Influence of Longterm Therapy with Methotrexate and Low Dose Corticosteroids on Type 1 and Type 2 Cytokine Production in CD4+ and CD8+ T Lymphocytes of Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is a chronic inflammatory disease with predominance of type 1 cytokine [interleukin 2 (IL-2), interferon-γ (IFN-γ)] production. In this prospective study, we evaluated the influence of longterm therapy with methotrexate (MTX) in combination with low dose corticosteroids on the type 1/type 2 cytokine balance in RA.

Methods. Peripheral blood mononuclear cells were isolated from 10 controls and 20 patients with RA before therapy and after 12 mo of therapy with MTX in combination with low dose corticosteroids. Using flow cytometry, the intracellular production of IL-2, IFN-γ, and IL-4 was measured in CD4+ and CD8+ T lymphocytes.

Results. Compared with healthy controls, patients with RA before therapy showed an increased percentage of IL-2 positive CD4+ and CD8+ T cells ($p = 0.002$, $p = 0.01$, respectively). An increased percentage of IFN-γ positive CD8+ T cells was found ($p = 0.0006$) compared with the control group. After 12 months of therapy, a significantly decreased percentage of IL-2 positive CD4+ T cells and IFN-γ positive CD4+ and CD8+ T lymphocytes was observed ($p = 0.0003$, $p = 0.0007$, $p = 0.001$). The percentage of IL-4/IFN-γ positive CD4+ and CD8+ T cells was significantly higher after 12 months of therapy ($p = 0.01$, $p = 0.02$). There was a positive correlation between the percentage of IFN-γ positive CD4+ T cells and disease activity variables (Ritchie Index and number of swollen joints) in RA patients before therapy ($r = 0.6$, $p = 0.04$ and $r = 0.4$, $p = 0.05$).

Conclusion. Longterm therapy with MTX in combination with low dose corticosteroids for RA influenced the predominance of type 1 cytokines toward normalization of the cytokine balance in both CD4+ and CD8+ T lymphocytes. (J Rheumatol 2001;28:1793–9)

Key Indexing Terms:

RHEUMATOID ARTHRITIS	CYTOKINE	METHOTREXATE
CORTICOSTEROIDS	CD4+ T CELLS	CD8+ T CELLS

T cell mediated autoimmune responses play an important role in the pathogenesis of rheumatoid arthritis (RA). At least 2 functional subpopulations have been distinguished, according to their cytokine profiles: type 1 lymphocytes mainly produce interleukin 2 (IL-2) and interferon-γ (IFN-γ) and type 2 lymphocytes secrete IL-4, IL-5, IL-13, and IL-10[1,2]. Collagen induced arthritis in mice, an animal model of RA, is accompanied by type 1 lymphocyte activation, while remission is accompanied by type 2 lymphocyte activation[3,4]. In several human studies, a predominance of type 1 cytokines was observed in both synovial tissue and peripheral blood cells of patients with RA[5-9]. Further evidence indicates that therapy (e.g., dexamethasone, anti-CD4) can induce a shift from a type 1 toward a type 2 cytokine profile, which was associated with disease amelioration[10-12].

We investigated the influence of longterm therapy with methotrexate (MTX) in combination with low dose corticosteroids on CD4+ and CD8+ T cell cytokine production, measured by flow cytometry identifying the producing cell type. We hypothesized that modulation of the cytokine balance, decreasing type 1 cytokines, could play an important role in the antiinflammatory and immunoregulatory action of MTX in combination with low dose prednisolone.

