patients with rheumatoid arthritis (RA).

Methods. Serum samples were collected from 40 patients with active RA refractory to conventional disease-modifying antirheumatic drugs who initiated therapy with etanercept plus methotrexate (MTX). Treatment response was assessed at Week 24 according to the European League Against Rheumatism response criteria. Serum levels of interleukin 6 (IL-6), TNF- α , IL-32, IL-23, IL-17A, IL-21, and IL-22 were measured in patients with RA and 25 healthy controls.

Results. Patients with RA had increased levels of IL-6 ($p < 0.001$), IL-32 ($p < 0.001$), IL-23 ($p < 0.001$), and a trend toward increased IL-21 in the sera compared to controls. At 24 weeks' posttreatment, followup serum samples of etanercept responders had decreased levels of IL-6 ($p < 0.001$) and increased IL-21 ($p < 0.05$) and IL-32 ($p < 0.001$), while there were no differences in cytokine levels in non-responders. Serum IL-6 levels were positively correlated with levels of erythrocyte sedimentation rate ($r = 0.458$, $p < 0.01$), C-reactive protein ($r = 0.593$, $p < 0.01$), and 28-joint Disease Activity Score ($r = 0.432$, $p < 0.01$) at baseline. Serum IL-21 levels were positively correlated with levels of rheumatoid factor ($r = 0.513$, $r = 0.633$, both $p < 0.01$) and antimutated citrullinated vimentin antibodies ($r = 0.515$, $p < 0.01$; $r = 0.428$, $p < 0.05$) at baseline and after 24 weeks of treatment with etanercept.

Conclusion. Multiple inflammatory pathways contribute to persistent chronic inflammation in RA. In contrast to nonresponders, etanercept therapy modulated serum cytokine levels and caused a marked decrease of IL-6 levels in responders. IL-21 might be involved in the regulation of autoantibody production in RA. (First Release Dec 1 2011; J Rheumatol 2012;39:18–21; doi:10.3899/jrheum.110697)

Key Indexing Terms:

RHEUMATOID ARTHRITIS
INNATE IMMUNITY

TUMOR NECROSIS FACTOR- α INHIBITORS
Th17 CYTOKINES

The inflamed synovial tissue in rheumatoid arthritis (RA) is characterized by a complex interplay between multiple immune cells and mediators¹. The important roles of innate inflammatory cytokines such as tumor necrosis factor- α

(TNF- α), interleukin 6 (IL-6), and IL-1 are well documented², and an autoinflammatory loop between TNF- α and IL-32 can contribute to chronic joint inflammation in RA³. IL-17 and Th17-associated cytokines could be crucial in the pathogenesis of RA, as IL-17 synergizes with TNF- α in the induction of proinflammatory cytokines and destruction of cartilage and bone⁴. Th17 cells predominantly produce IL-17, IL-21, and IL-22, while IL-23 is necessary for their survival and maintenance⁵. Therapy with etanercept, a TNF- α receptor antagonist, has led to successful control of RA⁶, but the reasons for inadequate response in a proportion of patients are not clear. Circulating cytokines may reflect disease activity and response to treatment with TNF- α inhibitors in RA⁷.

Our aim was to evaluate the pattern of innate inflammatory and Th17-associated cytokines in serum of patients with established active RA and to study the influence of anti-TNF therapy on levels of these cytokines in relation to clinical response.

MATERIALS AND METHODS

Patients. Forty patients with RA fulfilling the 1987 American College of Rheumatology (American Rheumatism Association) 1987 revised criteria for

From the Institute of Rheumatology and Institute of Medical Statistics, School of Medicine, University of Belgrade, Belgrade; and Center for Molecular Medicine and Stem Cell Research, Medical Faculty, University of Kragujevac, Kragujevac, Serbia.

Supported by grants from the Serbian Ministry of Science and Technological Development (175071 and 175069), Belgrade.

