

Interleukin 22 serum levels are associated with radiographic progression in rheumatoid arthritis

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Accepted 31 March 2011
Published Online First
18 May 2011

ABSTRACT

Objectives To study the role of interleukin 22 (IL-22) in rheumatoid arthritis (RA).

Methods IL-22 serum levels were measured in patients with early, treatment-naive RA (n=49) and in 45 age- and sex-matched healthy individuals as controls. Patients were assessed clinically and radiographically at baseline and followed up for 2 years. Correlations of IL-22 serum levels were sought with parameters of disease activity, serological markers, demographic factors and the incidence of erosions. IL-22 production by peripheral blood T cells was investigated by intracellular flow cytometry.

Results 24 of 49 patients with RA demonstrated elevated IL-22 levels compared with the range of healthy controls. At baseline, a high percentage of these patients (8/24, 33%) demonstrated bone erosions, whereas only one patient (4%) from the group with normal IL-22 had erosions. During the 2 years of follow-up, six additional patients with increased IL-22 at baseline developed erosions. In contrast, none of the patients in whom IL-22 levels were normal developed erosions despite similar treatment regimens. Multivariate regression analysis accounting for other parameters predictive for erosions, such as the presence of rheumatoid factor or anti-cyclic citrullinated peptide antibodies and disease activity, showed that elevated IL-22 baseline levels were independently and significantly associated with erosive RA. Cellular analysis demonstrated enhanced expression of IL-22 from CD4 T cells in RA.

Conclusion IL-22 is elevated in the serum of half of the patients with RA. Elevated serum IL-22 allows discrimination between patients with different radiographic progression and indicates a possible involvement of IL-22 in the pathophysiology of RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterised by persistent synovitis and the development of erosive joint destruction. Imbalance in the expression of certain cytokines promoting the disease is a key feature of RA.¹ Interleukin 22 (IL-22) was recently identified as a cytokine potentially contributing to joint pathology. IL-22-deficient mice develop decreased pannus formation and bone destruction in the course of collagen-induced arthritis as compared with their wild-type littermates.² The receptor for IL-22, IL-22R1, is expressed in both the lining and the sublining layers of RA synovial tissues.³ Further, IL-22 was found to be elevated in patients with early RA, particularly in the synovium.⁴ In vitro,

IL-22 increases the proliferation of human RA synovial fibroblasts and the production of monocyte chemoattractant protein 1, a critical chemokine for the recruitment of macrophages into the joints during rheumatoid inflammation.³⁻⁵ IL-22 has also been shown to promote osteoclastogenesis from macrophages, further contributing to bone destruction in the arthritic process.²

IL-22 is primarily produced by activated T cells and natural killer cells. Among T cells, the highest expression of IL-22 has been detected in CD4 memory cells.⁶⁻⁷ Th17 cells have been consistently shown to co-express IL-22, which led to the suggestion that IL-22 is a Th17 cytokine.⁸ However, in humans significant IL-22 production was also demonstrated by so called 'Th22' cells—a recently described CD4 T cell population that is negative for IL-17A and interferon γ (IFN γ)—and also by Th1 cells.⁹⁻¹⁰ In addition to these subsets, cells with a mixed phenotype capable of producing all of these cytokines, IL-22, IL-17 and IFN γ , can be detected in human blood.⁹⁻¹¹

In this study, we studied the association of IL-22 expression with clinical parameters and the radiological outcome in a cohort of treatment-naive patients with early RA. About half (49%) of the patients showed elevated levels of serum IL-22 at baseline. Accordingly, patients could be subdivided into two groups: one with IL-22 levels within the normal range ('IL-22 normal' group, 25/49 patients) and another with markedly elevated IL-22 serum levels ('IL-22 high' group, 24/49 patients). Whereas no correlation was detected between IL-22 serum level and measures of clinical disease activity or specific clinical feature, the association between the presence of elevated IL-22 in the serum at baseline and the development of erosions was striking and highly significant after 1 and 2 years. The data suggest a pathophysiological role for IL-22 in bone erosion. As a potential source of the elevated IL-22 serum level, increased frequencies of IL-22-producing CD4 T cells were detected in the patients with RA compared with controls.