MATERIALS AND METHODS

Study population. Twenty patients with active RA fulfilling the diagnostic criteria of the American College of Rheumatology[13] were evaluated before and after one year with a low dose of corticosteroids (5–10 mg/day) in combination with MTX (7.5–15 mg/week). The disease duration of RA was median 15 months (range 1.5–25). All patients received calcium 1000 mg daily and folic acid 5 mg weekly. Half the patients also received pamidronate 60 mg in 1000 ml 5% glucose over 3 h intravenously, starting

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at Month 0 and subsequently every 3 months. Vitamin D supplement was provided when baseline vitamin D level was < 15 ng/ml. Disease was considered active if the following criteria were present: morning stiffness > 20 min, presence of at least 2 painful joints and one swollen joint, and erythrocyte sedimentation rate (ESR) > 28 mm/h or C-reactive protein (CRP) at least 1 mg/dl. Patients previously treated with steroids or MTX were excluded.

A control group of 10 healthy volunteers, age and sex matched with the patients, was also evaluated.

Analysis of lymphocyte subsets. A quantity of 100 μl isolated mononuclear cells were incubated 15 min in the dark at 4°C with 10 μl of conjugated monoclonal antibody panels (CD3-PerCP + CD4-FITC + CD8-PE; CD45-PerCP + CD3-FITC + CD19-PE; Becton-Dickinson Immunocytometry Systems, Erembodegem, Belgium). The remaining red blood cells were treated with 2 ml of a lysing solution (155 mM NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2 EDTA) for 20 min at room temperature. Cells were pelleted at 400 g for 10 min and fixed with 1% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate buffered saline (PBS). The cells were resuspended in 1% paraformaldehyde in PBS and analyzed by FACScan flow cytometry (Becton-Dickinson) within 24 h.

Intracellular cytokine analysis in lymphocytes. T lymphocytes were isolated, cultured, and stained as described[14]. Briefly, mononuclear cells isolated by Ficoll-Paque density gradient (density 1.077 mg/ml; Amersham Pharmacia Biotech, Uppsala, Sweden) were cultured in AIM-V medium (Life Technologies, Gibco, Gent, Belgium) for 6 h at 37°C and 5% CO_2. The cells were stimulated in the presence of 50 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma, St. Louis, MO, USA), 1 μg/ml ionomycin (Sigma), and 2 μM monensin (Sigma). After stimulation, cells were labeled with surface antigens (anti-CD8-FITC PE anti-CD3-PerCP; Becton Dickinson) and fixed with 4% paraformaldehyde. After permeabilization with 0.3% saponin (Sigma), cells were incubated with anti-cytokine antibodies (mouse anti-Hu IL-2-PE, mouse anti-Hu IL-4-PE, mouse anti-IFN-PE; Becton Dickinson). Cells (10^5) were analyzed on a FACScan flow cytometer (Becton Dickinson) and data were processed with Win MDI 2.8 software (Windows Multiple Document Interface Flow Cytometry Application, provided by J. Trotter through http://facs.scripps.edu/software.html). Analysis gates were set on lymphocytes according to forward and side scatter properties. CD4+ T cells were defined as CD3+CD8– and CD8+ T cells as CD3+CD8+. According to PerCP and FITC emission, CD3+CD8– and CD3+CD8+ lymphocyte populations were gated out. Within this gate, intracellular cytokine production (PE emission) was measured in CD3+CD8– and CD3+CD8+ lymphocytes. Markers were set on the 99th percentile using isotype matched irrelevant antibodies (mouse IgG1 RPE; Serotec Ltd., Oxford, England) as reference. Results were expressed as the percentage of cytokine positive cells.

Statistics. Differences of cytokine production between controls and patients were assessed using the Mann-Whitney U test. The Wilcoxon rank test was applied for differences of cytokine production in patients before and after therapy. Correlations were calculated using the Spearman rank correlation and chi-square test. A p value < 0.05 was considered significant.

RESULTS

Clinical and inflammation variables. Table 1 shows the clinical effect of MTX in combination with low dose corticosteroids on disease activity variables. Twelve months of therapy with MTX and low dose corticosteroids resulted in a significant decrease of ESR, CRP, Ritchie Index score, number of swollen joints, Health Assessment Questionnaire score, visual analog scale score, and Disease Activity Score graded by physician and patient (p = 0.0002, p = 0.0007, p = 0.0001, p = 0.0001, p = 0.008, p = 0.0002, p = 0.001, p = 0.0007, respectively) (Table 1).

