# Interleukin-10 expression: Is there a neglected contribution of CD8+ T cells in rheumatoid arthritis joints?

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## Abstract

### Objective

*To search for RA specific processes among T cell accumulation, T cell activation, or cytokine expression in CD4+ and CD8+ synovial fluid (SF) T cells*

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### Methods

*Flow cytometry of CD4+, CD8+, CD45RA+, CD45RO+, CD69 double or triple stained peripheral blood (PB) and SF T cells. IL-2, IL-10, and IFN-γ expression was determined in PMA + ionomycin stimulated T cells on the single cell level. Concentrations of secreted IL-2, IL-4, IL-10, and IFN-γ were quantified in the sera and synovial fluids by enzyme linked immunosorbent assay (ELISA).*

### Results

*A preferential recruitment of CD45RO+ memory T cells was found for CD4+ helper T cells, and in similar also for CD8+ suppressor T cells. An elevated CD69 expression was detected in memory, but also in CD45RA+ naive CD4+ and CD8+ SF T cells, whilst IL-2 expression was only demonstrable in a minor proportion of T cells populations. Preferential recruitment of memory T cells, but incomplete activation of naive and memory, CD4+ and CD8+ T cells were in similar found in RA and control patients. In RA but not in the control patients, a relevant proportion of CD4+ and CD8+ PB and SF T cells expressed IL-10 and IFN-γ. High concentrations of IL-10, that were correlated with the amounts of secreted TNF-α, were only detected in RA joints.*

### Conclusion

*Memory and naive T cell state of CD4+ and CD8+ T cell accumulates in the joints, and early T cell activation occur in similar patterns in RA and control patients. High IL-10 SF concentrations in contrast, and elevated percentages of IFN-γ and IL-10 expressing CD4+ and CD8+ T cells in the PB and SF were characteristic for RA. Here, CD8+ T cells may contribute to high IL-10 concentrations in RA joints.*

**Key words**

Arthritis, interleukin-10, T cell, suppressor, cytotoxic.

*This work was supported by grants from the Riese and Klein foundations, Johann Wolfgang Goethe University of Frankfurt, Germany.*

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*Received on March 28, 2002; accepted in revised form on September 4, 2002.*



## Introduction

The synovial membrane in healthy individuals is composed of a thin lining layer with fibroblast-like (type B) synoviocytes (FLS). The sublining tissue is represented by FLS, lipocytes and blood vessels (1). In rheumatoid arthritis (RA), a disease defined by clinical classification criteria (2), this normal state undergoes dramatic changes. The morphology of synovitis may to some extent be heterogeneous in RA (1, 3), but cellular hyperplasia of the lining layer with highly activated macrophages (type A synoviocytes), and T and B lymphocytes infiltrating into the sublining tissue are common features of RA synovitis.

Lymphocyte infiltrates in the sublining acquire different forms of structural organization dependent on the predominant cytokine milieu (3). Organization into secondary lymphoid organs reflects B cell activation and is associated with rheumatoid factor (RF) production. This histologic RA variant of follicular synovitis indicates chronic aggressive disease, and it is correlated with high transcript levels for IFN-γ and IL-10. Diffuse T cell infiltration with low IL-10 levels in contrast is associated with a milder course of the disease (3). In accordance to the Th1 or Th0 predominance in RA (4, 5), single cell analyses confirmed CD4+ and CD8+ T cells being an important source of IFN-γ and IL-10 in RA synovial membranes and synovial fluids, whilst IL-4 and IL-13 expressing T cells are barely demonstrable (6, 7). Despite abundant infiltration of T cells into the RA synovial membranes, the major part of T cells is incompletely activated, and cytokine production in T cells thus appears relatively low (8). This discrepancy has led to a controversial discussion about the potential roles for T cells in RA pathogenesis (8-11). CD4+ helper T cells, in particular of the Th1 type, are claimed to propagate RA, whilst CD8+ suppressor T cells in RA joints might provide an anti-inflammatory capacity. However, CD8+ T cells do also have cytotoxic potential that might be of relevance for disease initiation or disease progression. In this context we were interested of whether CD8+ SF T cells differ from CD4+ SF T cells by their naive or memory composition, or their activation state, what might indicate a differential recruitment, or different modes of activation in these distinct T cell populations.

Here, we investigated the composition in separate for CD4+ helper and CD8+ suppressor T cells for their naive (CD45RA+) or memory (CD45RO+) state, their expression of the CD69 early activation marker, and cytokine production in the peripheral blood and synovial fluid in order to assign RA specific patterns of T cell recruitment and activation.

## Patients and methods

*Study population.* Simultaneously obtained PB and SF specimen were collected from 53 consecutive patients with a severe synovitis and joint fluid effusion of the knee joint (> 30 ml) worthy of arthrocentesis,and one of the following diagnoses: 35 patients fulfilled the ACR classification criteria for rheumatoid arthritis (2); 31 of them were RF positive. 18 pairs of sera and synovial fluids were obtained from control patients with an acute flare of psoriatic arthritis (n = 6), ankylosing spondylitis with peripheral joint involvement (n = 6), postinfectious arthritis with prior symptoms of diarrhea or urethritis (each n = 2), undifferentiated seronegative oligoarthritis (n = 2), juvenile idiopathic arthritis (oligoarticular, RF negative, n = 1), or Lyme´s disease (n = 1). Peripheral blood was also taken from 12 healthy donors. All human materials were obtained after receiving informed consent in accordance to the declaration of Helsinki principles.

Twenty-two of the 35 RA patients, and 4 of the 18 control patients received systemic corticosteroids (RA: 0-17.5 mg per day, non-RA control patients: 0-7.5 mg per day). 19 of 35 RA and 6 of 18 control patients received a single agent DMARD therapy with methotrexate (MTX), sulfasalazine (SSZ), intramuscular gold salts, or leflunomide. A triple combination DMARD therapy (MTX + SSZ + hydroxychloroquine) was given in 3 RA, and a TNF-α

blocking agent (etanercept) was administered in 2 RA patients.

*Methods*

*Cell isolation.* PBMC and synovial fluid mononuclear cells (SFMC) were obtained from 50 ml freshly drawn heparinized blood by Ficoll Hypaque (Seromed, Berlin, Germany) density centrifugation, or by centrifugation (1500 rpm at 4°C) from 30 to 180 ml synovial fluid samples. Separated mononuclear cells were thoroughly washed in phosphate buffered saline (PBS).

*T cell phenotype analysis.* 5 x $10^5$ unstimulated PBMC or SFMC were analyzed by triple-immunostaining for the following T cell surface markers: Helper T cells were stained with phycoerythrin (PE)-conjugated anti-CD4 $IgG_1$ monoclonal antibodies (mAbs), cytotoxic/suppressor T cells were detected by fluorescent isothiocyanate (FITC)-conjugated anti-CD8 $IgG_1$ mAbs (12). CD45RO+ T cells, which are referred to as memory T cells (13), were stained with Cy5-conjugated anti-CD45RO $IgG_2$ mAbs (Serotec, Oxford, UK). CD45RA+ T cells, determined by indo-cyanine dye (Cy5)-conjugated anti-CD45RA $IgG_2$ mAbs (Serotec), represented the naive T cells (13). An early state of T cell activation was assessed by immunostaining with FITC-conjugated anti-CD69 $IgG_1$ mAbs (14). Unless otherwise stated, mAbs were purchased from Becton Dickinson, Franklin Lane, NJ.

