Targeting IL-6 signalling in early rheumatoid arthritis is followed by Th1 and Th17 suppression and Th2 expansion

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Abstract

Objective

To investigate the in vitro and ex-vivo effect of IL-6 inhibition on the balance between Th1, Th2, Th17 and Treg cells.

Methods

Ten consecutive adult patients with active early rheumatoid arthritis (ERA) and ten healthy volunteers were included in the study. The percentages of Th1, Th2, Th17 and Treg cells were analysed by flow cytometry in the peripheral blood mononuclear cells obtained from controls and from RA patients at the time of first evaluation and just before the third TCZ infusion. The in vitro effect of TCZ on the different subsets of $CD4^+$ T cells and the expression levels of Th1, Th2, Th17 and Treg-related cytokines was also assessed.

Results

Treatment with TCZ, both ex vivo and in vitro, resulted in a significant reduction of the percentage of Th1, Th17 and Treg cells with a concomitant significant increase of Th2 cell subsets. The reduction of the different subsets of T lymphocytes was associated with an intense staining with Annexin V, suggesting an apoptotic-related cell reduction. A significant decrease of Th1, Th17 and Treg cytokines and a concomitant increase of IL-4 was also observed after TCZ treatment in PBMC isolated from RA patients.

Conclusion

TCZ could modify the immune imbalance in RA inducing apoptosis of Th1, Th17 and Treg cells and promoting the appearance of a Th2 response.

Key words

early rheumatoid arthritis, interleukin 6, tocilizumab, T-effector lymphpcytes

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Received on April 6, 2013; accepted in revised form on July 30, 2013.

Funding: this study was supported by a grant from the Ministero della Università e della Ricerca Scientifica of Italy.

Competing interests: none declared.

Introduction

IL-6 is a pleiotropic pro-inflammatory cytokine which is involved in the modulation of T cell proliferation, differentiation, and apoptosis (1-3). IL-6 in fact regulates the balance between Th1 and Th2 cells (4) and, together with TGF-β, promotes the differentiation of naïve T cell in the highly pathogenic Th17 cell (5), while inhibiting the generation of regulatory T cell (Treg) (6).

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disorder that may affect many tissues and organs, but principally leads to inflammation of the joints and surrounding tissues. In RA, articular inflammation seems to be caused by the expression of inflammatory cytokines and chemokines determining the activation and proliferation of the synovial lining and the recruitment of inflammatory cells and B cell activation with autoantibody production. Among several cytokines involved in RA pathogenesis, IL-6 has been demonstrated to promote synovial neovascularisation, infiltration of inflammatory cells, and synovial hyperplasia leading to RA joint destruction.

Blockade of IL-6 activity has been considered to be of both scientific and clinical interest in RA and tocilizumab (TCZ), a humanised anti-IL-6 receptor antibody, has been shown to be beneficial in decreasing RA disease activity, slowing radiographic progression and improving function thus being considered an effective treatment for RA (7-9). Conflicting data have, however, emerged on Treg and Th17 behaviour during TCZ treatment in both mice (10) and humans (11) and the role of IL-6 inhibition on Th1 and Th2 subset of T effector cells remains to be also elucidated.

The aim of the present study was to evaluate the *in vitro* and *ex vivo* effect of IL-6 inhibition on the balance between CD4 T effector subsets and Treg cells.

Patients and methods

Study population

Ten consecutive adult patients [8 females and 2 male; median ± Standard Deviation (SD) age 47 (3)] with active early rheumatoid arthritis (ERA) [median disease duration 7.5 (range 6–12 months)], diagnosed accordingly to the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) criteria for rheumatoid arthritis (12) and ten sex- and age-matched healthy volunteers (NC) were included in the study. Four of the patients had an inadequate response to methotrexate (MTX) and 6 failed to respond to an association of MTX and TNF-alpha blocking agents. Active disease was defined by at least 6 swollen and tender joint count, a C-reactive protein (CRP) over 10 mg/l and/or an erythrocyte sedimentation rate (ESR) over 30 mm/h and a DAS28 >3.8. All patients received low dose steroids (<7.5 mg daily prednisone). All participants gave their informed consent and the study was approved by the hospital ethics committee. TCZ was given intravenously at a dose of 8 mg/kg at baseline and thereafter every 4 weeks.