Lymphocyte subsets. In controls and in patients before therapy, the proportions of CD3, CD4+, CD8+ and CD19+ were similar (Table 2). Similarly, there was no difference in the proportion of these subsets in patients after 12 months of therapy (Table 2). Also the absolute number of CD4+ and CD8+ cells showed no difference between controls and patients before and after 12 months of therapy (Table 2).

Interleukin 2. Before therapy patients showed a significantly

Table 1. Clinical variables in patients with RA before and after 12 months of therapy with MTX and low dose corticosteroids. Values expressed as mean (range).

	Before Therapy, M0	After Therapy, M12*
ESR, (mm/h)	36 (5–118)	23 (4–62)
CRP, (mg/dl)	3 (1–17)	1 (0–15)
Ritchie Index score	12 (6–22)	1 (0–16)
Swollen joints	9 (2–17)	1 (0–12)
HAQ	53 (40–78)	7 (0–22)
VAS (mm)	58 (40–78)	10 (2–72)
DAS by physician	3 (2–4)	2 (1–3)
DAS by patient	4 (3–4)	2 (1–4)

HAQ: Health Assessment Questionnaire, VAS: visual analog scale, DAS: Disease Activity Score. DAS is expressed in categories 1 to 5. *p < 0.005 for all variables after 12 months of therapy.

Table 2. Lymphocyte subsets in controls and patients with RA before and after therapy.

	Controls	Patients	
		Before Therapy (m0)	After Therapy (m12)
CD3+ (%)	79 (65–89)	79 (52–89)	79 (60–90)
CD19+ (%)	4 (2–9)	5 (2–11)	5 (2–12)
CD4+ (%)	52 (32–68)	57 (28–74)	54 (31–74)
CD8+ (%)	17 (8–32)	16 (5–44)	19 (9–32)
CD3+ ($\times 10^9$/l)	1.85 (1.23–2.38)	1.64 (0.71–2.26)	1.73 (0.81–2.39)
CD19+ ($\times 10^9$/l)	0.08 (0.04–0.16)	0.08 (0.03–0.17)	0.10 (0.04–0.18)
CD4+ ($\times 10^9$/l)	1.27 (0.60–1.78)	1.04 (0.45–1.69)	1.10 (0.50–1.90)
CD8+ ($\times 10^9$/l)	0.45 (0.19–0.63)	0.41 (0.17–0.63)	0.43 (0.16–0.68)

Results are expressed as percentages (%) and as absolute numbers $\times 10^9$ per liter.

increased percentage and absolute number of IL-2 positive CD4+ and CD8+ T cells, compared to controls (p = 0.02, p = 0.01, p = 0.002, p = 0.01) (Figure 1, Table 3).

After 12 months of therapy with MTX and low dose corticosteroids, there was a significant decrease of the percentage of IL-2 positive CD4+ T cells in patients, but not of the percentage of IL-2 positive CD8+ T cells (p = 0.0003) (Figure 1). Moreover, the absolute number of IL-2 positive CD4+ and CD8+ T cells was lower after 12 months of therapy (p = 0.0003, p = 0.0002) (Table 3). After 12 months of therapy, patients with RA had a normalized percentage of IL-2 positive CD4+ T cells and did not differ significantly from the controls. The percentage of IL-2 positive CD8+ T cells was still significantly higher than in controls (p = 0.006) (Figure 1).