PATIENTS AND METHODS

Study population

Peripheral blood (PB) was obtained at the time of the first clinical evaluation from 49 patients (table 1) who fulfilled the American College of Rheumatology revised criteria for the diagnosis of RA.¹² The patients had active disease as defined by a Disease Activity Score in 28 joints (DAS28) of ≥ 3.2 . The patients had a mean RA duration of <5 months

Extended report

Table 1 Baseline clinical and demographic parameters of the study population*

Parameters	Healthy controls (n=45)	Patients with RA (n=49)
Age (years)	51.6±7.6	53.1±12.8
Female/male (n)	32/13	37/12
Smoking habit (%)	36.4	53.1
Disease duration (months)	NA [†]	4.0±3.4
NSAID use (%)	NA	72.9
RF positive (%)	ND [‡]	75.5
Anti-CCP positive (%)	ND	69.4
DAS28	NA	4.6±1.3
TJC (n)	NA	6.5±4.9
SJC (n)	NA	5.8±4.7
CRP (mg/dl)	ND	2.4±3.9
ESR (mm/h)	ND	31.5±33.3
Interleukin 22 (pg/ml)	3.3±1.4	15.1±15.9

*Data are shown as the mean±SD or absolute numbers.

[†]NA, not applicable.

[‡]ND, not determined.

anti-CCP, anti-cyclic citrullinated peptides; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; NSAIDs, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count.

since their initial clinical symptoms, and had never been treated with glucocorticoids or disease-modifying antirheumatic drugs (DMARDs). Demographic and clinical parameters such as age, gender, smoking, disease duration, use of non-steroidal anti-inflammatory drugs, tender joint count, swollen joint count, DAS28 values, erythrocyte sedimentation rate and levels of C-reactive protein, rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) antibodies were collected at the time of blood sampling. Radiographs of hands and feet were obtained at initial contact, and after 1 and 2 years after disease onset. For two patients baseline but no follow-up data were available as they were lost during follow-up. An independent experienced radiologist blinded to the clinical data and the study questions evaluated the radiographs for the presence of erosions.

Treatment of all patients was started with methotrexate (15 mg/week) often initially combined with glucocorticoids. If insufficient to control disease activity, the methotrexate dose was escalated and/or patients received a second DMARD (eg, leflunomide). In the case of conventional DMARD failure, biological agents, predominantly tumour necrosis factor (TNF) blocking agents were started (22.7% vs 23.8%, 'IL-22 normal' vs 'IL-22 high' group). The treating doctors were blinded to baseline IL-22 levels of the individual patient. For control, 45 age-matched healthy individuals were analysed (table 1). Written informed consent was provided by all patients and healthy donors. The study was approved by the ethics committee of the University of Munich.

Cell purification

Mononuclear cells from PB were isolated by Ficoll density-gradient centrifugation (Lymphoflot; Biotest AG, Dreieich, Germany). Untouched memory CD4 CD45RO T cells were isolated by negative selection using magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Purity was routinely controlled by flow cytometry. Purified T cells were more than 95% positive for CD3/CD4 and CD45RO.

Cell culture

Cell cultures were performed in RPMI 1640 medium supplemented with penicillin G (50 U/ml), 50 µg/ml streptomycin, 2 mM

L-glutamine (all from Gibco/Invitrogen, Carlsbad, California, USA), 10 IU/ml recombinant human IL-2 (Proleukin; Chiron, Emeryville, California, USA) and 10% normal human serum at 37°C in a humidified atmosphere containing 5% CO₂. Cells (0.5×10⁶ cells/ml) were activated in 48-well plates under non-priming conditions for 5 days by plate-bound anti-CD3 (1 µg/ml; OKT3; American Type Culture Collection, Manassas, Virginia, USA) and soluble anti-CD28 (1 µg/ml; 28.2; BD Bioscience, San Jose, California, USA), rested for 2 days and restimulated for 5 h with 1 mM ionomycin (Calbiochem, San Diego, California, USA) and 20 ng/ml phorbol myristate acetate in the presence of 2 µM monensin for intracellular cytokine detection (both from Sigma-Aldrich, St Louis, Missouri, USA).