Cell preparation, culture, and flow cytometry

Peripheral blood was obtained from controls and from ERA patients at the time of first evaluation and just before the third TCZ infusion. Peripheral blood mononuclear cells (PBMCs) were processed as previously described (13). The cells obtained at baseline (visit T0) were cultured *in vitro* with or without anti-IL-6R (Tocilizumab, Roche) (10 μg/ml) and incubated at 37°C in 5% CO_2 for 24h, 48h, and 72 h. Twenty-four hours before stopping the culture, PMA (50 ng/ml) and ionomycin (1 μg/ml) were added followed by Brefeldin A, 2 hours later (1 μg/ml; Sigma St. Louis, MO). PBMCs were also obtained from patients at the time of the third infusion with TCZ, and considered already treated *in vivo* with TCZ and for this reason stimulated only with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 24 h. After incubation, cells were collected and stained with the following monoclonal antibodies (mAb) anti-human CD4-PerCP (BD Biosciences, San Josè, CA), anti-human IFN-γ-PE (BD Biosciences, San Josè, CA), anti-human IL-17-APC (R&D system, Minneapolis, MN), anti-human IL-4-FITC (BD Biosciences, San Josè, CA), anti-human CD25-FITC APC

(BD Biosciences, San Josè, CA), anti-human FoxP3-PE (BioLegend, SanDiego, CA). Isotype-matched irrelevant antibodies were used as a negative control. Total cells were incubated with mAbs for 30 min on ice and washed twice in PBS, cointaining 0.1% (w/v) NaN3. After surface staining, the cells were fixed with 1% (w/v) paraformaldehyde (Sigma) for 30 min at 4°C and then were permeabilised with a permeabilisation solution (BD Biosciences) for 10 minutes at room temperature and stained with antibodies to intracellular antigens for 30 min at 4°C. Four colour flow cytometry analysis was performed using a FACSCalibur (BD Biosciences). At least 50,000 cells (events), gated on lymphocytes region, were acquired for each sample.

Aliquots of cells coming from *in vitro* experiments were incubated at 37°C 5% CO_2 for 72 hours to determine the percentage of dead cells utilising the Annexin V/PI kit (Roche).

RNA isolation and quantitative real-time polymerase chain reaction (RT-PCR)

RNA was extracted from PBMCs isolated from all the ERA patients, before and after 72 hours of *in vitro* treatment with TCZ as previously described (13). For quantitative TaqMan RT-PCR, master mix and TaqMan gene expression assays for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) control, for IL-10 (Hs00174086_m1), IL12 (Hs00168405_m1), IL-17 (Hs00174383_m1) and IL-4 (Hs00929862_m1) were obtained from Applied Biosystems.

Statistical analysis

The Mann Whitney U-test was used to compare paired patients before and after treatment and to compare patients before treatment (active RA) with the control group. One way Anova was used to compare whether the *in vitro* treatment with TCZ modified the percentage of T cell subsets at different time points. Results were expressed as median ± standard deviation (SD) and were considered statistically significant when $p<0.05$. All tests were two-tailed. Data are presented as histogrammes.

Table I. Baseline characteristics of RA patients and control subjects.

	Controls (n=10)	RA patients (n=10)
Age (years) (SD)	50 (5)	47 (3)
Female (%)	100	80
Disease duration (years), median (SD)	NA	7.5 (2.2)
DAS-28 CRP, median (SD)	NA	6 (1.3)
HAQ, median (SD)	NA	2 (0.8)
CRP (mg/l), median (SD)	NA	4 (1.6)
ESR mm/h, median (SD)	NA	53 (17)
RF, frequency, conc. (UI/L) (range)	NA	60 [84 (34-204)]
Anti-CCP, frequency, conc. (UI/L) (range)	NA	70 [120 (56-345)]

