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Decrease in peripheral type 1 over type 2 T cell cytokine production in patients with rheumatoid arthritis correlates with an increase in severity of disease

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Abstract

***Objectives*—To compare peripheral type 1 (T1) and type 2 (T2) T cell activities in rheumatoid arthritis (RA) patients with that found for osteoarthritic (OA) patients and healthy controls and to correlate peripheral T1/T2 cell activity in RA with parameters of the disease.**

***Methods*—Peripheral blood mononuclear cells were isolated from patients with RA (n=66), OA (n=19), and healthy controls (n=15). Primary T cell activity in these mononuclear cells was enhanced by means of anti-CD3/anti-CD28, which mimicks stimulation of T cells by activation of the T cell receptor and a major co-stimulatory signal. Interferon gamma (IFNγ) production and interleukin 4 (IL4) production in the three groups were quantified as measures of T1 and T2 cell activity, respectively, and compared. Serum tumour necrosis factor α (TNFα), erythrocyte sedimentation rate (ESR), C reactive protein (CRP), and joint destruction assessed radiographically of RA patients were determined as parameters of disease activity and correlated with T1/T2 cell activity.**

***Results*—Peripheral T cells from RA patients produced significantly less IFNγ and more IL4 than T cells from both age and sex matched OA patients and healthy controls. Moreover, in RA patients both a decrease in IFNγ and an increase in IL4 production correlated with an increase in serum TNFα, ESR, CRP, and joint destruction.**

***Conclusions*—These results suggest a role for differential T cell activity in RA. In view of the intra-articular T1 cell predominance the results might be explained by selective T1 cell migration into the joint or peripheral suppression of T1 cell activity.**

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It has been suggested that type 1 (T1) and type 2 (T2) T cells, defined by their characteristic cytokine profiles (interferon gamma (IFNγ) and interleukin 4 (IL4) being the major defining cytokines,[1 2]) play a significant regulatory part in rheumatoid arthritis (RA).[3] T1 cell activity effectively mediates cellular immunity, which can lead to proinflammatory responses. This activity includes the activation of macrophages, which is accompanied by the production of cytokines such as IL1 and tumour necrosis factor α (TNFα),[3] both of which are present in high concentrations in RA joints.[4–8] T2 cell activity mediates humoral immunity including the induction of IgE.[3] T1 and T2 cell activities are mutually inhibitory.[3] It thus has been suggested that the balance between T1 and T2 cell activity is important for regulation of the expression of the proinflammatory cytokines IL1, IL6, and TNFα in RA joints[3]; therefore, this balance is presumably instrumental in the control of joint damage in RA, which is caused largely by these cytokines.[9] It has been shown that systemic signs of inflammation, such as the acute phase response (that is, an increased erythrocyte sedimentation rate (ESR) and C reactive protein level (CRP)), largely depend on these proinflammatory cytokines.[9] Although the presence of T2 cell activity in RA joints has been shown by means of in situ production of IL4,[6] numerous studies have shown an intra-articular predominance of T1 cell activity.[10–14] Nevertheless, the association of these T cell activities with inflammation and joint damage in RA remains obscure.

Several groups have tried to correlate clinical parameters of RA and the spontaneous production of IFNγ[15 16] and IL4[16] by T cells from peripheral blood. These studies have revealed undetectable or low levels of IFNγ and IL4, which make it difficult to visualise the role of these cytokines in disease activity. Even under conditions of pronounced T2 cell activity, as in atopy and parasitic infections, spontaneously produced T2 cell cytokine levels are barely detectable both ex vivo and in vivo.[17 18] Nevertheless, in these latter studies, antigen or mitogen stimulation of peripheral blood T cells has shown clear differences in IFNγ and IL4 production, which correlate with disease markers.[17 18]

In this study we therefore chose defined co-stimulation (anti-CD3 with anti-CD28,[19–21]) to activate T cells to evaluate the capacity of peripheral T cells from patients with RA to produce IFNγ and IL4, which is indicative of T1 and T2 cell activity, respectively. IFNγ and

IL4 production by primary T cells from patients with RA was compared with that found for patients with osteoarthritis (OA) and healthy controls. Furthermore, the quantities of cytokines produced by T cells from patients with RA were correlated with severity of the disease, as indicated by the parameters of inflammation (serum TNFα, ESR, CRP) and radiographically detected joint destruction (Steinbrocker criteria).