Flow cytometry

For extracellular flow cytometry, cells were washed with 2% fetal calf serum in phosphate-buffered saline (FCS/PBS), stained with directly fluorochrome-labelled monoclonal antibodies against different surface molecules as indicated, and analysed on a Cytomics FC500 flow cytometer with CXP software (Beckman Coulter, Fullerton, California, USA). The antibodies used were as follows: fluorescein isothiocyanate (FITC)-labelled anti-CD3, phycoerythrin (PE)-labelled anti-CD4, FITC-labelled anti-CD27, PE-labelled anti-CD45RO (all from BD Biosciences).

For cytoplasmic cytokine staining, restimulated cells were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) and permeabilised with 0.1% saponin (Sigma-Aldrich) in PBS containing 2% FCS (Gibco/Invitrogen). The non-specific binding sites were blocked with 4% rat and mouse serum (both from Sigma-Aldrich). Afterwards, cells were incubated with FITC-labelled anti-IFN γ (BD Biosciences), PE-labelled anti-IL-22 and allophycocyanin-labelled anti-IL-17 (both from R&D Systems Minneapolis, Minnesota, USA) for 30 min at 4°C. After washing with 0.1% saponin in 2% FCS/PBS, cells were resuspended in 2% FCS/PBS and analysed for cytoplasmic cytokines by three-colour flow cytometry.

ELISA

Levels of IL-22, IL-17 and TNF in the serum samples were assessed using ELISA kits according to the manufacturer's instructions (R&D Systems eBioscience, San Diego, California USA, and Biolegend, San Diego, California, USA). Patients with RA were classified as 'IL-22 normal' or 'IL-22 high' if IL-22 serum levels did not or did, respectively, exceed the upper limit of the normal range as defined by the mean±3 SDs of 45 healthy individuals.

Statistical analysis

Differences between different cohorts of patients were determined by Mann-Whitney test, Student t test, or Fisher's exact test, where applicable. To account for confounding effects multivariate logistic regression analysis was performed. p Values <0.05 were considered statistically significant. The analyses were performed using SPSS 19 (SPSS, Chicago, Illinois, USA) and Prism 5.0 software (GraphPad Software, San Diego, California, USA).

RESULTS

IL-22 baseline levels in patients with RA are associated with radiological progression

To investigate the role of IL-22 in the systemic autoimmune disease RA, we first determined IL-22 levels in the PB of a cohort of patients with RA with very early, active disease (table 1). These patients had never been treated with glucocorticoids or

DMARDs to rule out possible influences of immunosuppressive drugs on cytokine expression. Serum IL-22 levels were higher in patients with RA than in healthy controls ($p < 0.0001$, table 1 and figure 1). Based on the IL-22 serum level, two groups of patients were identified within the RA cohort. A first group with IL-22 levels within the normal range was denoted as 'normal IL-22', and a second group with markedly elevated IL-22 serum levels was designated as 'high IL-22' (table 2). The two groups did not differ significantly for parameters of disease activity—for example, DAS28, tender joint count, swollen joint count, C-reactive protein levels and erythrocyte sedimentation rate at baseline or

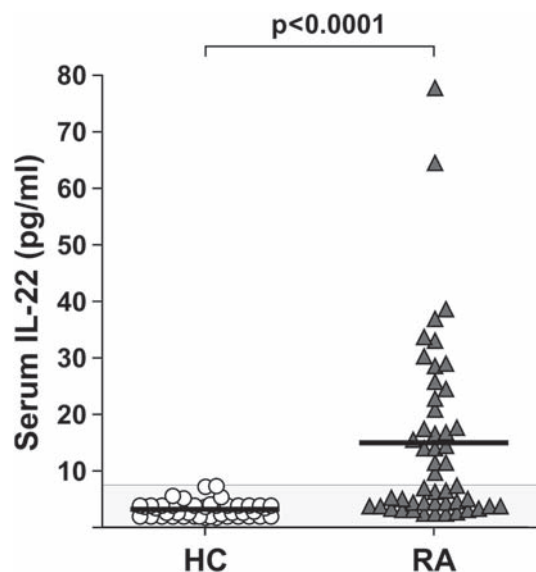


Figure 1 (A) Serum IL-22 concentration of healthy controls (HC; $n = 45$) and patients with RA ($n = 49$). The shaded area denotes the normal range (mean \pm 3 SDs) of healthy individuals. IL-22, interleukin 22; RA, rheumatoid arthritis.