*Intracellular cytokine staining*. PBMC or SFMC (2 x $10^6$ cells/ml) were stimulated in tissue culture tubes (Falcon) with phorbol myristate acetat (PMA, 5 ng/ml) and ionomycin (0.75 g/ml) in the presence of the secretion inhibiting agent monensin (3 µM) for 4 hours at 37°C in a 5% $C0_2$ containing humidified atmosphere. Stimulated cells were washed, suspended in PBS with 1% FCS and immunostained for 30 minutes with peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 $IgG_1$ mAbs (Becton Dickinson), and FITC or PE-conjugated anti-CD8 $IgG_1$ mAbs (Becton Dickinson). Surface marker stained cells were fixed in ice-cold paraformaldehyde/HBSS solution and stored at 4°C in the dark over night. Fixed cells were washed and incubated in PBS containing 0.5% saponin and 5% human AB-serum for 10 minutes at room temperature. Permeabilized cells were exposed for 10 minutes to either FITC-labeled anti-IFN-γ or anti-IL-2 $IgG_1$ mAbs, or to PE-labeled anti-IL-10 $IgG_2$ mAbs, and for control to isotype-matched control mAbs. Stained cells were suspended in FACS buffer solution containing PBS and 1% FCS prior to flow cytometry. The applied anti-cytokine mAbs were provided from Hölzel Diagnostika, Cologne, Germany.

*Flow cytometry*. The staining intensity of T cells was quantified by FACScan (Becton Dickinson) with Win MDI software (La Jolla, CA). The chosen gate for the entire T cell population based on the typical T cell characteristics in the forward and side light scatter. The percentage of stained cells was calculated on the basis of isotype-matched controls (<2% positive cells). In analyses requiring PMA+ionomycin stimulation, CD4+ T cells were indirectly characterized by their negativity for CD8 staining, a procedure demanded by the artificially reduced CD4+ expression in T cells through phorbol esters (6). Helper and suppressor T cells were gated on their characteristics for CD3 and CD8 immunostaining. The presented results base on at least 1x$10^4$ T cells per analysis.

*ELISA*. Commercial ELISA kits (Roche Diagnostics, Mannheim, Germany) were applied for quantitative IFN-γ, IL-2, IL-4, IL-10, and TNF-α analyses in the sera and synovial fluids, that were obtained by centrifugation of the cell pellets. Assays were performed in precise accordance to the manufacturers instructions.

*Statistics*. Results are given as the mean and standard error of mean (SEM). The statistical significance of differences between groups was determined by the non-parametric Wilcoxon's test for 2 tailed groups, or the Mann Whitney test for 2 untailed groups. Whenever appropriate, the test for tied groups was calculated. Correlations were calculated by Pearson´s coefficient.

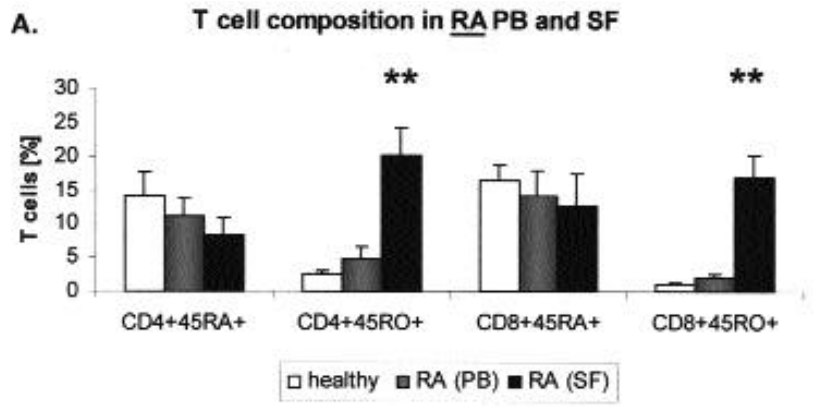
**Results**

*Composition of PB and SF T cells*

The proportion of CD4+ and CD8+ T populations, and the composition of CD45RA+ naive, and CD45RO+ memory T cells in the CD4+ and CD8+ T cell subsets were not significantly different in the PBMC from healthy donors, RA patients, and in control patients with other types of arthritis. In RA synovial fluids, the proportion of both the CD4+ and CD8+ T cells in SFMC was significantly increased when compared with matched RA PBMC (CD4: PB 34.6%; SF 42.1%, $n = 10$, $p < 0.05$; CD8: PB 19.6%, SF 48.1%, $n = 10$, $p < 0.01$). The CD4/CD8 ratio in RA SF T cells was thereby reduced in comparison with the CD4/CD8 ratio in RA PB T cells from average 1.78 to 0.88. In similar, a reduced CD4/CD8 ratio was also detected for the synovial fluid T cells in the non-RA control patient group when compared with the corresponding PB T cells. The proportion of CD45RO+ memory T cells was significantly increased in the RA joints (45.9%) when compared with corresponding peripheral blood specimen (CD45RO: 14.6%), and 2 color flow cytometry analysis showed a similar increase for CD4+ and CD8+ memory T cells in RA synovial fluids (Fig. 1A). In similar, a preferential accumulation of CD4+ and CD8+ memory T cells could be detected in the synovial fluids from control patients. The accumulation of CD8+CD45RO+ memory suppressor T cells in the inflamed joints was less in these patients (Fig. 1B).

*Activation of PB and SF T cells*

In RA, the proportion of CD69 expressing PB T cells was modestly increased in memory and naive cells of the helper and suppressor T cell compartment when compared with healthy donor PB T cells (Fig. 2A). In contrast to the preferential accumulation of memory T cells into inflamed RA joints, not only the memory T cells, but also significantly increased proportions of CD4+CD45 RA+ and CD8+CD45RA+ naive T cells expressed the CD69 T cell activation marker (Fig. 2A). In the control patients, the CD69 expression level was in