Seventeen of 20 patients with RA showing a decrease in CD4+ IL-2 positive cells also showed a decrease in CD8+ IL-2 positive cells. The ratio between CD4+ and CD8+ IL-

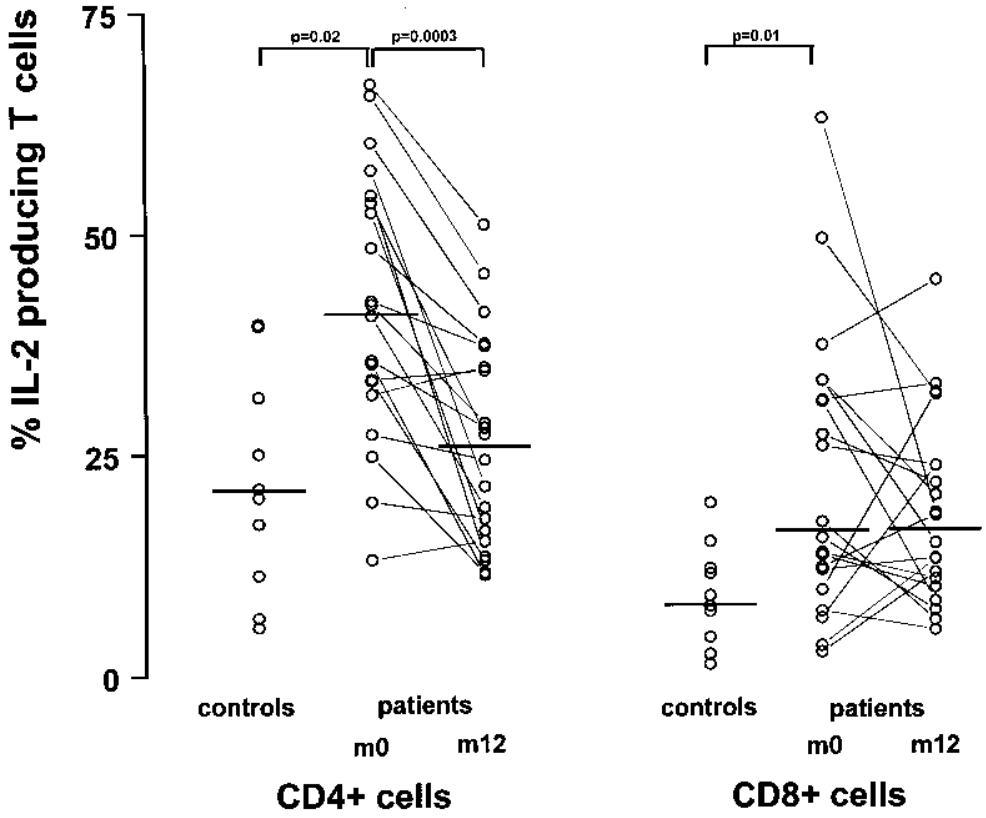

Figure 1. Percentage of IL-2 positive CD4+ T cells and CD8+ T cells in healthy controls and patients with RA before (m0) and after 12 months (m12) of therapy with MTX and low dose corticosteroids. Bars represent median values.

Table 3. Absolute number of cytokine positive CD4+ and CD8+ T lymphocytes.

	Controls	Patients	
		Before (m0)	After (m12)
CD4+ IL-2	0.18 (0.05–0.25)	0.36 (0.14–1.02)*	0.17 (0.07–0.63)††
CD8+ IL-2	0.04 (0.01–0.08)	0.06 (0.03–0.47)**	0.03 (0.01–0.24)††
CD4+ IFN-γ	0.14 (0.05–0.56)	0.25 (0.08–0.87)	0.15 (0.04–0.24)††
CD8+ IFN-γ	0.11 (0.02–0.17)	0.15 (0.06–0.70)†	0.07 (0.01–0.25)††
CD4+ IL-4	0.01 (0.01–0.04)	0.02 (0.01–0.08)	0.01 (0.01–0.03)
CD8+ IL-4	0.01 (0.01–0.02)	0.01 (0.01–0.05)	0.01 (0.01–0.14)

Results are expressed as median (range) of absolute number of cytokine positive T cells $\times 10^9$/l. *p = 0.002 vs controls, **p = 0.01 vs controls, †p = 0.06 vs controls, ††p < 0.001 vs m0.

2 positive T cells remained identical after 12 months of therapy.