Table 2 Clinical and serological parameters in patients with RA stratified according to the IL-22 serum level at baseline*

	'IL-22 normal' (n=25) [†]	'IL-22 high' (n=24) [‡]	p Value [§]
IL-22 (pg/ml)	4.3 \pm 1.4	26.2 \pm 16.1	<0.0001
Age (years)	54.7 \pm 11.1	51.3 \pm 13.8	NS
Female/male (n)	20/5	17/7	NS
Disease duration (months)	4.3 \pm 4.2	3.9 \pm 3.2	NS
NSAID use (%)	72.0	75.0	NS
Smoking habit (%)	44.0	62.5	NS
RF positive (%)	64.0	87.5	NS
Anti-CCP positive (%)	60.0	79.2	NS
DAS28	4.5 \pm 1.3	4.7 \pm 1.2	NS
TJC (n)	6.2 \pm 5.2	6.7 \pm 4.5	NS
SJC (n)	5.6 \pm 5.0	5.9 \pm 4.3	NS
CRP (mg/dl)	2.4 \pm 3.6	2.4 \pm 4.1	NS
ESR (mm/h)	31.8 \pm 33.0	31.3 \pm 33.7	NS
TNF (pg/ml)	0.2 \pm 1.0	0.5 \pm 1.9	NS
IL-17 (pg/ml)	2.8 \pm 3.9	8.7 \pm 13.9	<0.05

*Data are shown as mean \pm SD or absolute numbers.

[†]Patients with RA characterised by a normal IL-22 serum level (<7.5 pg/ml).

[‡]Patients with RA characterised by a raised IL-22 serum level (>7.5 pg/ml).

[§]p Values were calculated between the 'IL-22 normal' and 'IL-22 high' group by Mann-Whitney test or where applicable by Fisher's exact test.

anti-CCP, anti-cyclic citrullinated peptides; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; IL, interleukin; NSAIDs, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count; TNF, tumour necrosis factor.

during the follow-up (table 2 and data not shown). Although the proportion of autoantibody-positive (RF, anti-CCP) patients and the proportion of smokers was greater in the 'IL-22 high' group than in the 'IL-22 normal' RA group, this difference was not statistically significant. No correlation was detected, when serum levels of IL-17 and TNF were measured in order to assess whether other inflammatory cytokines correlated with the IL-22 serum level (table 2). However, although no direct correlation between serum IL-22 and IL-17 was evident, IL-17 concentrations were significantly greater in the 'IL-22 high' than in the 'IL-22 normal' group ($p < 0.05$).

The association of baseline IL-22 levels and radiographic progression as shown by the presence of erosions was assessed at baseline, and after 1 and 2 years of follow-up. To exclude confounding effects of other parameters associated with erosive disease we performed multivariate regression analysis correcting for the presence of anti-CCP antibodies and RF, and disease activity at baseline (table 3). Patients with high baseline IL-22 serum levels more often manifested bone erosions than patients from the 'IL-22 normal' group already at the onset of the disease (8/24 (33%) vs 1/25 (4%), $p = 0.05$) (table 3). Five additional patients had developed bone erosions at 1 year, and a sixth patient at 2 years of follow-up. All those patients were from the 'IL-22 high' group (table 3). As a consequence, at 1 and 2 years, the association of elevated IL-22 baseline serum levels with bone erosions was pronounced and significant (13/23 (57%) vs 1/24 (4%), $p < 0.01$, and 14/23 (61%) vs 1/24 (4%), $p < 0.005$, respectively) (table 3). Importantly, extending the regression model for confounding effects of smoking, age, gender, disease duration, each alone or in combination, did not alter the significant association of IL-22 baseline level with erosive disease.