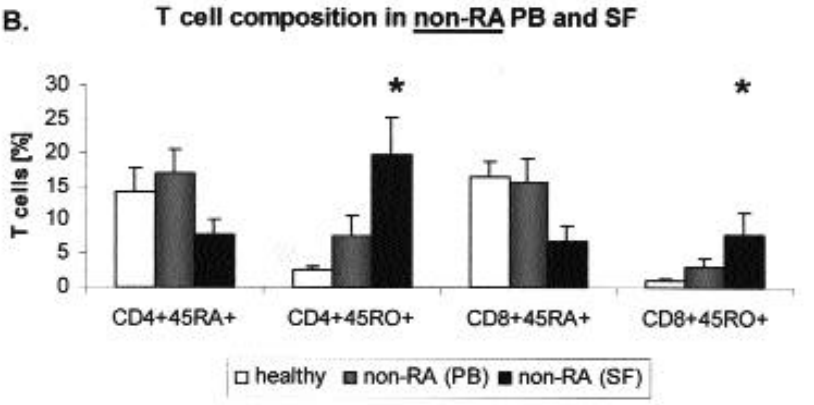
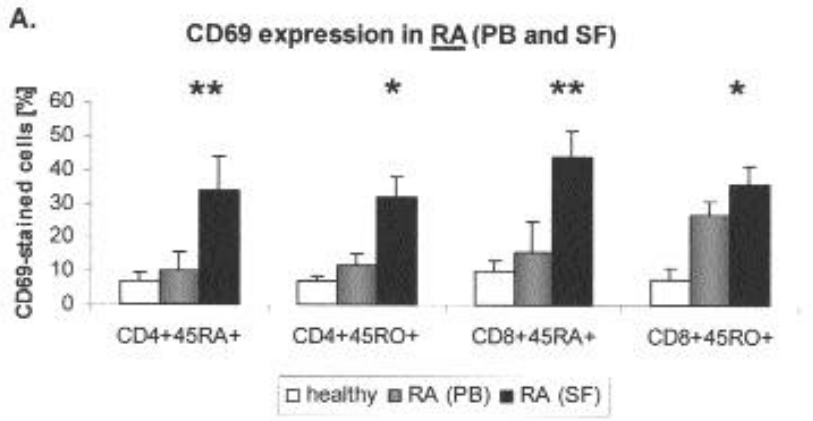




**Fig. 1.** Composition of peripheral blood and synovial fluid T cells in (**A**) RA and (**B**) control patients. A preferential accumulation of CD45 RO+ memory T cells in the joints could be shown for the CD4+ and the CD8+ SF T population (black bars) when compared with the peripheral blood T cells (gray shadowed bars) irrespective from the underlying type of arthritis. Bars represent the quantity (mean ± SEM) of T cells, determined in each $5x10^4$ mononuclear cells by triple immunostaining in corresponding PB and SF specimen (RA:n = 8, non-RA:n = 9, healthy: n = 7).



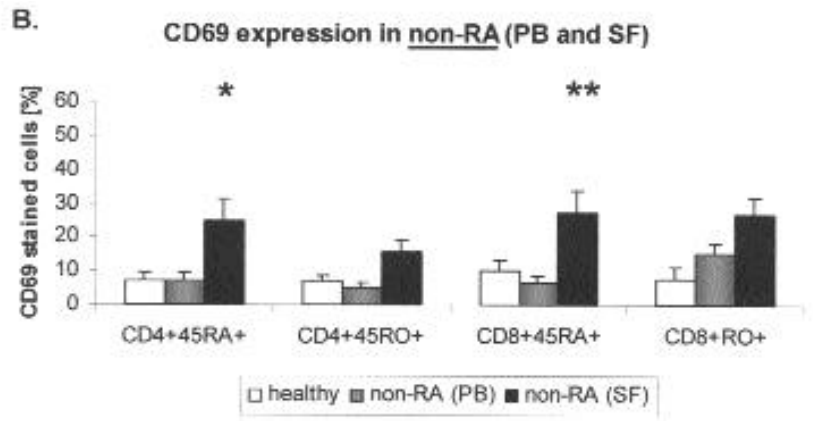




**Fig. 2.** CD69 T cell activation marker expression in peripheral blood and synovial fluid T cells in (**A**) RA and (**B**) control patients. CD69 expression is increased in both, the memory and naive T cells of the CD4+ and CD8+ T cell populations in the synovial fluid (black bars) when compared with the peripheral blood (gray shadowed bars) in RA and control arthritis patients. Bars represent the mean ± SEM of CD69+ T cells, determined in each $5x10^4$ mononuclear cells by triple immunostaining in corresponding PB and SF specimen (RA:n = 8,non-RA:n = 9, healthy: n = 7).

average lower for all PB and SF T cell subsets than in RA patients. However, qualitatively similar results, with an increased CD69 expression in all distinguished SF T cell populations when compared with the corresponding PB T cells were also detected in the control patients (Fig. 2B). In particular, statistical significance of this result could be shown for the CD4+CD45RA+, and the CD8+CD45RA+ naive SF T cell populations when compared with the low CD69 expression levels in CD45RA+ PB T cells (Fig. 2B). In respect of these similar T cell accumulation and activation patterns in the joint fluids from RA and control patients, we next focused on the T cell cytokine expression in RA and non-RA patients.

*Cytokine expression in PB T cells*

The proportion of IFN-γ expressing PB T cells, and the number of IL-10 expressing PB T cells was significantly increased in RA patients when compared with healthy donor PB T cells (Fig. 3). Low amounts of IL-2 expressing PB T cells were likewise detected in RA patients and in healthy donors. In the control patients in contrast, the proportion of IFN-γ and IL-10 producing PB T cells was essentially the same as determined in healthy donors, whilst the proportion of IL-2 expressing PB T cells was significantly reduced in the control patients when compared with healthy donor PBMC (n = 12, $p < 0.01$).

*Cytokine expression in SF T cells*

The proportion of IFN-γ expressing T cells was elevated in the SF when compared with matched PB T cells in 13 of 21 analyses performed in RA patients. The proportion of IL-10 producing SF T cells was consistently elevated in all experiments in comparison with the corresponding PB T cells (Fig. 3). Analyses performed in parallel to these experiments revealed negative IL-10 staining results for CD14+ SF monocytes and CD19+ SF B cells (data not shown). Neither in the RA nor in the control patient population, significant differences were detected for the average number of cytokine expressing CD4+ or CD8+ SF T cells in the entire populations, although some statistically significant differences were found in PB T cell analyses (Table I). Irrespective from the underlying health state, the proportion of IL-2 expressing cells was in general higher in CD4+ PB T cells, and the average number of IFN-γ and IL-10 expressing cells was higher in CD8+ PB T cells. In particular, a significantly higher proportion of IFN-γ expressing CD8+ than CD4+ T cells was detected in healthy donor and RA PB T cells, but this difference was equalized in RA SF T cells (Table I). Of note, the proportion of IFN-γ and IL-10 expressing CD4+ and CD8+ SF T cells could be most different within the same joint: for example, a strong IL-10 expression in CD8+, but a low frequency of IL-10 expressing CD4+ T cells was seen within the same joint fluid aspirate obtained from a patient suffering from unequivocal RF-positive, longstanding, but non-erosive RA (Fig. 4A-D).