Interferon-γ. The percentage and absolute number of IFN-γ positive CD8+ T cells was significantly higher in patients with RA before therapy compared to controls, but not in CD4+ T cells (p = 0.0006, p = 0.06) (Figure 2, Table 3). After 12 months of therapy, the percentage of IFN-γ positive CD4+ and CD8+ T cells was not significantly different from controls. A lower percentage and absolute number of IFN-γ positive CD4+ and CD8+ T cells was observed in patients after 12 months of therapy (p = 0.0007, p = 0.0001, p = 0.00009, p = 0.0001) (Figure 2, Table 2). In only 11 of 20 patients was the decrease in CD4+ IFN-γ positive T cells associated with a decrease in CD8+ IFN-γ positive T cells. The ratio between CD4+ and CD8+ IFN-γ T cells remained identical after 12 months of therapy.

Interleukin-4. Although no significant differences were found in percentage of IL-4 positive CD4+ and CD8+ T lymphocytes (data not shown), the ratio of IL-4/IFN-γ positive CD4+ and CD8+ T cells was significantly higher in patients [0.15 (0.09–0.50) (median (minimum–maximum)) and 0.13 (0.06–0.48)], respectively, in comparison with controls [0.10 (0.01–0.29) and 0.08 (0.01–0.26)] (p = 0.02, p = 0.03).

The percentage of IL-4 positive CD4+ and CD8+ T cells was not significantly different after 12 months of therapy with MTX and low dose corticosteroids. The ratio of IL-4/IFN-γ CD4+ and CD8+ T cells was significantly decreased [0.15 (0.09–0.50) (median (minimum–maximum)) and 0.13 (0.06–0.48) vs 0.11 (0.01–0.14) and 0.09 (0.01–0.10)] (p = 0.01, p = 0.02).

The absolute numbers of IL-4 positive CD4+ and CD8+ T cells did not differ between controls and patients before and after therapy (Table 3).

Correlation between disease activity variables and cytokines. There was a positive correlation between the percentage of IFN-γ positive CD4+ T cells and disease activity variables (Ritchie Index and number of swollen joints) in patients before therapy (r = 0.69, p = 0.01 and r = 0.67, p = 0.01) (Figures 3 and 4).

After 12 months of therapy, there was no correlation between disease activity variables and IFN-γ positive CD4+ T cells.

DISCUSSION

Applying a flow cytometric technique, the intracellular cytokine production in CD4+ and CD8+ T lymphocytes of patients with active RA was evaluated before therapy with