Increased IL-22-producing CD4 T cells in RA

Next, we assessed the frequencies of different CD4 T cell populations producing IL-22 in patients with RA. Memory CD4 T cells were expanded for 5 days and analysed for intracellular cytokine production. CD4 T cells from four patients with RA contained substantially higher frequencies of IL-22-producing cells as compared with control cells (figure 2A,B). Three-colour analysis revealed that CD4 T cells from patients with RA contained significantly higher frequencies of 'pure' IL-22 producers ('Th22' cells; IL-22+IL-17-IFN γ -) (figure 2B). Moreover, in RA increased frequencies of IL-22-producing cells were found within the Th17 (IL-22+IL-17+IFN γ -) and the Th1 (IL-22+IL-17-IFN γ +) cell populations (figure 2B). The triple-producing cells (IL-22+IL-17+IFN γ +) were also increased in patients with RA (figure 2B). Interestingly, whereas the IL-22-producing fraction within the Th17 cells was of comparable size

Table 3 Association of baseline IL-22 serum levels with erosive disease

	'IL-22 normal' (n=25)*	'IL-22 high' (n=24) [†]	p Value [‡]
Erosive disease at baseline	1/25 (4)	8/24 (33)	0.05
Erosive disease after 1 year	1/24 [§] (4)	13/23 [§] (57)	<0.01
Erosive disease after 2 years	1/24 [§] (4)	14/23 [§] (61)	<0.005

Results are shown as number (%).

*Patients with RA characterised by a normal IL-22 serum level (<7.5 pg/ml).

[†]Patients with RA characterised by a raised IL-22 serum level (>7.5 pg/ml).

[‡]Multivariate regression analysis was performed to control for confounding effects of anti-CCP and RF positivity, baseline CRP and DAS28.

[§]One patient from each group was lost during follow-up.

anti-CCP, anti-cyclic citrullinated peptides; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints; IL-22, interleukin 22; RA, rheumatoid arthritis; RF, rheumatoid factor.

