Interleukin 10 (IL-10), Not IL-4 or Interferon-γ Production, Correlates with Progression of Joint Destruction in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Both interleukin 4 (IL-4) and IL-10, separately and in combination, and under *in vitro* and *in vivo* conditions in animals, have been reported to inhibit characteristics of rheumatoid arthritis (RA) and experimentally induced arthritis. We investigated if IL-10 and IL-4 production, as well as interferon-γ (IFN-γ) production, opposing IL-4, were related to RA disease variables. A method was chosen to exclude the influence of age and disease duration.

Methods. We selected RA patients with mild and severe disease. Inclusion criteria were erythrocyte sedimentation rate (ESR) ≤ 28 mm/h and ≥ 50, C-reactive protein (CRP) ≤ 20 and ≥ 30, Thompson joint score ≤ 60 and ≥ 100 and radiographic joint damage score, Sharp score ≤ 30 and ≥ 40. Age and disease duration were restricted: 30 to 70 years and 5 to 15 years, respectively. Peripheral blood mononuclear cells were isolated and the *ex vivo* 48 h production of T cell IL-10, IL-4, and IFN-γ (after CD3-CD28 stimulation) was assessed and was correlated to clinical variables.

Results. Only IL-10 production differed significantly between the 2 groups of RA patients, being highest in the "mild" group. Taking all patients together, a strong negative correlation was found between IL-10 production and radiographic joint damage ($r = –0.53$, $p < 0.001$) as well as progression of joint damage ($r = –0.56$, $p < 0.0001$). Similar negative correlations, although less powerful, were found between IL-10 production and ESR, CRP, and Thompson joint score. No correlation was found for IFN-γ, IL-4, or the ratio of the 2 with disease activity variables or joint damage.

Conclusion. The findings suggest that the high IL-10 production found in patients with RA may be protective, especially against progression of joint destruction in RA. (J Rheumatol 2001;28:1960–6)

Key Indexing Terms:
RHEUMATOID ARTHRITIS JOINT DAMAGE INTERLEUKIN 10
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Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology characterized by destruction of joints. There is increasing evidence that T lymphocyte directed macrophage activity plays a central role in the pathogenesis of the disease[1]. Although T cell activity in general seems to be suppressed in the RA joint, studies suggest a relative predominance of T1 cell activity[2-4]. T1 cell activity, characterized by interferon-γ (IFN-γ) production, participates in macrophage activation and thereby causes joint destruction[1]. T2 cell activity, characterized by interleukin 4 (IL-4) production, has been reported to counteract T1 cell activity and may thus diminish disease activity and joint destruction[5-8]. The balance between T1 and T2 cells regulates production of pro- and antiinflammatory cytokines produced by macrophages, granulocytes, and fibroblasts. Among them, IL-1, IL-6, tumor necrosis factor-α (TNF-α), IL-8, IL-10, and transforming growth factor-ß (TGF-ß) are the most extensively reported[9-11]. Systemic clinical signs of RA, such as the acute phase response [erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)], in turn depend on the balance between these pro- and antiinflammatory cytokines[12].

T2 cell activity is thought to be relatively low if not absent in the rheumatoid joint[2,3,13,14]. This is in contrast to IL-10, which is also seen as an immune suppressive cytokine in RA and is found to be elevated in RA synovial fluid and peripheral blood compared to controls[15,16]. IL-10 is produced by macrophages and, in humans, by both T2 and T1 cells as well as by B cells[17-20]. When administered in several human *in vitro* and animal *in vivo* experiments IL-4 and IL-10 have been found to be immunosuppressive, the combination of the 2 being the most effective[6,21,22]. *In vitro*

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experiments have revealed that both cytokines are able to downregulate a number of macrophage functions, including the production of IL-1ß, IL-6, and TNF-α[6,15,16,22]. In this context, it has been suggested that the elevated levels of IL-10 in RA are insufficient to downmodulate the "derailed" immune response in this disease.