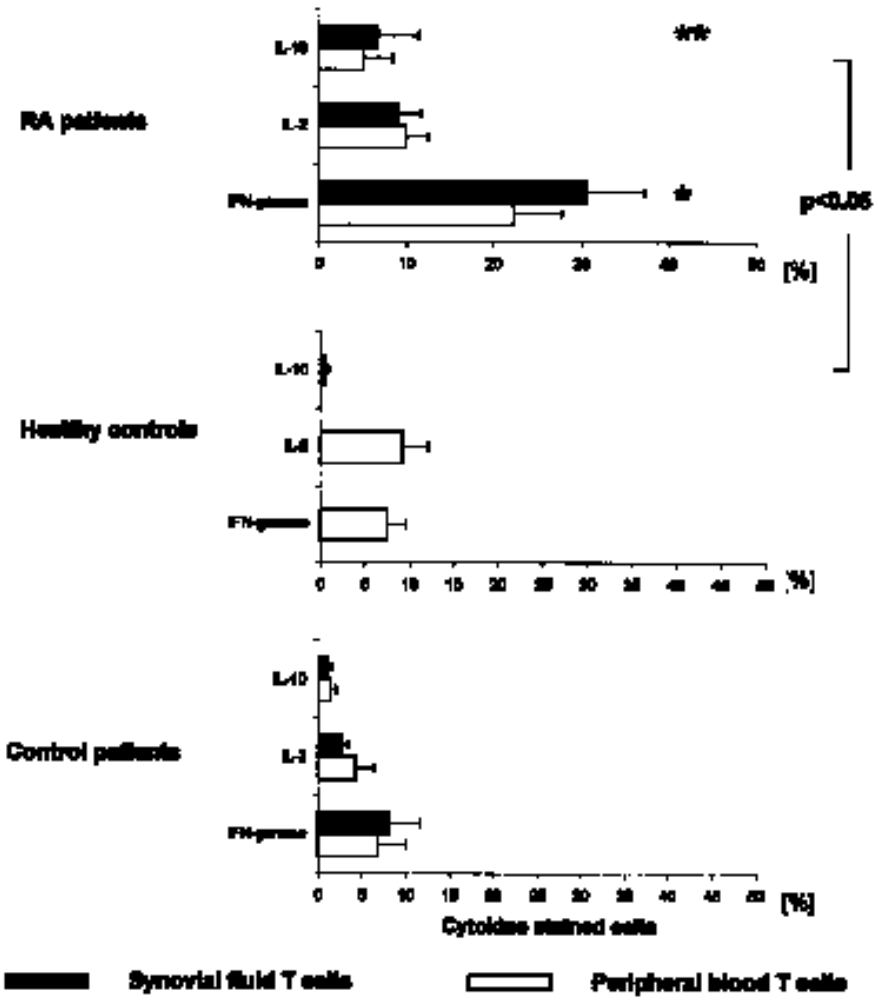



**Fig. 3.** Proportion of IL-2, IL-10 and IFN-γ expressing PB and SF derived CD3+ T cells in RA (n = 10),control patients (n = 12),and in healthy donor PBMC (n = 10). Bars represent the mean ± SEM of the pooled data. Statistical analyses between different populations were performed with Mann Whitney test of 2 unpaired groups. Comparative analyses between the corresponding PB and SF T cells were calculated with Wilcoxon´s test of 2 paired groups. The proportion of IFN-γ, and the counts for IL-10 producing T cells were significantly elevated in RA synovial fluids when compared with corresponding PB specimen. *$p < 0.05$; **$p < 0.01$.

*Serum and synovial fluid cytokine concentrations*

The serum concentrations in both arthritis patient groups did not significantly differ from the results obtained in healthy donors (Table II). In the synovial fluids, all T cell cytokines investigated could be detected in a higher percentage of, and when positive in

**Table I.** Cytokine staining in PB T cells from healthy donors (n=6), and in paired PB and SF T cells from RA patients (n = 7) and control patients (n = 9). Analyses were performed in triplicate. Results are presented as the mean ± SEM. Wilcoxon test for 2 tailed groups (CD4+ versus CD8+ T cells): *p < 0.05, **p < 0.01.

| | | PB (healthy) | PB (RA) | SF (RA) | PB (non-RA) | SF (non-RA) |
|---|---|---|---|---|---|---|
| IL-2 | CD4+ | 11.2 ± 4.7 | 5.0 ± 3.5 | 11.5 ± 3.7 | 7.9 ± 5.0 | 3.3 ± 1.8 |
| | CD8+ | 7.2 ± 3.8 | 1.5 ± 0.5 | 4.1 ± 2.1 | 3.1 ± 2.0 | 1.6 ± 0.7 |
| IL-10 | CD4+ | 0.3 ± 0.1 | 0.5 ± 0.3 | 2.7 ± 0.9 | 0.2 ± 0.1 | 0.6 ± 0.4 |
| | CD8+ | 0.8 ± 0.6 | 0.8 ± 0.2 | 17.6 ± 15.7 | 1.7 ± 1.3* | 1.4 ± 1.0 |
| IFN-γ | CD4+ | 5.4 ± 2.3 | 13.1 ± 10.2 | 31.1 ± 11.8 | 6.1 ± 3.8 | 7.5 ± 5.0 |
| | CD8+ | 9.7 ± 3.8* | 25.6 ±15.3** | 35.8 ± 14.5 | 11.3 ± 7.5 | 8.6 ± 5.2 |

average higher concentrations than in corresponding sera. A significant increase of IL-2, and in particular of the IL-10 concentrations was observed in RA, but interestingly not in the control arthritis patients (Tab. 2, Fig. 4E). The average IL-10 concentrations were increased to higher levels in the synovial fluids from RF positive (90.9 ± 14.3pg/ml, n=31) than RF negative RA patients (40.0 ± 21.1 pg/ml, n = 4), and they were correlated with the IL-2 (r = 0.36, n = 35, p < 0.05), and IFN-γ (r = 0.46, n = 35, p < 0.01) concentrations. In similar to IL-10, the average TNF-α synovial fluid concentrations were also significantly higher in RA than in control patients, and the TNF-α concentrations in RA patients were significantly correlated with the IL-10 concentrations (r = 0.63, n = 35, p < 0.05).

RA patient subset analyses were based on their immunosuppressive treatment in order to estimate putative influences of immunosuppressive drugs on cytokine expression. No significant differences were obtained when comparing IFN-γ, IL-2, IL-4, or IL-10 serum and synovial fluid concentrations in steroid-treated (n=13) and steroid-free RA patients (n = 7, Mann Whitney test of 2 untailed groups was performed). In similar, no significant differences were detected for these same parameters when comparing RA patients on (n = 14) and off DMARD treatment (n = 7). In contrast, a comparison of RA patients (n=7) and control patients (n= 9) that were all off steroid and DMARD treatment confirmed in average higher IL-10 synovial fluid concentrations in RA patients (mean ± SEM = 52.7 ± 14.6 pg/ml) than in control patients (12.2 ± 2.8 pg/ml, p = 0.01) as already seen for