S.M. Zivojinovic, MD, Institute of Rheumatology, N.N. Pejnovic, MD, PhD, Professor, Institute of Rheumatology, Center for Molecular Medicine and Stem Cell Research, Faculty of Medicine, University of Kragujevac; M.N. Sefik-Bukilica, MD, PhD, Assistant Professor, Institute of Rheumatology, Faculty of Medicine, University of Belgrade; L.V. Kovacevic, MD, Institute of Rheumatology; I.I. Soldatovic, MD, Institute of Medical Statistics, Faculty of Medicine, University of Belgrade; N.S. Damjanov, MD, PhD, Professor, Head, Institute of Rheumatology, Faculty of Medicine, University of Belgrade.

Dr. Zivojinovic and Dr. Pejnovic contributed equally to this study.

Address correspondence to Prof. N.N. Pejnovic, Center for Molecular Medicine and Stem Cell Research, Medical Faculty, University of Kragujevac, Svetozara Markovica 69, 34000 Kragujevac, Serbia.
E-mail: nadap@ikomline.net

Accepted for publication August 18, 2011.

the classification of RA⁸ (37 women, 3 men, mean age 48 yrs, range 22–70 yrs), with a mean duration of disease of 7 years (range 2–20 yrs), with an unsatisfactory response to conventional disease-modifying antirheumatic drugs including methotrexate (MTX) and presenting with active RA were involved in our study. Patients received subcutaneous etanercept 50 mg/week with MTX 10–25 mg/week and were permitted to continue taking low-dose oral corticosteroids (prednisone < 10 mg/day) and/or nonsteroidal antiinflammatory drugs at a stable dose. The control group consisted of 25 sex- and age-matched healthy individuals. The criterion of clinical response was a decrease of the 28-joint Disease Activity Score (DAS28) index by > 1.2 points with reference to baseline according to the European League Against Rheumatism (EULAR) response criteria⁹.

Our study was approved by the ethics committee at the Institute of Rheumatology, School of Medicine, Belgrade, and was conducted according to The Helsinki Declaration. All patients gave written informed consent to participate.

Clinical and laboratory assessments. Patients underwent clinical and laboratory assessments just before and after 24 weeks of treatment with etanercept, including DAS28, Health Assessment Questionnaire-Damage Index (HAQ-DI), patient's global assessment of disease (by visual analog scale; VAS), serum C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), rheumatoid factor (RF) titers; and antimutated citrullinated vimentin (anti-MCV) antibodies (Organon, Oberschleissheim, Germany) were determined.

Serum samples. Serum samples were obtained at baseline and at 24 weeks posttreatment followup and stored at –80°C until assayed.

Measurement of innate inflammatory and Th17 cytokines. Serum levels of IL-6, IL-32, TNF- α , IL-17A, IL-21 (Biologend Inc., San Diego, CA, USA), IL-23, IL-22 (Bender MedSystems, Vienna, Austria) were measured using commercial ELISA kits.

Statistical analysis. Statistical analysis was performed using the SSPS 12.0 program. Data were compared with the Mann-Whitney U test or Wilcoxon matched-pairs signed-rank test. Correlations were assessed by Spearman's method. A p value < 0.05 was considered significant and p < 0.01 as highly significant.

RESULTS

Circulating cytokine profiles in patients with RA. Patients with RA had increased levels of IL-6, IL-32, IL-23, and a trend toward increased IL-21, and decreased levels of IL-17A and TNF- α in sera samples compared with healthy controls (Figure 1).

Serum cytokine levels and disease activity before and after etanercept treatment. There was a significant improvement of laboratory and clinical measures in patients with RA from baseline to 24 weeks after treatment with etanercept plus MTX, as follows: ESR (mm/h; p < 0.001), CRP (mg/l; p < 0.001), tender joint count (p < 0.001), DAS28 (p < 0.001), HAQ-DI (p < 0.001), patient's global assessment (VAS; p < 0.05), IgM RF (IU/ml; p < 0.01), and anti-MCV antibodies (U/ml; p < 0.01). A good/moderate EULAR response was acquired in 32 patients and 8 patients were nonresponders. In responders, serum IL-6 levels decreased significantly, while IL-32 and IL-21 levels increased at the 24-week posttreatment followup (Figure 2). No significant change in serum cytokine levels was found in nonresponders (Figure 2).