Several studies have been undertaken to relate the clinical condition of RA patients with cytokine levels or *ex vivo* cytokine production[23-29]. Some studies have found such relations, whereas others were less conclusive. With respect to IL-4, IFN-γ, and IL-10 in relation to RA disease activity variables, reports are scarce. It has been reported that mononuclear cells (MC) from patients with active RA produced significantly less IFN-γ than mononuclear cells from control subjects or from patients with inactive RA[30]. Van Roon, *et al* recently showed that in patients with RA a decrease in IFN-γ and an increase in IL-4 production by peripheral blood mononuclear cells (PBMC) correlated with an increase in TNF-α, ESR, CRP, and joint destruction (Steinbrocker criteria)[27]. However, in none of these studies were age or disease duration taken into account. Yet some reports describe the influence of age on cytokine production in controls. In mice, IFN-γ production was higher in old compared to young animals[31]. In autoimmune mice IL-4 mRNA production of spleen cells increased with age, whereas IFN-γ remained unchanged[32]. In healthy humans both IFN-γ and IL-4 production by activated T cells was lower in old compared to young persons[33]. But in another study of healthy humans, IL-4 production increased with age[34]. With respect to IL-10, both in mice and in humans IL-10 production upon stimulation was higher in the older subjects[35,36].

This study was designed to determine if in RA patients the production of IL-10 and IL-4, as well as IFN-γ opposing IL-4, upon T cell costimulation of isolated blood MC, is related to disease activity variables and radiographic joint damage. To circumvent the influence of age and disease duration in the regulation of cytokine production, a study design was chosen comparing groups of RA patients with "mild" and "severe" disease that did not differ for age or disease duration.

MATERIALS AND METHODS

Patients. We selected 14 RA patients with mild disease and 23 with severe disease. The diagnosis of RA was based on the 1987 revised American College of Rheumatology criteria. Inclusion criteria were ESR (Westergren) ≤ 28 and ≥ 50 mm/h, CRP ≤ 20 and ≥ 30 U/ml, Thompson joint score (maximum score 534) ≤ 60 and ≥ 100, and joint damage on radiographs of hands and feet assessed according to the modified Sharp score (maximum score 448) ≤ 30 and ≥ 40[37,38]. Patients were between 30 and 70 years of age and disease duration was restricted to between 5 and 15 years. In this respect, the groups were comparable.

Cytokine measurement. The *ex vivo* 48 h production of IL-10, IL-4, and IFN-γ by an isolated PBMC population was evaluated. Peripheral blood was diluted 1:1 with Dulbecco's modified Eagle's medium (DMEM; Gibco 074-01600) and MC isolated by density centrifugation using Ficoll-Paque (Pharmacia Biotech, Roosendaal, The Netherlands). Isolated cells (5×10^5/ml) were cultured 48 h in DMEM supplemented with PSG (penicillin 100 U/ml, streptomycin sulfate 100 µg/ml, glutamine 2 mmol/l) and 10% pooled human adult male AB+ serum. IL-4 and IL-10 were assessed as a measure of potentially immune suppressive cytokine activity. IFN-γ production as a measure of T1 cell activity and opposing IL-4 (T2 cell activity) was measured as well. The ratio of IFN-γ to IL-4 production was calculated for every patient as a measure of relative T1/T2 cell activity. Because of the undetectably low spontaneous production of these cytokines and to measure predominantly T cell produced cytokines the PBMC were stimulated with anti-CD3-anti-CD28 monoclonal antibodies (both diluted 1:1000; CLB, Amsterdam, The Netherlands)[39,40]. IL-10, IL-4, and IFN-γ levels were determined by ELISA according to the manufacturer's instructions (Medgenix, Fleurus, Belgium). The detection limits of the assays were 5, 10, and 5 pg/ml for IL-10, IL-4, and IFN-γ, respectively. For healthy individuals such measurements showed stable cytokine production over time during several weeks[41].

Statistical analysis. For comparing patients with mild and severe RA, the Mann-Whitney U test was used. The production of cytokines was correlated to clinical variables by means of the Spearman correlation test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics. Table 1 shows the characteristics of RA patients with mild and severe disease. There was no statistically significant difference in mean age or disease duration, as we restricted for these criteria. As expected, a higher percentage of RF+ patients was found in the severe RA group. Disease activity is also reflected in the use of disease modifying antirheumatic drugs (DMARD); use of methotrexate and DMARD in general is higher in the group with severe disease. The number of previously used DMARD was also higher in the severe disease group. These statistics are shown in Table 1. The mean doses of the different DMARD in the mild and in the severe disease group were not statistically significantly different. No patient used oral corticosteroids at the time of the study. Figure 1 illustrates the clinical variables of both groups (ESR, CRP, Thompson joint score, and Sharp radiographic scores). Progression of joint damage was calculated for each patient by dividing radiographic joint score by disease duration. All these variables were statistically significantly lower in the mild RA group compared to the severe RA group.