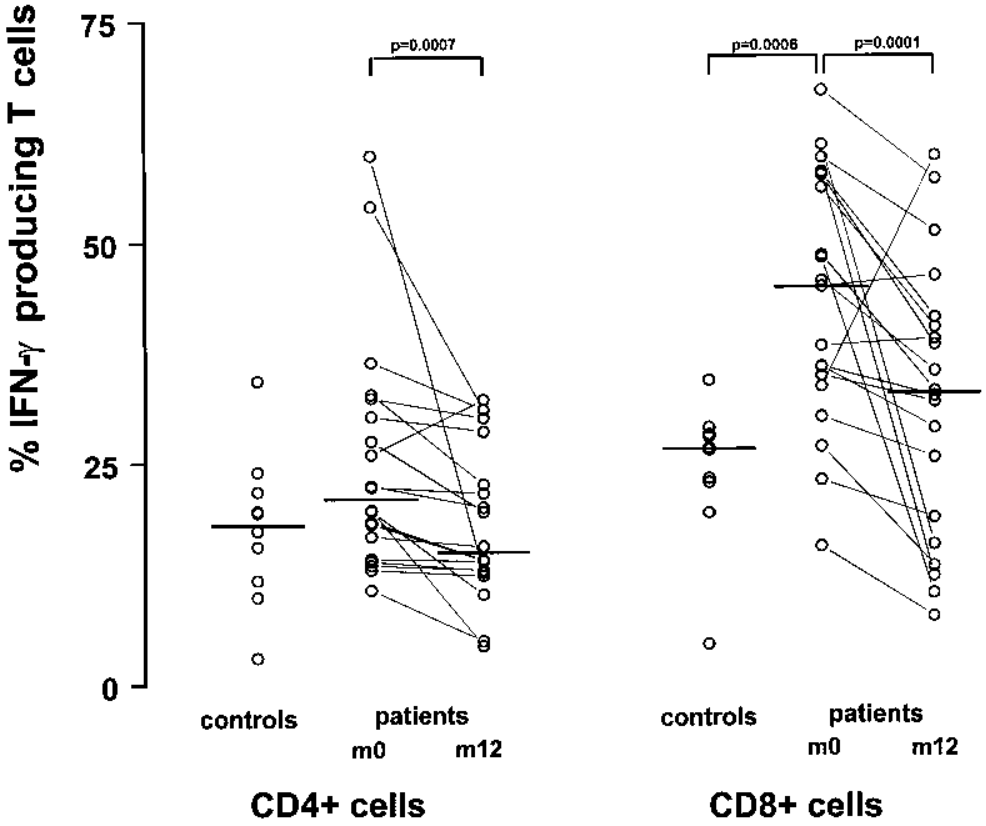

Figure 2. Percentage of IFN-γ positive CD4+ T cells and CD8+ T cells in healthy controls and patients with RA before (m0) and after 12 months (m12) of therapy with MTX and low dose corticosteroids. Bars represent median values.

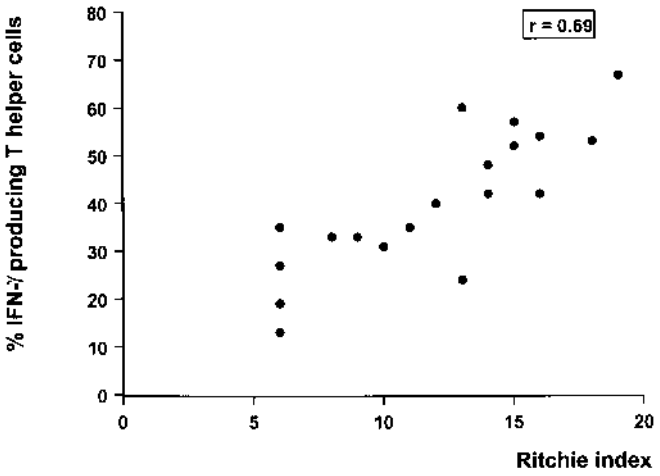

Figure 3. Correlation between percentage of IFN-γ positive CD4+ T lymphocytes and Ritchie Index of patients with RA before therapy (r = 0.69, p = 0.01).

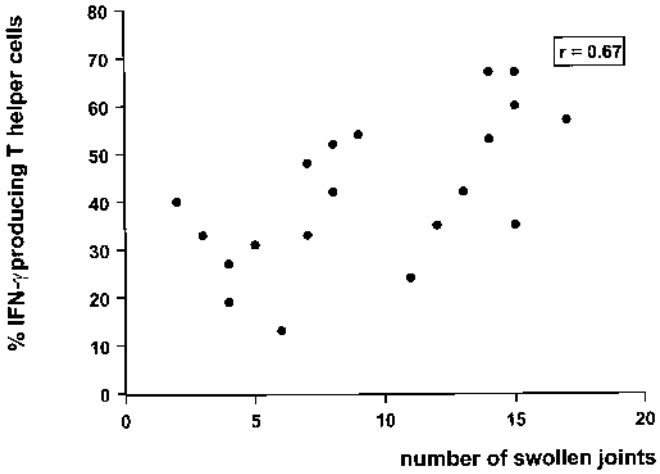

Figure 4. Correlation between percentage of IFN-γ positive CD4+ T lymphocytes and number of swollen joints of patients with RA before therapy (r = 0.67, p = 0.01).