Methods

PATIENTS AND CONTROLS

For analysis of T1 and T2 cell activity, mononuclear cells (MNC) were isolated from heparinised peripheral blood from 66 randomly selected patients with RA. RA was defined according to the 1987 revised ACR criteria.[22] Patients (50 women and 16 men) ranged in age from 25 to 84 years with a mean (SD) age of 60 (12). Mean (SD) disease duration was 13 (11) years with a range of 1 to 48. Fifty patients were rheumatoid factor positive and 16 were negative. Fifty nine patients received non-steroidal anti-inflammatory drugs, 48 took slow acting anti-rheumatic drugs, and 20 were taking low dose prednisone; three did not receive any medication. None of the patients had received recent bolus injection of corticosteroids. In addition, MNC were isolated from 19 randomly selected patients (16 women and three men) with OA (mean (SD) age 67.4 (6.9) years) and 15 healthy controls (12 women and three men; mean (SD) age 66.3 (6.9)). In the OA patient group two patients were taking NSAIDS, whereas 17 did not receive medication. To compare RA patients with OA patients and healthy controls an age and sex matched group was selected from the RA group (mean (SD) age 67.4 (7.0) years, n=19).

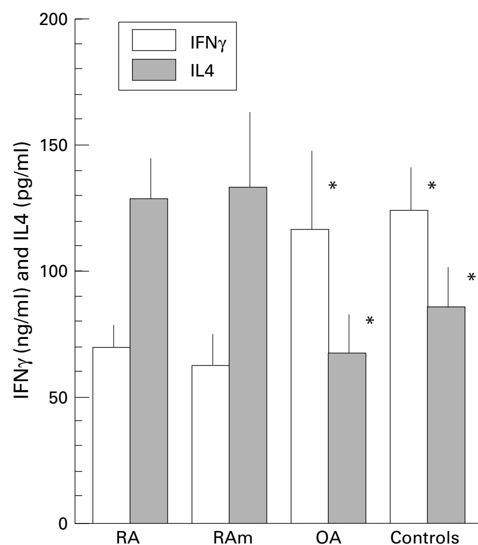

*Figure 1 IFNγ and IL4 production by anti-CD3/CD28 stimulated peripheral blood T cells (mononuclear cells) from patients with RA (n=66) and OA (n=19) and healthy controls (C, n=15). Data for a group of patients with RA matched for age and sex with OA patients and healthy controls are also shown (RAm). Means (SEM) are given. * Indicates a statistically significant difference between OA, controls, and RA patients ($p \leqslant 0.05$).*

Serum TNFα values and ESR of the RA patients were assessed. Serum TNFα was determined by ELISA (EASIA, Medgenix, Flerus, Belgium) according to the manufacturer's guidelines. In addition, CRP values for 29 patients and radiographs of both hands of all RA patients, taken within the last 12 months, were evaluated retrospectively. Radiographs were scored according to the Steinbrocker criteria[23] by two independent rheumatologists who were not aware of the patient's characteristics. The Steinbrocker criteria for hand radiographs were defined as follows: I–no destructive changes, but periarticular osteoporosis may be present, II–osteoporosis and slight cartilage and/or subchondral bone destruction are present, III–osteoporosis and cartilage and/or bone destruction, IV–stage III plus fibrous or bony ankylosis.