Correlation of serum cytokine levels and RA disease activity markers. Baseline serum IL-6 levels correlated positively with DAS28 (r = 0.432, p < 0.01) and levels of ESR (r = 0.458, p < 0.01) and CRP (r = 0.593, p < 0.01). Moreover, serum IL-21 levels correlated positively with levels of RF and anti-MCV antibodies both at baseline and at the 24-week post-treatment followup (Table 1).

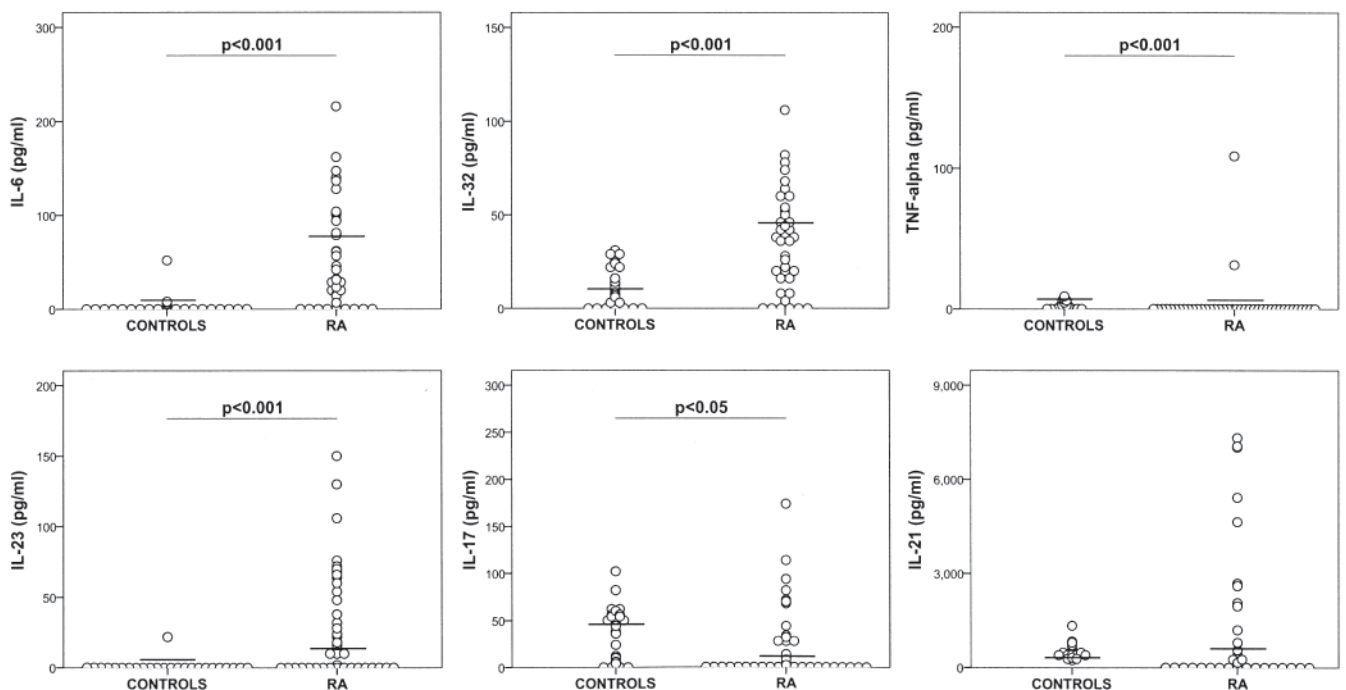


Figure 1. Baseline serum levels of innate inflammatory cytokines interleukin (IL)-6, IL-32, and tumor necrosis factor (TNF)- α , and Th17-associated cytokines IL-23, IL-17A, and IL-21 in healthy controls (n = 25) and patients with rheumatoid arthritis (RA; n = 40). Horizontal bars indicate the median.