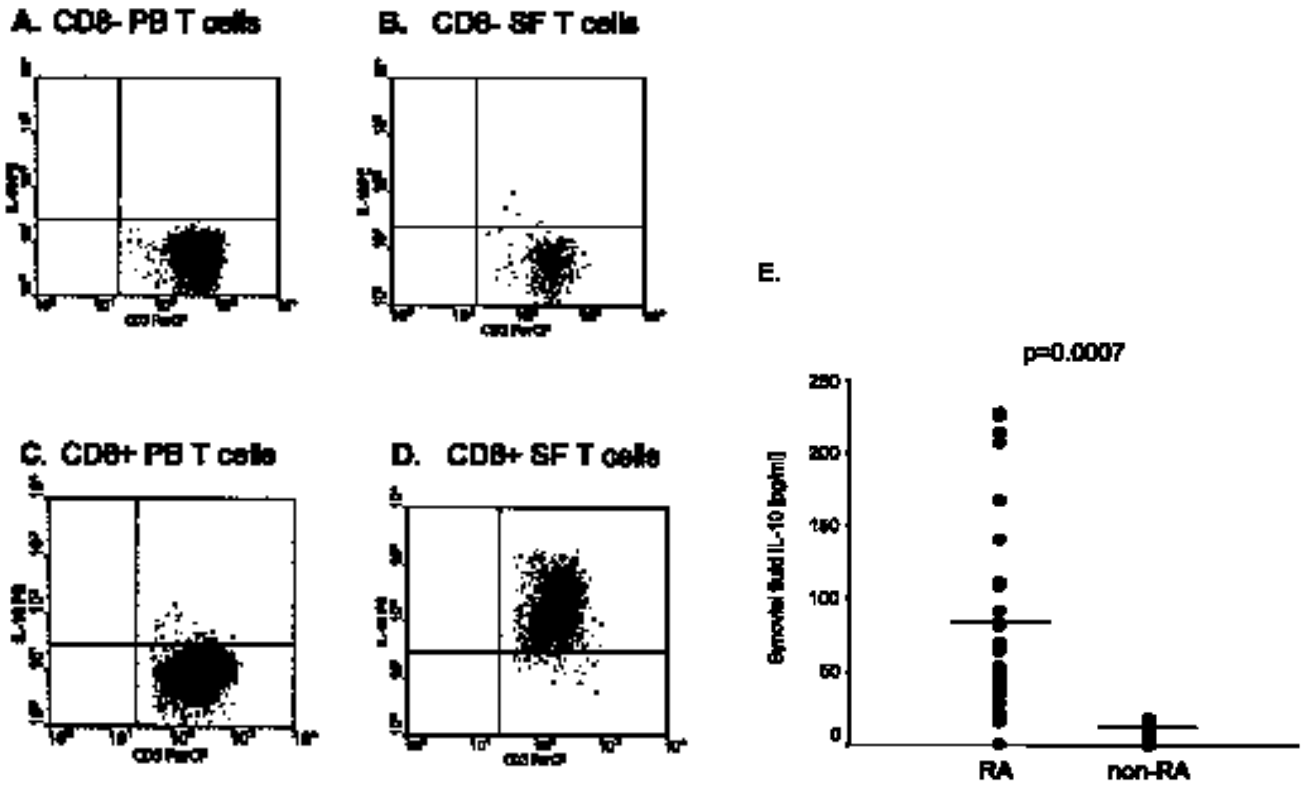



**Fig. 4.** IL-10 expression in T cells obtained from one individual RA patient that was representative for divergent IL-10 expression in CD4+ and CD8+ T cells (**A**-**D**). Scatter plots of simultaneously obtained, PMA and ionomycin stimulated T cells that were triple-stained for CD3, CD8, and IL-10: (**A**) PB CD8- T cells, (**B**) SF CD8- T cells, (**C**) PB CD8+ T cells,and (**D**) SF CD8+ T cells. The thresholds for positive cells were defined by isotype-matched controls (< 2% positive cells). In this RA patient,intensive intracellular IL-10 staining (right upper quadrant) was only detected in CD8+ SF T cells. (**E**) Average IL-10 synovial fluid concentrations are significantly higher in RA than in control patients (n = 53,p = 0.0007). Horizontal lines represent the mean of the pooled data in each patient population. Statistical analyses between the populations were performed with Mann Whitney test of 2 unpaired groups.

**Table II.** Cytokine concentrations in the sera from healthy donors (n = 11), and in pairs of sera and synovial fluids from RA (n = 35) and control patients (n = 18). Results are given as the proportion of positive tests (on top), and the mean ± SEM of cytokine concentrations measured by ELISA (on bottom). ** $p < 0.01$, *** $p < 0.001$.

| Parameter | Healthy donors | RA patients | Control patients |
|---|---|---|---|
| Serum IFN-γ | 0/11 | 1/35<br>(86 pg/ml) | 0/18 |
| IFN-γ (SF) | | 9/35<br>(13.1 ± 3.2 pg/ml) | 9/18<br>(16.7 ± 3.3 pg/ml) |
| Serum IL-2 | 0/11 | 15/35<br>(17.9 ± 4.1 pg/ml) | 10/18<br>(37.5 ± 13.5 pg/ml) |
| IL-2 (SF) | | 21/35<br>(82.4 ± 37.4 pg/ml) | 16/18<br>(21.4 ± 2.2 pg/ml) |
| Serum IL-4 | 2/11<br>(10.6 ± 4.4 pg/ml) | 1/35<br>(39.4 pg/ml) | 1/18<br>(6.3 pg/ml) |
| IL-4 (SF) | | 3/35<br>(6.5 ± 0.5 pg/ml) | 1/18<br>(58 pg/ml) |
| Serum IL-10 | 5/11<br>(25.2 ± 9.3 pg/ml) | 7/35<br>(22.3 ± 7.1 pg/ml) | 7/18<br>(12.1 ± 1.8 pg/ml) |
| IL-10 (SF) *** | | 26/35<br>(88.4 ± 13.3 pg/ml) | 13/18<br>(9.2 ± 1.2 pg/ml) |
| Serum TNF-α | 5/11<br>(46.5 ± 12.9 pg/ml) | 15/35<br>(77.5 ± 23.6 pg/ml) | 10/18<br>(66.4 ± 16.7 pg/ml) |
| TNF-α (SF) ** | | 25/35<br>(96.6 ± 13.7 pg/ml) | 14/18<br>(45.7 ± 12.2 pg/ml) |

the whole patient populations. IL-10 serum concentrations however, as well as IFN-γ, IL-2, and IL-4 serum and synovial fluid concentrations were not significantly different in these patient groups.

Additional analyses focused on RA patients that were refractory to TNF-blocking agents as indicated by more than 6 swollen joints, severe synovitis and at least one joint effusion. Notably, the average proportion of IFN-γ expressing PB and SF T cells was the highest (PB-CD4: 48.6 ± 14.8%, SF-CD4: 59.1 ± 21.8%, PB-CD8: 73.8 ± 10.4%, SF-CD8: 74.9 ± 21.5%, (mean ± SEM)) in these two available patients as compared to all the other investigated RA and control patients (RA (n = 12): PB-CD4: 7.4 ± 4.1%, SF-CD4: 14.3 ± 7.3%, PB-CD8: 8.7 ± 3.5%, and SF-CD8: 15.5 ± 6.0% IFN-γ positive T cells). IL-2 and IL-10 T cell immunostaining analyzes in contrast revealed no obvious peculiarities of these 2 patients refractory to TNF-α blockage.