MONONUCLEAR CELL CULTURES

Peripheral blood was diluted 1:1 with Dulbecco's modified Eagle's medium (DMEM, Gibco 074-01600; 24 mM $NaHCO_3$) supplemented with glutamine (2 mM), penicillin (100 U/ml), and streptomycin sulphate (100 mg/ml; $DMEM^+$) and MNC were isolated by density centrifugation using Ficoll-Paque (Pharmacia). Viability of the cells, checked by trypan blue exclusion, was always more than 95%. Subsequently, MNC were cultured for 48 hours in $DMEM^+$ supplemented with 10% human male AB^+ serum (Red Cross Blood Transfusion Centre, Utrecht, the Netherlands). Spontaneous production of IFNγ and IL4 by these cells was increased by anti-CD3 and anti-CD28 antibodies (1:1000 v/v, CLB-T3/4.E, CLB-CD28, respectively, CLB, Amsterdam, the Netherlands). This stimulus activates T cells through the CD3 complex together with a costimulatory signal via the CD28 molecule.[19–21] After 48 hours of culture, conditioned media were harvested and freed of cellular material by centrifugation (five minutes, 900 × *g*), frozen in liquid nitrogen, and stored at −80°C. IFNγ and IL4 were assessed by ELISA (Medgenix, Flerus, Belgium) according to the manufacturer's guidelines. Detection limits were 50 pg/ml for IFNγ and 16 pg/ml for IL4.

STATISTICAL ANALYSIS

Statistical evaluation of differences between RA and OA patients was performed by the Student's *t* test for unpaired data. Correlations between cytokine production and disease parameters were evaluated with Pearson regression analysis.

Results

IFNγ AND IL4 PRODUCTION BY BLOOD T CELLS

Figure 1 shows IFNγ and IL4 production of RA patients compared with OA patients and healthy controls. Because OA patients and healthy controls did not match with RA patients for age and sex, a group was selected from the RA population that was age and sex matched (RAm). This matched RA population also was compared with the OA and healthy control groups. IFNγ production of peripheral

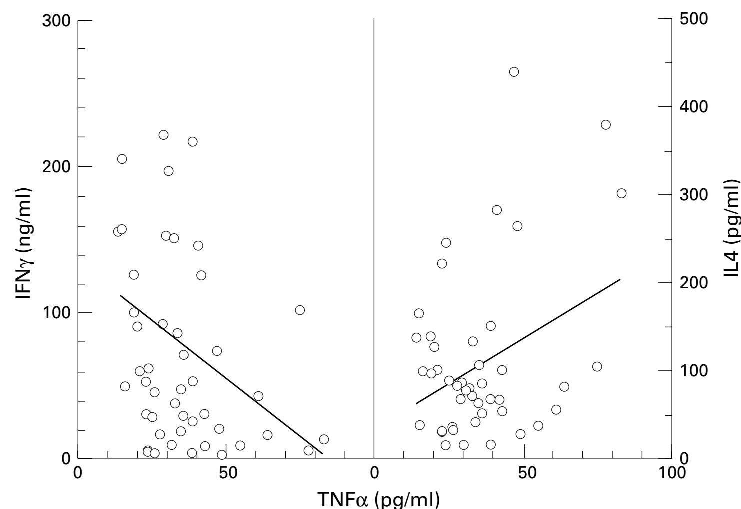

Figure 2 Correlation of IFNγ and IL4 production by anti-CD3/CD28 stimulated peripheral blood T cells (mononuclear cells) from RA patients with serum TNFα.

blood T cells from patients of both RA groups was significantly lower than that found for patients with OA (both $p \leq 0.05$) and healthy controls (both $p \leq 0.05$). The opposite was found for IL4 production, which was significantly higher for RA T cells than T cells from patients with OA ($p \leq 0.05$) and healthy controls ($p \leq 0.05$). Medication or the presence of rheumatoid factor did not reveal subpopulations of the RA group that differed in IFNγ and IL4 production.

RELATION BETWEEN CYTOKINE PRODUCTION AND DISEASE PARAMETERS OF RA PATIENTS

Figure 2 depicts the correlation between IFNγ and IL4 production by RA peripheral blood T cells and serum TNFα. With increasing serum TNFα, a decrease in IFNγ production was found ($r=-0.37$, $p \leq 0.01$), whereas IL4 production increased ($r=+0.33$, $p \leq 0.01$). The ratio of IFNγ and IL4 production decreased with increasing serum TNFα ($r=-0.48$, $p \leq 0.001$; not shown).