Cytokine production. Figure 2 shows the *ex vivo* cytokine production of both groups. Although there was a trend toward lower IFN-γ concentration in the severe RA group, comparing both groups no statistically significant difference in IFN-γ and IL-4 production from PBMC was seen. Also, the IFN-γ/IL-4 ratios, reflecting T1/T2 cell balance, showed no statistically significant difference. On the other hand, *ex vivo* IL-10 production was statistically significantly higher in the patient group with mild disease ($p \leq 0.01$).

Because it was specifically relevant for this study, we tested the source of IL-10 production in the CD3-CD28 stimulated mixed MC population. For this purpose, CD3+ T cells were isolated using MACS cell sorting, and IL-10 production upon CD3-CD28 stimulation was compared with

Table 1. Characteristics and medication of the 2 selected groups of patients with mild and severe disease. Disease activity variables are given in Figure 1. The groups were compared by Mann-Whitney U test.

	Mild RA, n = 23	p	Severe RA, n = 14
Patient characteristics			
Mean age, yrs (range)	50.9 (30–69)		52.1 (35–68)
Mean disease duration, yrs (range)	8.5 (5–14)		10.9 (5–15)
Male/female	10/13		4/10
RF positive	13	≤ 0.01	13
DMARD medication			
Methotrexate	4	≤ 0.05	8
Sulfasalazine	2	NS	3
OH-chloroquine	5	NS	2
Penicillamine	1	NS	
Oral gold	1	NS	
Cyclosporine		NS	2
Combination therapy	0	NS	4
No DMARD	10	NS	3
No. of previous DMARD	0.9	≤ 0.0002	3.3

IL-10 production from the non-T cells and the mixed MC population. Indeed, it appeared that more than 95% of the IL-10 produced in the mixed MC population was T cell derived: 210 ± 77 and 10 ± 0 pg/ml IL-10 for the CD3+ and CD3– MC, respectively; 290 ± 21 and 196 ± 17 pg/ml IL-10 for the original MC population and the combination after isolation of CD3+ and CD3– cells (mean ± SD; n = 2).

A strong negative correlation was found between IL-10 production and Sharp radiographic joint damage score (r = –0.53, $p < 0.001$; Figure 3A). Thus, the more destructive the RA the lower the *ex vivo* IL-10 production by PBMC. Also the progression of joint damage (Sharp score/disease duration) correlated strongly with IL-10 production (r = –0.56, $p < 0.0001$; Figure 3B). Similar negative correlations, although less profound, were observed for IL-10 production and the other disease variables (Table 2). No correlation was found between IFN-γ, IL-4, or the ratio of both and the disease variables measured (Table 2).