## Discussion

A huge body of evidence has been accumulated for the involvement of T cells in RA pathogenesis (reviewed in 8), but it is still a matter of controversial debate, whether these cells are the masterminds of an immune mediated disease, or simple bystanders of a pathologic inflammatory process dominated by macrophages or fibroblast-like synoviocytes (8-11). Here we sought to demonstrate RA specific mechanisms of T cell recruitment or activation in separate for the CD4+ and CD8+ populations. Based on the hypothesis of RA being an autoimmune mediated disease induced by pathogenic CD4+ helper T cells, with a focus on proinflammatory Th1 cells, the possible role of CD8+ suppressor T cells attracted minor attention in the past. However, recent work indicated the presence and cytokine expression of CD8+ T cells in RA synovitis (7, 8).

A preferential recruitment of pre-activated T cells, and an additional T cell activation by cell-cell contact with endothelial cells is an established concept for T cell involvement in RA pathogenesis (15). Differences in the recruitment of helper and suppressor T cells might lead to an imbalance of pro- and anti-inflammatory activities, and we thus studied their naive and memory state, and also the early activation state of CD4+ and CD8+ T cell subsets. Here we found that CD4+ and CD8+ memory T cells become likewise accumulated in RA joints. As most similar results were also found in the synovial fluid T cells from control arthritis patients, the preferential accumulation of CD4+ and CD8+ memory T cells was not specific for the inflammation in RA joints. It appears noteworthy in this context that T cells of the RA synovial membranes, and in particular those adjacent to the cartilage pannus junction may differ from those of the synovial fluids (15, 16). The presented data indicate similar CD4 and CD8 T cell recruitment into RA joints. T cell analyses of the pannus site, that were not available in this trial, may give additional insights in the CD4/CD8 balance in RA.

*In vitro* challenged T cells express activation markers in a clearly defined sequence with expression of early antigens such as CD69, a tyrosine phosphatase, and subsequent IL-2 and IL-2 receptor (CD25) expression within 1-2 days (17). CD69 expression is a sensitive marker for early T cell activation, and CD25 or IL-2 expression differentiates these activated T cells from an incomplete activation state (CD69+, CD25 or IL-2 negative) of T cells called anergy (17, 18). RA synovial fluid T cells express CD69 and HLA-DR molecules, a late antigen, but only the minor proportion of these T cells is IL-2 or CD25 positive (8). As shown here for RA synovial fluid T cells, and as previously published for synovial membrane T cells, most CD4+ and CD8+ T cells in RA joints exhibit a memory state (19). Although up to 50 percent of the SF memory T cells in RA were CD69 positive, less than 10% of the PMA stimulated T cells expressed IL-2. Anergy is thus a phenomenon of CD4+ and CD8+ SF T cells in RA joints.

A relevant proportion of naive CD4+ and CD8+ SF T cells expressed the early T cell activation marker CD69, either suggesting accumulation of pre-activated, but antigen naive T cells in the inflamed joints, or early activation

of antigen naive, however joint invading T cells by the inflammatory environment. As an increased proportion of CD69 expressing CD4+ and CD8+ SF, to a major part IL-2 negative cells was also found in the non-RA joint fluids, T cell anergy is not specific for RA. Although the obtained data on T cell recruitment and activation negated RA disease specificity, one aspect of the presented results should be highlighted: Preferential T cell recruitment and T cell activation occur in different T cell subsets, so fare indicating that T cell invasion and activation might at least to a part be independent processes of RA synovitis. Here, 2 recent publications on the similar expression of chemokine receptors in CD4+ and CD8+ RA PB and SF T cells, and the role of pro-inflammatory cytokines (TNF-α, IL-6 and IL-2) for the antigen-independent activation of T cells in RA joints should be considered for possible explanations (20, 21).

Increased proportions of IL-10 and IFN-γ producing PB T cells, and furthermore elevated numbers of IL-10 and IFN-γ producing SF T cells were only observed in RA patients. Among the SF cytokine concentrations investigated by ELISA, IL-10 was the predominant T cell cytokine in RA synovial fluids, but it was more or less absent in the synovial fluids obtained from the control patients. As steroid and methotrexate treatment, the most common DMARD in the RA patient population in this trial may have account for some of the obtained results (22-24), comparative subset analyses were performed in steroid-treated and steroid-free RA patients, and in DMARD-treated or DMARD-free RA patients, respectively. Here, we did not detect significant differences in the IFN-γ, IL-2, IL-4, or IL-10 serum or synovial fluid concentrations between patients on or off these immunosuppressive agents. In contrast, when these same comparative analyses were performed in those RA and control patients all off immunosuppressive agents, IL-10 again was the predominant T cell cytokine in RA synovial fluids as compared to the control patients. The IFN-γ synovial fluid concentrations in contrast were not significantly different between the RA and non-RA group, and IL-4 was below the detection limit in almost all RA and control patients. Interestingly, very high proportions of PB and SF CD4+ and CD8+ T cells were found in those few RA patients investigated here that appeared refractory to a TNF-α blocking therapy. The statistical analyses have to be considered preliminary due to the small number of these patients, but it is tempting to speculate that very high percentages of IFN-γ expressing CD4+ and CD8+ T cells in the peripheral blood and synovial fluid might be causative for the treatment refractory course of disease in these patients.

High IL-10 SF levels appear more or less RA specific. In the RA patients analyzed here, IL-10 SF concentrations were detected in a similar quantitative range, and they were moreover correlated with the SF TNF-α concentrations. High average IL-10 SF concentrations in RA were also detected in previous ELISA studies (25, 26), although the results of comparative IL-10 expression analyses in RA and PsA synovium gave controversial results (27, 28). Strong IL-10 expression in RA patients is obviously localized to the inflamed joints (6, 7, 29), either suggesting that yet unknown factors may be relevant for a preferential recruitment of IL-10 expressing cells into RA joints, or alternatively that IL-10 expression in RA joints is induced by articular factors.

Controversial data on IL-10 gene and IL-10 promoter polymorphisms in arthritis patients however are difficult to interpret in respect of the resulting quantitative IL-10 expression capacity and the observed phenotype (30-34). Hence, other regulatory mechanisms of IL-10 gene expression, e.g. secondary responses to the inflammatory environment in RA joints need to be considered. IL-10 expression in RA synovitis is located to CD3+ lymphocytic aggregates (35), but also to lining layer macrophages and FLS (36,37). Following the presented results, both the CD4+ and CD8+ populations appear to have a relevant contribution to high IL-10 concentrations in RA synovitis, although to a variable extent and with remarkable individual differences. IL-10 expression in CD8+ T cells can be predominant, and in this circumstance, neither Th2 cells nor regulatory Tr1 T cells (38) would be essential for IL-10 expression in suchlike CD8+ dominated RA joints.

IL-10 reduces the cellular infiltration into the rheumatoid synovium and prevents cartilage degradation (39). IL-10 inhibits the expression of class II MHC and co-stimulatory molecules (40), and impairs IL-1β and TNF-α expression in macrophages (39, 41). IL-10 furthermore inhibits dendritic cell (DC) maturation (42), and the antigen dependent proliferation of RA T cells (43). Finally, the net effect of IL-10 administration is beneficial in different animal models of RA (39, 40). It can be therefore suggested that elevated IL-10 production in RA joints is part of an unsuccessful repair mechanism, and missing IL-10 induction in the control patients may be also of pathogenic relevance in these types of arthritis. However, only scarce effects were observed in a clinical RA trial with therapeutic administration of recombinant IL-10 (44). High endogenous, saturating IL-10 concentrations as observed here in many RA joints may be the reason for this treatment failure, and exuberant endogenous IL-10 expression may also explain the capacity of IL-4 administration, but no anti-inflammatory effect of IL-10 on the Th1/Th2 balance in cultured RA SF T cells (45).