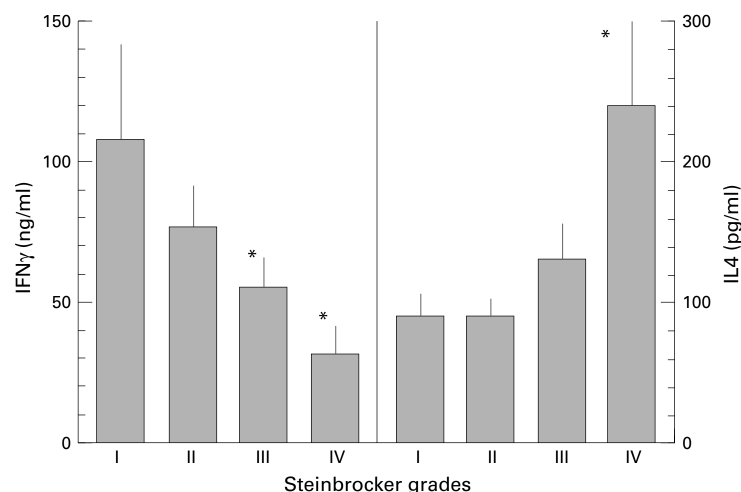

*Figure 3 Production of IFNγ and IL4 by anti-CD3/CD28 stimulated peripheral blood T cells (mononuclear cells) from RA patients divided according to the Steinbrocker radiological damage scores on hand radiographs. The numbers of patients per group were: I=14, II=19, III=25, and IV=8. * Indicates a statistically significant difference between group III or IV and group I (p≤0.05).*

Table 1 Correlations (r; and p values) of IFNγ, IL4, and the ratio of IFNγ to IL4 with disease parameters of patients with RA

	IFNγ		IL4		IFNγ/IL4	
	r	p ≤	r	p ≤	r	p ≤
TNFα	−0.37	0.01	+0.33	0.01	−0.48	0.001
ESR	−0.26	0.05	+0.38	0.01	−0.30	0.01
CRP	−0.41	0.01	+0.43	0.01	−0.49	0.01

Similarly, a decrease in IFNγ and an increase in IL4 production correlated with an increase in ESR and CRP. The ratio of IFNγ to IL4 production also decreased with an increase in ESR and CRP (table 1).

The relation of IFNγ and IL4 production by peripheral blood T cells and radiographically determined joint destruction in patients with RA is shown in figure 3. IFNγ production decreased whereas IL4 production increased with increasing joint damage, statistical significance for both cytokines being found for the group of patients with the most severe joint damage (both $p \leq 0.05$). No correlations were found between IFNγ and IL4 production and duration of RA.

Discussion

In general, spontaneous cytokine production by T cells in peripheral blood is low, even when T cell predominance is evident.[17 18] Anti-CD3/anti-CD28 co-stimulation of T cells is a frequently described[21] T cell stimulus, which considerably enhances both the IFNγ and the IL4 signal in T cells (correlation of IFNγ and IL4, $r=0.50$, $p \leq 0.001$), which may consist of CD4+, CD8+ as well as naive (CD45RA+) and memory (CD45RO+) T cells. This allowed us to study the overall cytokine secreting potential of peripheral T cells. Although there may be potential interactions of the used antibodies with Fc receptors on monocytes we were not able to note any difference in T cell stimulation using PBMC or monocyte depleted PBMC (data not shown, n=9). We furthermore have shown that this defined co-stimulation of T cell cytokine production by anti-CD3/anti-CD28 significantly correlated with that induced by ionomycin/PMA, a stimulus, which has been used to detect differences in T1 and T2 cell activity under several conditions— including RA.[12 17 18]