Results

An increase in Th1 and Th17 and a decrease in Treg occur in active RA patients

Clinical characteristics of patients are summarised in Table I. We first assessed the percentages of the different subsets of T-effector lymphocytes in ERA patients and in normal controls. As expected, a significant increase in the percentage of circulating Th17 ($CD4^+$/IFN-γ^+/$IL\text{-}17^+$) (6.5±0.4 in ERA and 3±0.3 in controls, $p<0.004$). and Th1 effector cells ($CD4^+$/IFN-γ^+/IL-17) (9±0.8 in ERA and 3 ± 0.3 in controls, $p<0.004$) was observed in ERA patients. Conversely, the percentage of Treg, defined as $CD4^+/CD25^+/Foxp3^+$ cells, was significantly reduced in active ERA patients compared to controls (0.7±0.12 *vs*. 1.5±0.18, $p<0.006$). The percentage of Th2 cells ($CD4^+$/$IL\text{-}4^+$) was not different between patients and controls (3.03±0.4 *vs*. 3.18±0.3, *p=ns*), confirming the predominance of Th1/Th17 polarisation in ERA patients.

Effect of TCZ treatment on ERA patients

After three months of therapy all patients were in remission (DAS28 <2.6). As shown in Figure 1A, treatment with TCZ resulted in a significant reduction of the percentage of Th1 (9±0.8 *vs*. 3.4±0.2 $p<0.004$), Th17 (6.5±0.4 *vs*. 2.7±0.3, $p<0.003$) and Treg cells (0.7±0.12 *vs*. 0.35±0.06, $p<0.02$). On the other hand a significant increase in the percentage of Th2 cells (4.5±0.29 *vs*. 3.03±0.4, $p<0.01$) was observed in TCZ-treated patients.

In agreement with the variations of the percentages of the different subsets observed after TCZ therapy by flow cytometry, rt-PCR analysis demonstrated that TCZ treatment induced a significant reduction of the expression levels of IL-10 (0.7±0.04 *vs*. 0.2±0.04, $p<0.02$), IL-12 (1±0.08 *vs*. 0.4±0.04, $p<0.003$) and IL-17 (1±0.12 *vs*. 0.47±0.04, $p<0.007$). A significant increase in the expression of IL-4 (0.8±0.04 *vs*. 0.4±0.05, $p<0.006$) also followed TCZ treatment (Fig. 1B).

In vitro experiments

The effect of TCZ on PBMCs was also investigated in *in vitro* studies. Incubation of PBMC with TCZ determined a significant time dependent decrease in the percentage of Th1 and Th17 in both patients and controls at 24h, 48h and 72h respectively. Treg cells showed a time dependent response to TCZ with an initial rapid increase occurring after 24-hour incubation, followed by a rapid decrease to baseline levels after 48–72 hour in both patients and controls. On the other hand a progressive increase of Th2 lymphocytes after TCZ incubation was observed in both patients and controls (Fig. 2A).

Finally TCZ incubation (Fig. 2B-C) resulted in a significant increase of apoptotic cells in Th1 (6.7±0.5 *vs*. 2.1±0.9 $p<0.05$), Th17 (4,1±0.6 *vs*. 1±0.4 $p<0.05$) and Treg (0.98±0.1 *vs*. 0.2±0.9, $p<0.05$) compartments but not in Th2 cells (0.5±0.7 *vs*. 0.6±0.9 *p=ns*).

Discussion

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by the imbalance between effector and regulatory T cells (14). Among the various cytokines involved in immune regulation, IL-6 seems to play a fundamental role in the pathogenesis