The localization of IL-10 producing T cells in rheumatoid synovitis resembles that of CD8+ T cell accumulates adjacent to germinal centers (46). Here, IL-10 is a candidate to augment RF synthesis in lymphoid follicles (47, 48), and to activate RA synovial B cells, that may anew be critical for the activation of CD4+ T cells (49). In this context, IL-10 may to some extent contribute to the chronic progressive course of RA, and both the anti-inflammatory and the immune modulating aspects of this ambivalent cytokine need to be regarded in RA pathogenesis.

In summary, memory T cells of both the CD4+ and CD8+ populations exhibit a preferential accumulation in

the joints, but early T cell activation is not strictly limited to memory T cells. Memory T cell accumulation and the activation state of the different SF T cell subsets occur in similar patterns for RA and control patients. High IL-10 SF concentrations in contrast, and elevated percentages of IFN-γ and IL-10 expressing CD4+ and CD8+ T cells in the PB and SF were characteristic for RA. Here, CD8+ T cells may contribute to high IL-10 concentrations in RA joints. The cellular mechanism of this disease associated pathologic process appears worthy of further investigation.

**Acknowledgements**

We thank Julia Bauer for her technical assistance, and the Riese and Klein foundations for the financial support of this work.

**References**

1. TAK PP: Examination of the synovium and synovial fluid. *In* FIRESTEIN GS, PANAYI GS, WOLLHEIM FA (Eds.): *Rheumatoid Arthritis:New Frontiers in Pathogenesis and Treatment.* Oxford University Press 2000: 55-68.
2. ARNETT FC, EDWORTHY SM, BLOCH DA, *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315–24.
3. KLIMIUK PA, GORONZY JJ, BJÖRNSSON J, BECKENBAUGH RD, WEYAND CM: Tissue cytokine patterns distinguish variants of rheumatoid synovitis. *Am J Pathol* 1997; 151: 1311-9.
4. VAN DER GRAAFF WL, PRINS AP, DIJKMANS BA, VAN LIER RA: Prognostic value of Th1/ Th2 ratio in rheumatoid arthritis. *Lancet* 1998; 351: 1931.
5. MASUKO-HONGO K, KATO T, NISHIOKA K: Prognostic value of Th1/Th2 ratio in rheumatoid arthritis. *Lancet* 1998; 352: 988-9.
6. MORITA Y, YAMAMURA M, KAWASHIMA M, *et al.*: Flow cytometric single-cell analysis of cytokine production by $CD4^+$ T cells in synovial tissue and peripheral blood from patients with rheumatoid arthritis. *Arthritis Rheum* 1998; 41: 1669-76.
7. BERNER B, AKCA D, JUNG T, MULLER GA, REUSS-BORST MA: Analysis of Th1 and Th2 cytokines expressing CD4+ and CD8+ T cells in rheumatoid arthritis by flow cytometry. *J Rheumatol* 2000; 27: 1128-35.
8. FOX DA: The role of T lymphocytes in rheumatoid arthritis. *In* FIRESTEIN GS, PANAYI GS, WOLLHEIM FA (Eds.): *Rheumatoid Arthritis: New Frontiers in Pathogenesis and Treatment*. Oxford University Press 2000: 55-68.
9. FIRESTEIN GS, ZVAIFLER NJ: How important are T cells in chronic rheumatoid synovitis ?:II. T cell-independent mechanisms from beginning to end. *Arthritis Rheum* 2002; 46: 298-308.
10. GEILER T, KRIEGSMANN J, KEYSZER GM, GAY RE, GAY S: A new model for rheumatoid arthritis generated by engraftment of rheumatoid synovial tissue and normal human cartilage into SCID mice. *Arthritis Rheu*m 1994; 37: 1664-71.
11. KLIMIUK PA, YANG H, GORONZY JJ, WEYAND CM: Production of cytokines and metalloproteinases in rheumatoid synovitis is T cell dependent. *Clin Immunol* 1999; 90: 65-78.
12. JULIUS M, MAROUN CR, HAUGHN L: Distinct roles for CD4 and CD8 as co-receptors in antigen receptor signalling. *Immunol Today* 1993; 14: 177-83.
13. CLEMENT LT: Isoforms of the CD45 common leukocyte antigen family: markers for human T-cell differentiation. *J Clin Immunol* 1992; 12: 1-10.
14. SANTIS AG, LOPEZ-CABRERA M, SANCHEZ-MADRID F, PROUDFOOT N: Expression of the early lymphocyte activation antigen CD69,a C-type lectin,is regulated by mRNA degradation associated with AU-rich sequence motifs. *Eur J Immunol* 1995; 25: 2142-6.
15 KIRKHAM B, PORTEK I, LEE CS *et al.*: Intraarticular variability of synovial membrane histology, immunohistology, and cytokine mRNA expression in patients with rheumatoid arthritis. *J Rheumatol* 1999; 26: 777-84.
16. GERLI R, PITZALIS C, BISTONI O *et al.*: CD 30+ T cells in rheumatoid synovitis. Mechanisms of recruitment and functional role. *J Immunol* 2000; 164: 4399-407.
17. IANNONE F, CORRIGALL VM, KINGSLEY GH, PANAYI GS: Evidence for the continuous recruitment and activation of T cells into the joints of patients with rheumatoid arthritis. *Eur J Immunol* 1994; 24: 2706-13.
18. CORRIGALL, VM, SOLAU-GERVAIS E, PANAYI GS: Lack of CD80 expression by fibroblast-like synoviocytes leading to anergy in T lymphocytes. *Arthritis Rheum* 2000; 43: 1606-15.
19. MAMOUNE A, DURAND V, LE GOFF P, PENNEC YL, YOUINOU P, LE CORRE R: Abnormal distribution of CD45 isoforms expressed by CD4+ and CD8+ T cells in rheumatoid arthritis. *Histol Histopathol* 2000; 15: 587-91.
20. RUTH JH, ROTTMAN JB, KATSCHKE KJ Jr *et al.*: Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthritis Rheum* 2001; 44: 2750-60.
21. BRENNAN FM, HAYES AL, CIESIELSKI CJ, GREEN P, FOXWELL BM, FELDMANN M: Evidence that rheumatoid arthritis synovial T cells are similar to cytokine-activated T cells: Involvement of phosphatidylinositol 3-kinase and nuclear factor kappaB pathways in tumor necrosis factor alpha production in rheumatoid arthritis. *Arthritis Rheum* 2002; 46: 31-41.
22. VERHOEF CM, VAN ROON JA, VIANEN ME, LAFEBER FP, BIJLSMA JW: The immune suppressive effect of dexamethasone in rheumatoid arthritis is accompanied by upregulation of interleukin 10 and by differential changes in interferon gamma and interleukin 4 production. *Ann Rheum Dis* 1999; 58: 49-54.
23. CONSTANTIN A, LOUBET-LESCOULIÉ P, LAMBERT N *et al.*: Antiinflammatory and immunoregulatory action of methotrexate in the treatment of rheumatoid arthritis. *Arthritis Rheum* 1998; 41: 48–57.
24. SEITZ M, ZWICKER M, WIDER B: Enhanced *in vitro* induced production of interleukin 10 by peripheral blood mononuclear cells in rheumatoid arthritis is associated with clinical response to methotrexate treatment. *J Rheumatol* 2001; 28: 496-501.
25. PARTSCH G, WAGNER E, LEEB BF, BROLL H, DUNKY A, SMOLEN JS: T cell derived cytokines in psoriatic arthritis synovial fluids. *Ann Rheum Dis* 1998; 57: 691-3.
26. CUSH JJ, SPLAWSKI JB, THOMAS R *et al.*: Elevated interleukin-10 levels in patients with rheumatoid arthritis. *Arthritis Rheum* 1995; 38: 96-104.
27. DANNING CL,ILLEI GG, HITCHON C,GREER MR, BOUMPAS DT, MCINNES IB: Macrophage-derived cytokine and nuclear factor kappaB p65 expression in synovial membrane and skin of patients with psoriatic arthritis. *Arthritis Rheum* 2000; 43: 1244-56.
28. RITCHLIN C, HAAS-SMITH SA, HICKS D, CAPPUCCIO J, OSTERLAND CK, LOONEY RJ: Patterns of cytokine production in psoriatic synovium. *J Rheumatol* 1998; 25: 1544-52.
29. COHEN SB, KATSIKIS PD, CHU CQ *et al.*: High level of interleukin-10 production by the activated T cell population within the rheumatoid synovial membrane. *Arthritis Rheum* 1995; 38: 946-52.
30. COAKLEY G, MOK CC, HAJEER AH *et al.*: Interleukin-10 promoter polymorphisms in rheumatoid arthritis and Felty´s syndrome. *Br J Rheumatol* 1998; 37: 988-91.
31. HAJEER AH, LAZARUS M, TURNER D *et al.*: IL-10 gene promoter polymorphisms in rheumatoid arthritis. *Scand J Rheumatol* 1998; 27: 142-5.
32. CANTAGREL A, NAVAUX F, LOUBET-LESCOULIE P *et al.*: Interleukin-1beta, interleukin-1 receptor antagonist, interleukin-4, and interleukin-10 gene polymorphisms: relationship to occurrence and severity of rheumatoid arthritis. *Arthritis Rheum* 1999; 42: 1093-100.
33. CROWLEY E, KAY R,SILLIBOURNE J, PATEL P, HUTCHINSON I, WOO P: Polymorphic haplotypes of the interleukin-10 5´flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. *Arthritis Rheum* 1999; 42: 1101-8.
34. HUIZINGA TW, KEIJSERS V, YANNI G *et al.*: Are differences in interleukin-10 production associated with joint damage ? *Rheumatology (Oxford)* 2000; 39: 1180-8.
35. VERHOEF CM, VAN ROON JA, VIANEN ME, BIJLSMA JW, LAFEBER FP: Interleukin-10 (IL-10), not IL-4 or interferon-γ production, correlates with progression of joint destruction in rheumatoid arthritis. *J Rheumatol* 2001; 28: 1960-6.
36. KATSIKIS PD, CHU CQ, BRENNAN FM, MAINI RN, FELDMANN M: Immunoregula-