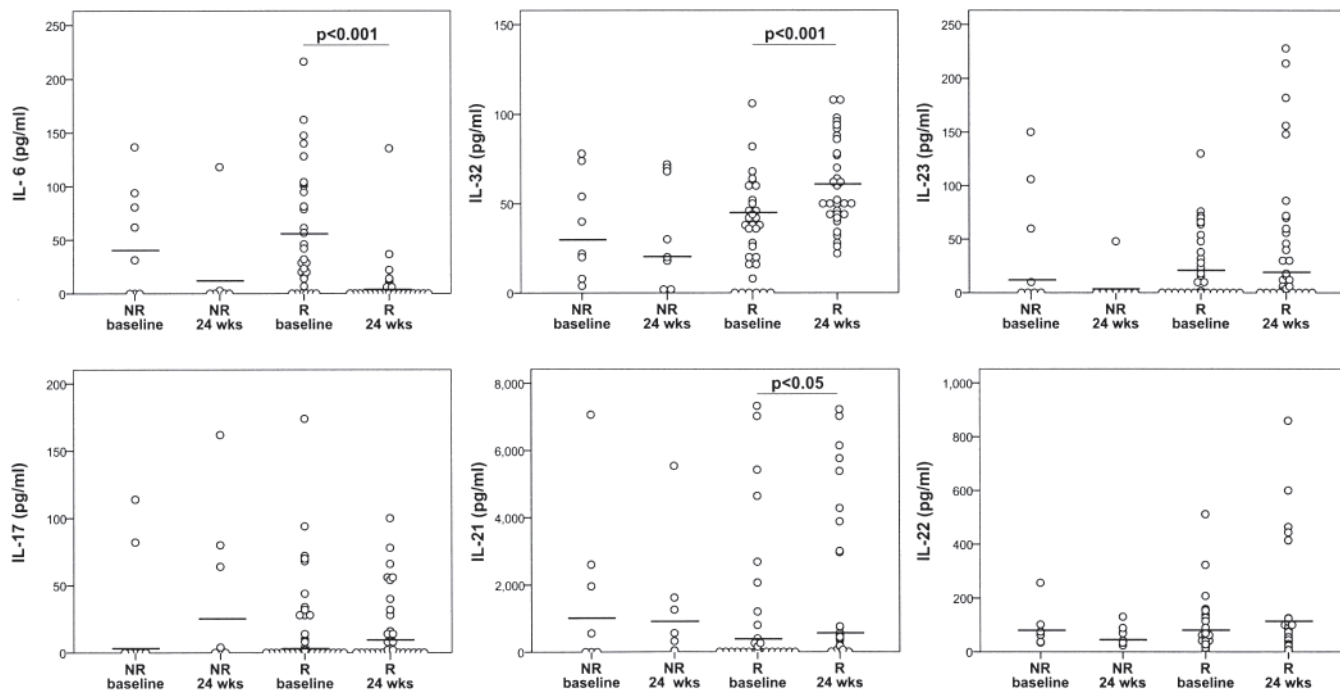


Figure 2. Serum levels of interleukin (IL)-6, IL-32, IL-23, IL-17A, IL-21, and IL-22 in patients with rheumatoid arthritis (n = 40) according to clinical response after 24 weeks of etanercept treatment. Horizontal bars indicate the median. NR: nonresponders; R: responders.

Table 1. Correlations (r_s) between serum IL-21 cytokine levels versus rheumatoid factor (RF) and antimutated citrullinated vimentin (anti-MCV) antibody levels in patients with rheumatoid arthritis, at baseline and at 24 weeks after treatment with etanercept.

	Baseline RF	Anti-MCV	24 Weeks RF	Anti-MCV
IL-21	0.513**	0.515**	0.633**	0.428*

Spearman's rank correlation; * $p < 0.05$; ** $p < 0.01$. IL: interleukin.

DISCUSSION

We demonstrated that a heterogeneous pattern of systemic multiple proinflammatory cytokines exists in patients with chronic active RA. The cytokines that regulate TNF- α production, such as IL-32, or Th17 cell function, such as IL-23 and IL-6, were elevated in patients with RA. The findings of lower serum IL-17A and TNF- α levels in patients with RA compared to healthy controls are similar to those of another recent study¹⁰. In addition, Th17 cells that coexpress TNF are recruited to the inflamed synovial tissue of affected joints in RA¹¹. In therapy responders, etanercept caused a marked decrease of serum IL-6 levels, which is at variance with the study showing decreased serum IL-23 levels and unchanged serum IL-6 levels in patients with RA receiving etanercept therapy¹². We recorded a notable increase in serum levels of one of the Th17-related cytokines, IL-21, which might be in accord with results of the recent study that showed increased IL-17 production by peripheral blood Th17 cells after

anti-TNF treatment¹³. In relation to increased IL-32 levels, the dissociated TNF from unstable complexes between TNF and etanercept¹⁴ could possibly affect its production.