disease modifying antirheumatic drugs, in comparison with healthy controls. In previous studies, *in vitro* type 1 cytokine production by RA peripheral blood and synovial T lymphocytes was found to be deficient[15-21], normal[22-25], or higher[5,8,9,11,14,15,25,26] compared with lymphocytes from controls. These discrepancies may be partially due to methodological differences, but they may also reflect the broad clinical and pathological spectrum of RA. In our study, cytokine production was assessed at the single cell level by flow cytometry after stimulation with PMA and ionomycin, clearly identifying the proportion of cytokine positive CD4+ and CD8+ T lymphocytes, in contrast to others[17-24,27], in which supernatants of stimulated mononuclear cells after phytohemagglutinin (PHA) stimulation were used to determine cytokine production.

In this study, a type 1 cytokine profile was observed in CD4+ and CD8+ T lymphocytes in patients with RA with active disease (i.e., before starting therapy with MTX and corticosteroids). Schulz-Koops, *et al* also described an increased IFN-γ production in patients with active RA treated at the time of evaluation only with nonsteroidal anti-inflammatory drugs[8]. In contrast, RA patients under treatment for at least 3 months with disease modifying antirheumatic drugs (DMARD) or prednisolone showed no difference in type 1 cytokine (IL-2, IFN-γ) production by peripheral blood lymphocytes compared to healthy controls[9,14,25]. Indeed, the disease activity variables of the patients in our study and that of Schulz-Koops were higher; together with the absence of DMARD therapy, this could explain the increased type 1 cytokine production in active RA. In addition, the positive correlation between disease activity variables (Ritchie Index and number of swollen joints) and IFN-γ supports the hypothesis that the imbalance between type 1 and type 2 cytokines can be related to the disease activity and our therapy.

Some studies investigated the short term *in vitro* effects of MTX alone on T cell derived cytokines, concluding that MTX enhances IL-2 production by T lymphocytes and IL-2 mediated cytotoxity[28,29]. Others found evidence *in vitro* for decreased IFN-γ production after PHA and lipopolysaccharide stimulation in combination with MTX[30,31]. In adjuvant and streptococcal cell wall arthritis, MTX normalized defective IL-2 production[32,33]. In patients with RA receiving one single dose of MTX, serum IL-2 levels were increased[34]. Moreover, longterm evaluation of patients treated with MTX showed increased serum IL-2 or IFN-γ concentrations, associated with lower disease activity[35-38]. All these *in vivo* studies have in common that the therapeutic agent was MTX alone for the majority of the patients.

Glucocorticoids interfere with cytokine production not only by suppressing their transcription, but also by decreasing messenger RNA stability and effecting other posttranscriptional events, resulting in inhibited *in vitro* IL-2, IL-4, and IFN-γ production[39-43]. After pulse therapy with high dose corticosteroids in patients with RA, a strong decrease of IFN-γ was seen, while IL-4 production decreased only slightly[11].

Since therapy with MTX in combination with low dose corticosteroids has proven to be more effective in reducing symptoms of inflammation in RA, combination therapy is more commonly used in early RA than MTX alone[44-46]. Thus we examined the longterm effect of MTX in combination with low dose corticosteroids on T cell cytokine profiles. To our knowledge, the influence of combination therapy with MTX and corticosteroids on T cell cytokines in RA has not yet been reported. Although the number of patients and controls is small, these preliminary results nevertheless showed that combination therapy with MTX and low dose corticosteroids resulted in a decreased percentage of IL-2 and IFN-γ positive CD4+ T cells.

Further, the data suggest a role for type 1 cytokine (IFN-γ) positive CD8+ T lymphocytes, since we also found a decreased percentage of IFN-γ positive CD8+ T cells after therapy. In conclusion, RA therapy with MTX in combination with low dose corticosteroids reduces the predominance of a type 1 cytokine profile and leads to a normalized balance between type 1 and type 2 cytokines.

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