tory role of interleukin 10 in rheumatoid arthritis. *J Exp Med* 1994; 179: 1517-27.

37. RITCHLIN C, HAAS-SMITH SA: Expression of interleukin-10 mRNA and protein by synovial fibroblastoid cells. *J Rheumatol* 2001; 28: 698-705.
38. YUDOH K, MATSUNO H, NAKAZAWA F, YONEZAWA T, KIMURA T: Reduced expression of the regulatory CD4+ T cell subset is related to Th1/Th2 balance and disease severity in rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 617-27.
39. JORGENSEN C, APPARAILLY F, COURET I, CANOVAS F, JACQUET C, SANY J: Interleukin-4 and interleukin-10 are chondroprotective and decrease mononuclear cell recruitment in human rheumatoid synovium *in vivo*. *Immunology* 1998; 93: 518-23.
40. VAN ROON JA, LAFEBER FP, BIJLSMA JW: Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. *Arthritis Rheum* 2001; 44: 3-12.
41. ISOMAKI P, LUUKKAINEN R, SAARIO R, TOIVANEN P, PUNNONEN J: Interleukin-10 functions as an antiinflammatory cytokine in rheumatoid synovium. *Arthritis Rheum* 1996; 39: 386-95.
42. MOTTONEN M, ISOMAKI P, SAARIO R, TOIVANEN P, PUNNONEN J, LASSILA O: Interleukin-10 inhibits the capacity of synovial macrophages to function as antigen-presenting cells. *Br J Rheumatol* 1998; 37: 1207-14.
43. SMEETS TJ, KRAAN MC, VERSENDAAL J, BREEDVELD FC, TAK PP: Analysis of serial synovial biopsies in patients with rheumatoid arthritis:Description of a control group without clinical improvement after treatment with interleukin 10 or placebo. *J Rheumatol* 1999; 26: 2089-93.
44. YIN Z,SIEGERT S, NEURE L *et al.*: The elevated ratio of interferon gamma-/interleukin-4-positive T cells found in synovial fluid and synovial membrane of rheumatoid arthritis patients can be changed by interleukin-4 but not by interleukin-10 or transforming growth factor beta. *Rheumatology* 1999; 38: 1058-67.
45. CORRIGALL VM, GARYFALLOS A, PANAYI GS: The relative proportions of secreted interleukin-2 and interleukin-10 determine the magnitude of rheumatoid arthritis T-cell proliferation to the recall antigen tuberculin purified protein derivative. *Rheumatology (Oxford)* 1999; 38: 1203-7.
46. WAGNER UG, KURTIN PJ, WAHNER A *et al.*: The role of CD8+ CD40L+ T cells in the formation of germinal centers in rheumatoid synovitis. *J Immunol* 1998; 161: 6390-7.
47. PEREZ L, ORTE J, BRIEVA JA: Terminal differentiation of spontaneous rheumatoid factor-secreting B cells from rheumatoid arthritis patients depends on endogenous interleukin-10. *Arthritis Rheum* 1995; 38: 1771-76.
48. REPARON-SCHUIJT CC, VAN ESCH WJ, VAN KOOTEN C, LEVARHT EW, BREEDVELD FC, VERWEIJ CL: Functional analysis of rheumatoid factor-producing B cells from the synovial fluid of rheumatoid arthritis patients. *Arthritis Rheum* 1998; 41: 2211-20.
49. TAKEMURA S, KLIMIUK PA, BRAUN A, GORONZY JJ, WEYAND CM: T cell activation in rheumatoid synovium is B cell dependent. *J Immunol* 2001; 167: 4710-8.