Our findings suggest that IL-6 could serve as a good marker of disease activity in RA. This is the first report to demonstrate the significant association between serum IL-21 levels and levels of RF and anti-MCV autoantibodies in RA. IL-21 is known to promote B cell activation and differentiation, and an important role for IL-21 in the pathogenesis of RA in animal models was recently revealed¹⁵.

We showed that multiple immune pathways are activated in chronic active RA. Serum cytokine levels were not modulated by etanercept in nonresponders, while responders showed a marked reduction of serum IL-6 levels and increased levels of IL-21 and IL-32. Our data indicate that IL-21 might be involved in the regulation of pathogenic autoantibodies in RA.

ACKNOWLEDGMENT

We thank Prof. Miodrag L. Lukic for helpful comments on the manuscript.

REFERENCES

- Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. *J Clin Invest* 2008;118:3537-45.
- Petrovic-Rackov L, Pejnovic N. Clinical significance of IL-18, IL-15, IL-12 and TNF-alpha measurement in rheumatoid arthritis. *Clin Rheumatol* 2006;25:448-52.
- Heinhuis B, Koenders MI, van Riel PL, van de Loo FA, Dinarello CA, Netea MG, et al. Tumour necrosis factor alpha-driven IL-32 expression in rheumatoid arthritis synovial tissue amplifies an

- inflammatory cascade. *Ann Rheum Dis* 2011;70:660-7.
4. Van Bezooijen RL, van der Wee-Pals L, Papapoulos SE, Lowik CW. Interleukin 17 synergises with tumour necrosis factor alpha to induce cartilage destruction in vitro. *Ann Rheum Dis* 2002;61:870-6.
 5. Romagnani S, Maggi E, Liotta F, Cosmi L, Annunziato F. Properties and origin of human Th17 cells. *Mol Immunol* 2009;47:3-7.
 6. Caporali R, Bobbio Pallavicini F, Filippini M, Gorla R, Marchesoni A, Favalli EG, et al. Treatment of rheumatoid arthritis with anti-TNF-alpha agents: A reappraisal. *Autoimm Rev* 2009;8:274-80.
 7. Chandra PE, Sokolove J, Hipp BG, Lindstrom TM, Elder JT, Reveille JD, et al. Novel multiplex technology for diagnostic characterization of rheumatoid arthritis. *Arthritis Res Ther* 2011;13:R102.
 8. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315-24.
 9. van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism criteria. *Arthritis Rheum* 1996;39:34-40.
 10. Takeuchi T, Miyasaka N, Tatsuki Y, Yano T, Yoshinari T, Abe T, 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.
 11. Notley CA, Inglis JJ, Alzabin S, McCann FE, McNamee KE, Williams RO. Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells. *J Exp Med* 2008;205:2491-97.
 12. Kageyama Y, Ichikawa T, Nagafusa T, Torikai E, Shimazu M, Nagano A. Etanercept reduces the serum levels of interleukin-23 and macrophage inflammatory protein-3 alpha in patients with rheumatoid arthritis. *Rheumatol Int* 2007;28:137-43.
 13. Aerts NE, De Knop KJ, Leysen J, Ebo DG, Bridts CH, Weyler JJ, et al. Increased IL-17 production by peripheral T helper cells after tumour necrosis factor blockade in rheumatoid arthritis is accompanied by inhibition of migration associated chemokine receptor expression. *Rheumatology* 2010;49:2264-72.
 14. Scallon B, Cai A, Solowski N, Rosenberg A, Song X-Y, Shealy D, et al. Binding and functional comparisons of two types of tumor necrosis factor antagonists. *J Pharmacol Exp Ther* 2002;301:418-26.
 15. Young DA, Hegen M, Margery HL, Whitters MJ, Albert LM, Lowe L, et al. Blockade of the interleukin-21/interleukin-21 receptor pathway ameliorates disease in animal models of rheumatoid arthritis. *Arthritis Rheum* 2007;56:1152-63.