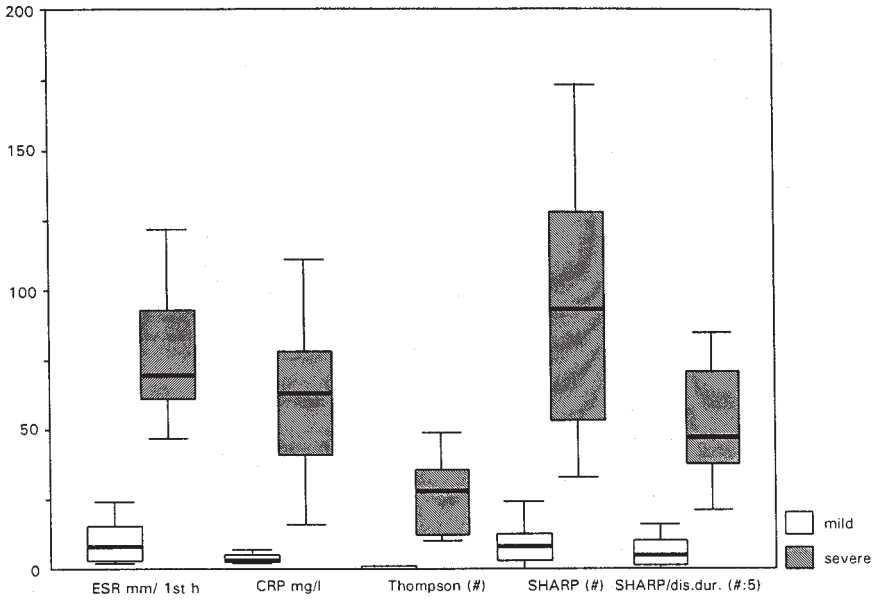

Figure 1. Median values and 25–75th and 10–90th percentiles for ESR, CRP, Thompson joint score, modified Sharp score, and modified Sharp score normalized for disease duration (Sharp/disease duration; calculated for every patient and averaged) of the 37 patients with RA. Averages of patients with mild disease (n = 23; white boxes) and with severe disease (n = 14; shaded boxes) are shown. All variables were statistically significantly different.

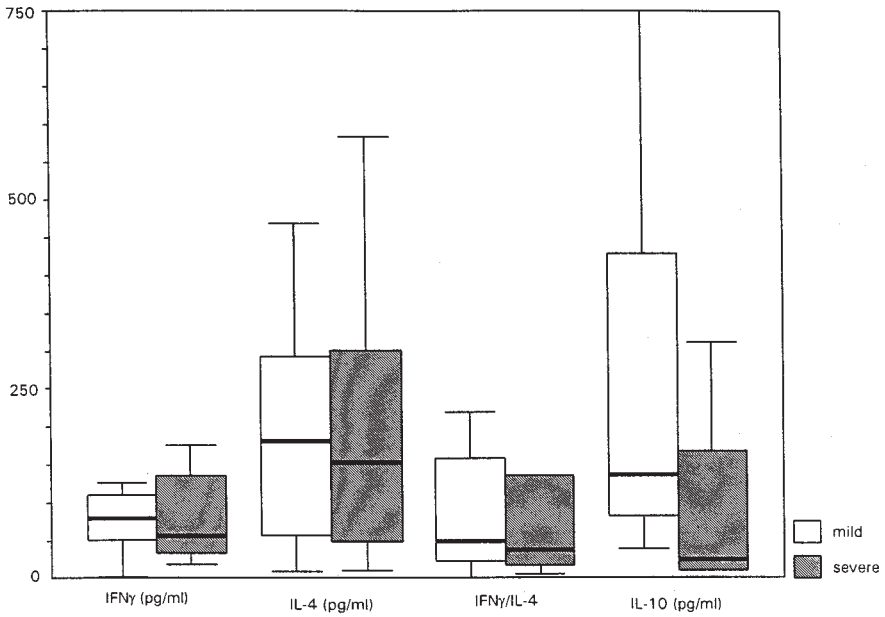

Figure 2. Median values and 25–75th and 10–90th percentiles for production of IL-10, IL-4, and IFN-γ and their ratio (calculated for every donor and averaged) of 23 RA patients with mild disease (white boxes) and 14 with severe disease (shaded boxes). No statistically significant differences were found between mild and severe RA except for IL-10 ($p \leq 0.01$).

Although we circumvented the influence of age by comparing groups of RA patients that did not differ in this respect, the window between 30 and 70 years of age enabled us to correlate production of cytokines with age. IFN-γ production correlated negatively with age of the RA patients ($r = –0.365$, $p \leq 0.031$), whereas IL-4 correlated positively with age ($r = 0.363$, $p \leq 0.038$). The IFN-γ/IL-4 ratio correlated negatively with age ($r = –0.280$, $p \leq 0.098$). Similarly, an age and sex matched group of healthy volunteers [n = 29; 18 women, 11 men, age 51 (35–62) yrs] showed a negative correlation for IFN-γ ($r = –0.405$, $p \leq 0.029$) and IFN-γ/IL-4 ratio ($r = –0.420$, $p \leq 0.026$) with age. In this population of healthy individuals IL-4 production showed no significant correlation with age.