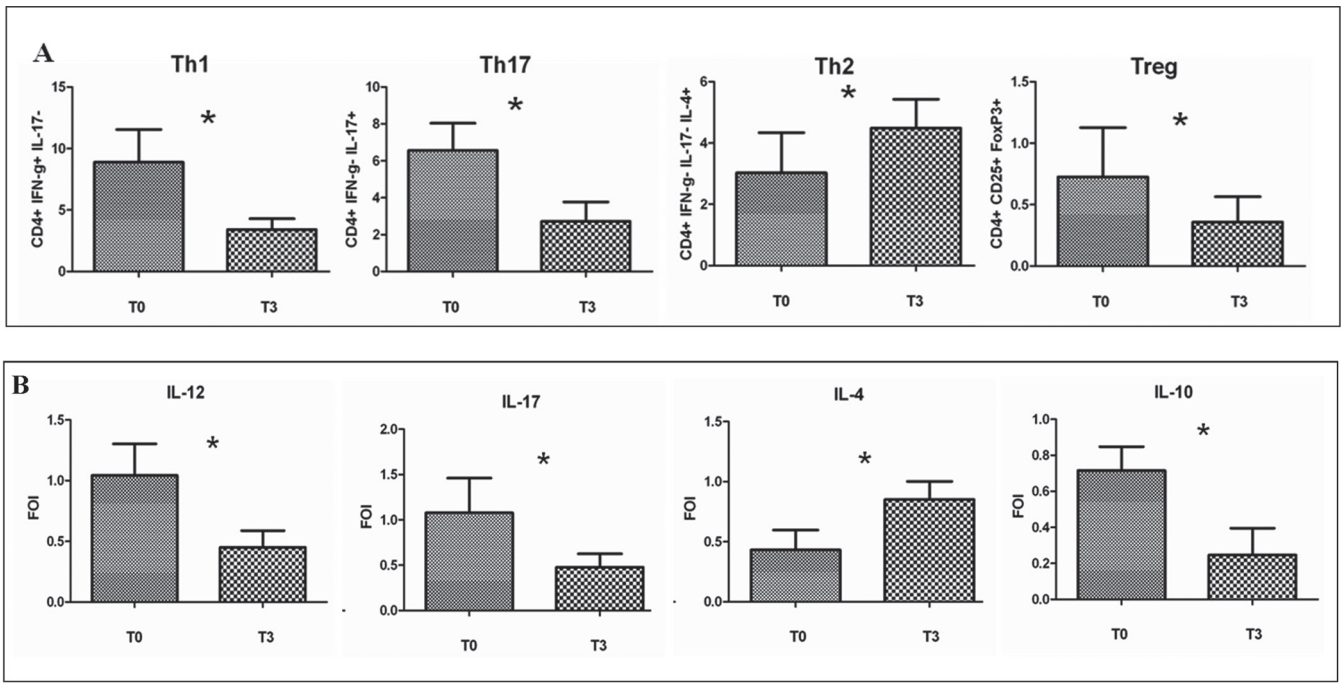

Fig. 1. *Ex vivo* effect of tocilizumab (TCZ) therapy in patients with early rheumatoid arthritis. **A**. Mean percentage of different CD4 T cell subsets in peripheral blood of ERA patients before and after 3 month of TCZ therapy. **B**. Relative mRNA quantification of IL-12, IL-17, IL-4, IL-10 was assessed by quantitative reverse transcriptase PCR in PBMC obtained from patients before and after 3 month of therapy. *$p<0.05$.