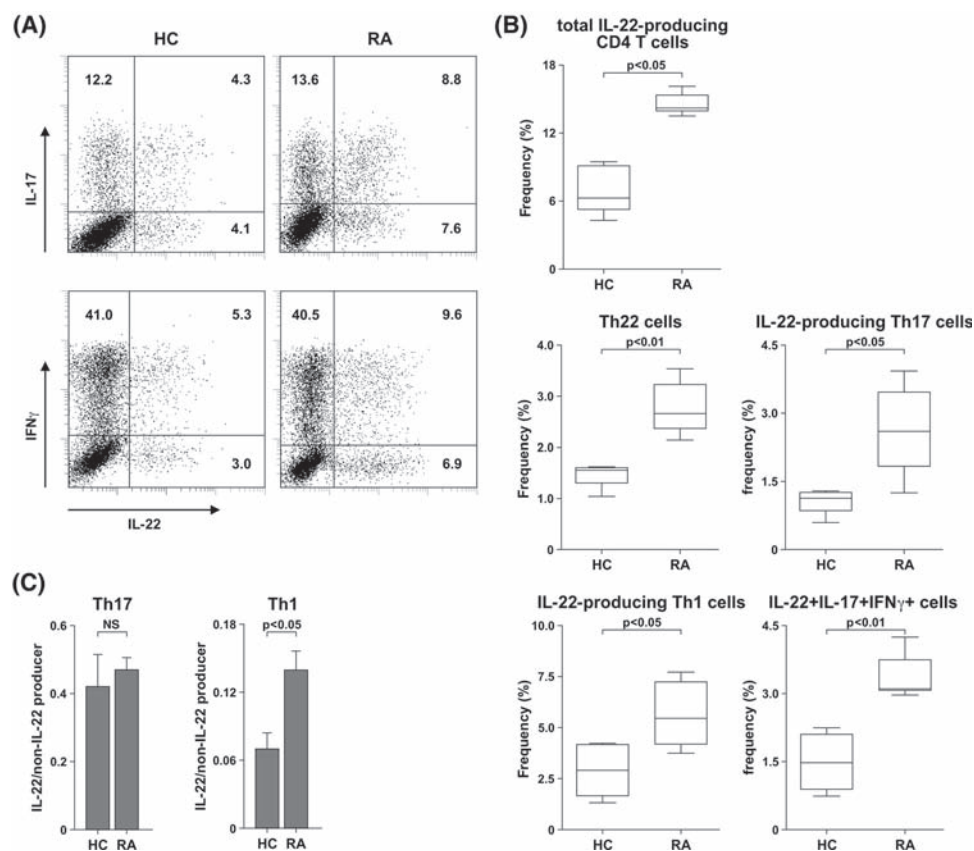


Figure 2 High interleukin 22 (IL-22) levels associated with synovitis in early rheumatoid arthritis (RA) derive from different CD4 T cell subpopulations. (A) Representative flow cytometry dot plots for expression of IL-22 and IL-17 (upper panels), and IL-22 and IFN γ (lower panels). Values indicate the cell frequencies in the respective quadrants. (B) Frequencies of IL-22-producing cells within CD4 T cells after 5 days of expansion, followed by stimulation with phorbol myristate acetate and ionomycin, and intracellular cytokine staining for IL-22, IL-17 and IFN γ . Box-and-whisker plots show the median, IQR, and 95% CI of five healthy controls (HC) and four patients with RA. (C) Ratios of IL-22-producing to IL-22-non-producing cells within Th17 cells (left panel) and Th1 cells (right panel). Bars show the mean + SEM of five HC and four patients with RA. IFN γ , interferon γ .

in patients with RA and in controls, it was specifically enriched in Th1 cells of patients with RA (figure 2C). Together, the data reveal an enhanced IL-22 expression by RA CD4 T cells. However, the increase of IL-22-producing Th17 cells in patients with RA is probably due to the general increase of Th17 cells as reported previously by us¹⁵—for example, both IL-22-producing and non-producing Th17 cells, while within Th1 cells, IL-22-producing cells were specifically enriched.

DISCUSSION

IL-22 has been recently suggested to be involved in joint pathology in autoimmune arthritis. Here, we have analysed IL-22 in patients with early, untreated RA. IL-22 serum levels were elevated in about 50% of the patients. Of potential implication for clinical rheumatology, elevated serum IL-22 at baseline was significantly associated with the development of bone erosions early in the course of the disease and might, thus, provide a novel means for predicting aggressive disease. As a potential source of elevated IL-22 levels in RA, increased frequencies of IL-22-producing CD4 T cells could be detected.

Whereas a murine study of autoimmune arthritis has pointed towards a role for IL-22 in arthritic joint destruction,² the knowledge about IL-22 in human autoimmune arthritis is limited. In RA, IL-22 mRNA was detected in synovial tissue directly as well as in synovial fluid mononuclear cells.³ The presence of increased Th17 cell-associated cytokines including IL-22, particularly in the synovium of patients with early RA, further supports the

idea that IL-22 might play a role in RA pathophysiology.⁴ With regard to the source of IL-22, IL-22 expression was observed in CD4 T cells infiltrating the inflamed synovial tissue in patients with juvenile idiopathic arthritis.¹⁴ Increased numbers of IL-22-positive CD4 T cells in the peripheral circulation of patients with RA have been reported.^{15 16} However, in those studies the authors did not investigate in detail the source of IL-22-producing cells within the CD4 T cell subsets—for example, Th1, Th17, or Th22. Based on our findings, elevated IL-22 production appears to be a general feature of inflammatory CD4 T cells in RA that is embedded in every CD4 T effector cell subset. This observation of elevated IL-22 production irrespective of a particular CD4 T cell subset emphasises the altered phenotype of CD4 T cells in RA and is in line with findings associating CD4 T cells with the disease development.¹⁷