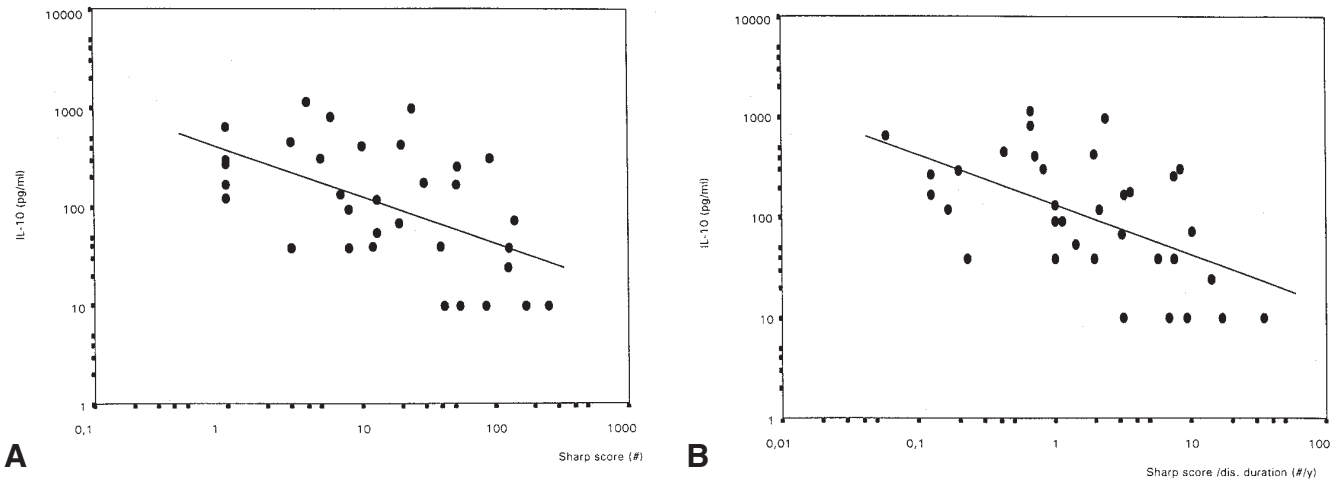

Figure 3. A. Correlation between IL-10 production of 37 RA patients (mild and severe disease together) and radiological damage by modified Sharp score (mild disease < 30, severe disease > 40). Spearman correlation of the absolute values revealed $r = –0.531$, $p < 0.001$. B. Correlation between IL-10 production and progression of joint destruction calculated from the individual Sharp scores normalized for disease duration (Sharp score/disease duration). Spearman correlation of the absolute values revealed $r = –0.554$, $p < 0.0001$ (n = 37; see Table 2). Detection limit for IL-10 was 10 pg/ml.

Table 2. Spearman correlations between *ex vivo* cytokine production of 37 patients with RA and disease activity variables. Sharp/disease duration was calculated as a measure for progression of joint destruction.

	IL-10		IL-4		IFN-γ		IFN-γ/IL-4	
	r	p	r	p	r	p	r	p
ESR	–0.374	≤ 0.012	0.065	≤ 0.351	–0.075	≤ 0.330	–0.073	≤ 0.334
CRP	–0.393	≤ 0.010	0.083	≤ 0.316	–0.143	≤ 0.202	–0.147	≤ 0.196
Thompson joint score	–0.279	≤ 0.050	0.194	≤ 0.125	0.047	≤ 0.392	–0.068	≤ 0.345
Joint damage (Sharp)	–0.531	≤ 0.001	–0.054	≤ 0.379	–0.111	≤ 0.262	–0.082	≤ 0.320
Sharp/disease duration	–0.554	≤ 0.000	–0.034	≤ 0.422	–0.171	≤ 0.163	–0.059	≤ 0.368

DISCUSSION

Our study clearly shows a relation between *ex vivo* peripheral IL-10 production and severity of joint damage as well as disease activity in RA. Such a relation was not found between *ex vivo* peripheral IL-4 or IFN-γ production and RA disease activity variables and severity of RA disease.