In this study it is shown that in RA, the cytokine pattern of blood T cells shows relative predominance of IL4 over IFNγ compared with that found for patients with OA and healthy controls. This shows that the potency of T1 over T2 cell activity is lower in the blood of RA patients than in that of patients with OA and healthy controls. Although surprising in view of the intra-articular T1 cell predominance,[10–14] our findings corroborate previous reports on a lower IFNγ production by mononuclear cells from the peripheral blood of patients with RA compared with controls, stimulated with phytohaemagglutinin or anti-CD3.[15 16] In the latter study that used anti-CD3, no significant difference in IL4 production by peripheral blood MC between RA

patients and healthy controls was found, although RA patients clearly tended to produce higher levels of IL4.[16] Lack of significance may be the result of the low number of patients in this study and the considerable variation in IL4 production by RA patients. Our data on the overall T cell cytokine production are also supported by the recent report that anti-CD3-stimulated IFNγ production by isolated RA peripheral blood CD4+ T cells is strongly reduced, whereas IL4 production by both CD4+ and CD8+ T cells is strongly increased compared with those of healthy controls.[24]

Importantly, this study shows an inverse correlation between ex vivo determined peripheral T1 cell activities and disease parameters of RA as well as a positive correlation of these parameters of disease with T2 cell activity, most clearly expressed by a decrease in T1/T2 cell cytokine ratio. These findings were surprising, as it was expected that predominant intra-articular T1 cell activtiy,[10–14] leading to activation of macrophages[4–8] and subsequently inflammation (monitored by serum TNFα, ESR, and CRP), would be found in the periphery as well. These findings may be explained by a selective migration of T1 cells from peripheral blood into the inflamed joint and consequently a decrease in IFNγ producing cells in peripheral blood. Recently, selective migration of T1 cells, compared with T2 cells, into inflamed joints has been shown in mice.[25] Furthermore, selective migration of T1 cells has been suggested after treatment of RA patients with antibodies against the intercellular adhesion molecule ICAM-1, which prevents trans-endothelial migration of T cells to the inflammatory site.[26] Prevention of the migration of T cells resulted in a specific increase in the number of IFNγ producing cells in peripheral blood from these patients, whereas no increase IL4 producing cells was seen. The increase in IFNγ producing cells in peripheral blood was related to clinical improvement,[26] which shows that migration of T1 cells, causing low T1 cell activity and relatively high T2 cell activity in peripheral blood, is responsible for the severity of the arthritis. The increased T2 cell activity could be caused by the decreased T1 activity and thereby decreased inhibition of T2 cell activity. This assumption is supported by the finding that the percentage IFNγ producing T cells and IFNγ production within the population of synovial fluid MNC were significantly higher than that within the paired peripheral blood MNC, whereas the number of IL4 producing cells and the IL4 production were higher in peripheral blood than in synovial fluid.[12]

Alternatively, there may be a specific RA related peripheral T2 cell activation, which can down regulate T1 cell activity. Recently, increased serum values of IL4 in RA patients were reported.[27] Although in this study higher production was associated with more severe disease activity, no significant correlations were found between serum IL4 and disease activity.[27] This finding as well as the lack of a clear association of T2 cell manifestations, like IgE and eosinophilia with RA disease activity, do not support the idea that increase T2 cell activity is primarily linked to RA.

Low peripheral T1 cell activity and high T2 cell activity might also be the consequence of factors, such as IL10 and transforming growth factor β,[28–31] which can change the T1/T2 cell balance in favour of T2 cell activity. In RA these factors have been shown to be produced by the intra-articularly activated macrophages. These mediators might occur in the periphery.[31] Intra-articular T1 cell activating signals may overcome these suppressive signals, maintaining a predominance of T1 cell activity. This idea is supported by significant production of IL1 and TNFα (as well as cartilage degradation) in the presence of high amounts of IL10 and transforming growth factor β.[28 30 32 33] However, in the absence of T1 cell activating signals in the periphery, these suppressive factors may lead to a change in the T cell balance in favour of T2 over T1 cell activity.

Finally, the observed differences in T cell balance may be because of differences in medication of the patients. However, in our study we did not observe significant differences in T1 or T2 cell activity by RA patients receiving different treatments. The number of patients in groups receiving different medication may however be too small to permit such an analysis.

These data show that the balance of peripheral T1/T2 cell cytokine production is directly associated with the severity of RA, pointing to a role for these differentiated T cell activities in RA. Future experiments may reveal the origin of the T1/T2 cell imbalance in RA.

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