An indication for the pathophysiological role of IL-22 in autoimmune diseases was initially provided by the analysis of patients with psoriasis. In those patients, elevated IL-22 serum levels correlated with disease severity.¹⁸ Data from a murine model of psoriasis-like autoimmune disease further support a pathogenic role of this cytokine in psoriasis as neutralisation of IL-22 prevented the development of skin manifestations.¹⁹ An association between IL-22 levels and disease activity has also been reported in patients with Crohn's disease.²⁰ In our study, we show for the first time an association between IL-22 and disease outcome in RA, implicating a potential pathophysiological role of this cytokine also in the course of RA. The

mechanisms by which IL-22 contributes to RA pathogenesis are not completely clear. IL-22 has no direct effects on immune cells. It targets fibroblasts and osteoclasts and might thereby mediate structural joint changes. The overall proinflammatory effect of IL-22 in arthritis seems, therefore, to be rather mild.²¹ This is in line with our observation, that patients with high and normal IL-22 serum levels did not differ in their clinical inflammatory parameters, such as disease activity, but did display a significant difference in erosive joint changes (table 3). Consistent with this, IL-22 knockout mice demonstrated reduced bone destruction in collagen-induced arthritis but their disease activity did not differ from that of their wild-type littermates.²

A recent study has demonstrated elevated expression of IL-22 in the bronchial mucosa of healthy smokers compared with healthy non-smokers, suggesting a link between smoking and increased expression of IL-22.²² Although the proportion of autoantibody-positive (RF, anti-CCP) patients as well as smokers was greater in the 'IL-22 high' group compared with the 'IL-22 normal' RA group, this difference was not significant. When we stratified the patients with high levels of IL-22 into smokers and non-smokers (to exclude the possibility that the difference in IL-22 expression between the two cohorts was biased owing to smoking), no significant difference in IL-22 serum levels was found. Even more importantly, the addition of the variable 'smoking habit' into the multivariate regression analysis did not change the significant and independent association between IL-22 baseline levels and erosive disease.

Manifestation and long-term course of RA are characterised by a considerable heterogeneity making it difficult to establish optimal treatment strategies. Although a number of studies have demonstrated the prognostic value of demographic, clinical, laboratory and genetic characteristics for the development of erosive RA, some of the results are conflicting, which indicates the need for further predictive factors.²³ Further biomarkers reflecting the outcome for joint damage would be clinically helpful to identify patients with a potentially aggressive disease course. The association of elevated IL-22 serum levels with bone erosions in patients with RA early in their disease suggests the potential of this cytokine as a predictive marker early in the course of the disease. Of the analysed patients, 24/49 demonstrated increased IL-22 serum levels very early in the disease (average disease duration of 4 months). Of those 24 patients, 33% had developed erosions of hands and feet already at their first visit to a rheumatologist. In contrast, only 4% of the patients with IL-22 serum levels within the normal range had developed erosions at baseline. In the course of the disease, elevated serum IL-22 at baseline became even more prognostic as in the group of 'IL-22 high' patients nearly two out of three patients had developed erosions after 2 years, in contrast to only one out of 24 'IL-22 normal' patients despite comparable treatment regimens. Thus, IL-22 might be useful as a serological marker for early predictions of joint erosions and might contribute to treatment stratification in patients with early RA. The data provide novel insight into the pathophysiology of RA, suggesting a possible role of IL-22 in arthritic joint destruction and defining a potential for this cytokine as a predictive marker of destructive disease.

Acknowledgements The authors thank Christine Schnabel for her expert technical assistance. The authors are grateful to Andreas Schramm, Stefan Schewe, Klaus Krüger, Veronika Brumberger and Christine Strasser for their help in recruiting patients. The authors are indebted to all patients and healthy individuals for their invaluable willingness to donate blood.

Funding This work was supported by the Deutsche Forschungsgemeinschaft, grants SK59/4-1, Schu 786/2-5, the Sonderforschungsbereich 571 (project D9), the Graduiertenkolleg 1202 (project E2) and by the FöFoLe programme of the medical faculty of LMU Munich.

Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the ethics committee University of Munich.

Provenance and peer review Not commissioned; externally peer reviewed.

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Ann Rheum Dis 2011 70: 1453-1457 originally published online May 18, 2011

doi: 10.1136/ard.2011.152074

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