The lack of a relation between disease activity variables and *ex vivo* peripheral IL-4 and IFN-γ production following a specific T cell stimulus, as measures for differential T cell activity, was unexpected. Several studies have been undertaken to relate cytokine profiles with disease variables, but they were inconclusive. With respect to IL-4 and IFN-γ production as indicators of T2 and T1 cell activity, studies are particularly scarce[23,26,27]. Kanik, *et al* described increased production of IFN-γ in RA patients with new onset synovitis, in contrast to patients with chronic RA who had increased IL-10 production[23]. A correlation between IFN-γ and IL-4 production and disease variables was reported by Van Roon, *et al*[27]. However, in these studies the influence of age and disease duration in relation to disease activity could not be excluded. The design of our study was chosen to circumvent these variables. We compared 2 populations with severe and mild disease activity matched for age and disease duration. With this method no correlations between disease variables and IL-4 or IFN-γ production were found. That age and disease duration are indeed interfering with production of these T cell differentiating cytokines is confirmed by our RA population. Moreover, similar correlations between age and IL-4 as well as IFN-γ production were evident in a matched group of healthy volunteers. This corroborates findings of similar age related change in IFN-γ production in healthy individuals[33]. In this study, low IL-4 correlated with older age, whereas in another study high IL-4 correlated with older age[34]. In our group of healthy individuals no correlation with IL-4 and age was found.

On the other hand, the use of medication might explain the absence of a correlation between IL-4 and IFN-γ production and the disease variables. The patients in our study who were selected for active as well as destructive disease used more DMARD and had already failed therapy with several DMARD medications. From studies of several DMARD it is known that cytokine profiles change and correlate with response[42,43]. With respect to IFN-γ and IL-4 production, no influence of MTX could be shown[44]; on the other hand, this study showed an increase of IL-10 with MTX treatment. Thus in our study the higher number of MTX treated patients in the severe group seems to be in contrast with the low IL-10 production found in this group. Moreover, in our (relatively small) group the *ex vivo* peripheral IFN-γ, IL-4, or IL-10 production could not be attributed to DMARD use (data not shown).

In contrast to IL-4, as a potential immune suppressive therapeutic cytokine in RA, there was a strong correlation between disease variables and *ex vivo* peripheral IL-10 production. That we found higher *ex vivo* IL-10 production in the patients with milder disease plus the negative correlation between disease variables and IL-10 production is in accord with the immune suppressive activity of this cytokine in RA. In human *in vitro* and animal *in vivo* experiments, exogenously added IL-10 has been shown to be protective and suppressive in the course of RA, including cartilage and joint destruction[6,21,22]. Moreover, it has been reported that IL-10 is induced during the spontaneous remission of collagen induced arthritis[21]. The present study corroborates the finding that IL-10 production upon T cell stimulation in RA is related to disease suppression. In addition to disease activity measured by ESR, CRP, and Thompson joint score, the severity of RA measured by radiographic joint destruction and progression of joint destruction was even more negatively correlated with *ex vivo* peripheral IL-10 production.

There are probably many factors behind the differences in peripheral IL-10 production in RA. It has been suggested that low IL-10 production in patients with RA was associated with specific haplotypes. These patients had a more severe disease course, as indicated by the occurrence of a large number of erosions in the first 3 years of disease[45]. Similarly, in families of patients with meningococcal disease, variation in IL-10 production is genetically determined and is reported to be related to recovery of the disease[46].

In this study we were interested in T cell produced IL-4 and IL-10 as markers for suppressive T cell activity in comparision to IFN-γ production as a marker for T1 cell activity. We realize, however, that *ex vivo* IL-10 production may originate from more than just T cells. The enhanced T

cell activity within the mononuclear cell population will have its influence on monocytes and B cells as well. However, it has been reported that IL-10 after CD3-CD28 costimulation is mainly produced by T cells[47]. Moreover, a check on this revealed that indeed more than 95% of the IL-10 that is produced is T cell derived. Recently Yudoh, *et al*[48] showed that the frequency of a regulatory subset of T cells producing IL-10, but not IL-4, in RA synovium correlated negatively with disease activity scores. It is possible that our finding of increased IL-10 production, upon CD3-CD28 stimulation as a specific T cell stimulus, in RA patients with mild disease is due to an increased proportion of such regulatory T cells compared to severe RA. Thus, such a regulatory IL-10 producing T cell population, in addition to IL-10 in general, may have a disease controlling effect.

In summary, we observed that, for RA patients with similar age and disease duration, after specific T cell stimulation of blood mononuclear cells, *ex vivo* peripheral IL-4 and IFN-γ production does not correlate with disease activity variables. On the other hand, *ex vivo* IL-10 production correlates strongly and negatively with both the activity and severity of disease, a correlation not caused by differences in age or disease duration. Assuming a beneficial influence for endogenous IL-10 suggests that IL-10 in general or produced by specific T cell subsets might influence the course of RA.

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