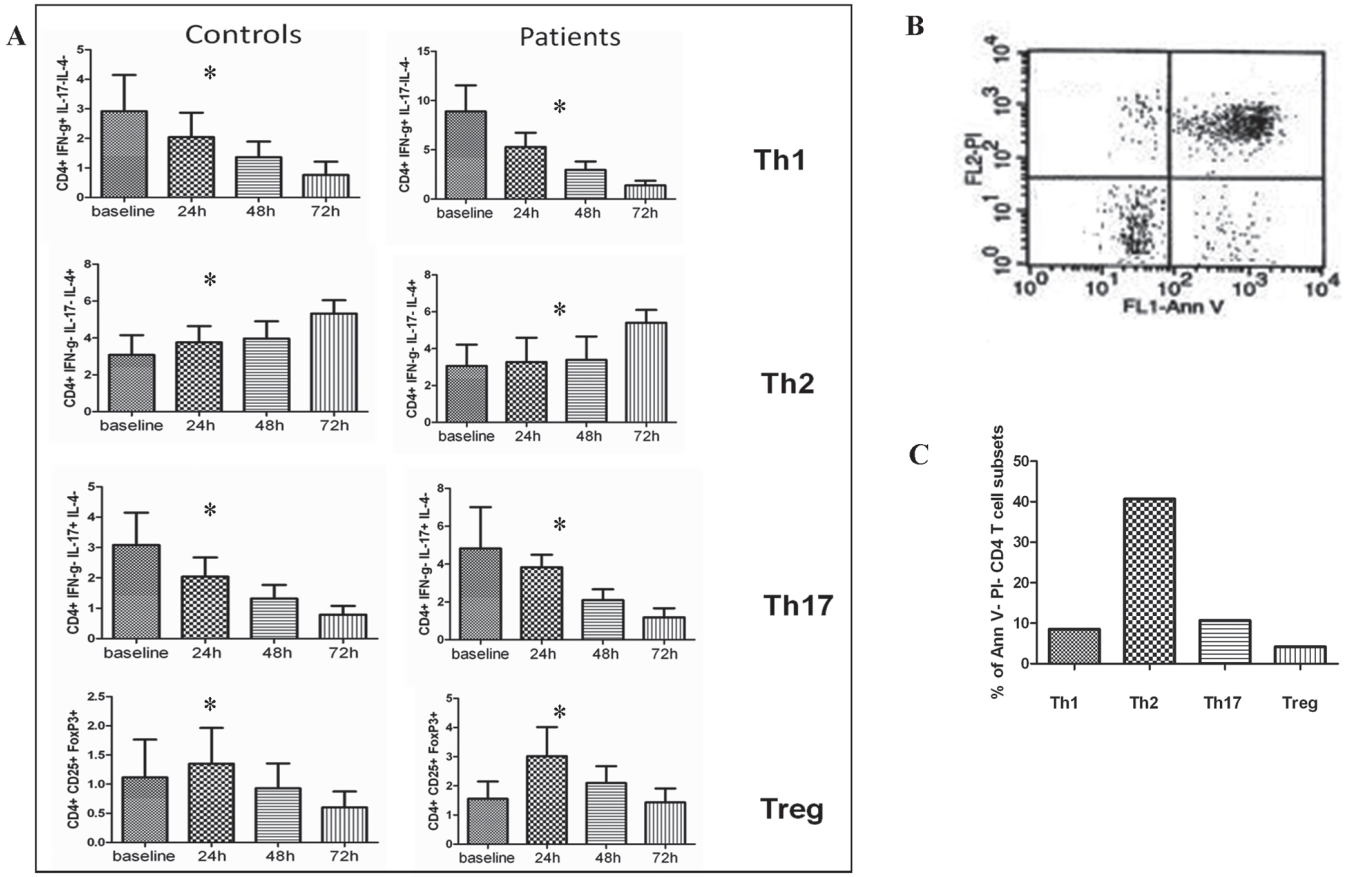

Fig. 2. *In vitro* effect of tocilizumab (TCZ) therapy in patients with early rheumatoid arthritis (ERA). **A**. Mean percentage of different CD4 T cell subsets, obtained from ERA patients before TCZ therapy, treated *in vitro* with TCZ and analysed at different time points. **B**. Viable cells (annexin-V– PI– (14%)); non-viable, including late apoptotic or necrotic cells (annexin-V+PI+(80%) or annexin-V– PI+(2.7%)); and apoptotic cells (annexin-V+ PI–(3.3%)) were detected on lymphocyte region. **C**. Percentage of CD4 T cell subsets evaluated on viable cells region (annexin-V– PI–) represented as a histogramme. * $p<0.05$.

of RA regulating the balance between pathogenic and protective T effector cells, modulating cell proliferation, differentiation, and apoptosis (6, 15). Since the important immunological role of IL-6, its block appears to be an effective therapeutic strategy for the treatment of rheumatoid arthritis. IL-6 block could in fact result in restoring the immune balance in RA patients as suggested by a recent study demonstrating that treatment with TCZ was able to restore the imbalance between Th17 and Treg cells (11).

In our study a significantly *in vivo* and *in vitro* exposure to TCZ induced a profound depletion of Th1, Th17 and Treg lymphocytes and their related cytokines (IL-12, IL-17, IL-10). This immunologic effect seems to be related to the capacity of TCZ in inducing late apoptosis of Th1/Th17 and Treg cell subsets, as suggested by the intense staining for annexin V we observed after *in vitro* exposure of PBMC of patients and controls to TCZ. In agreement with this hypothesis is, in our opinion, the demonstration that IL-6 inhibits the Fas-mediated death of T cells (16) and that blocking of IL-6 in Crohn's disease patients results in the intense apoptosis of lamina propria T lymphocytes (17).

These results are partially different from a recent study demonstrating a significant increase of Treg cell subset in RA patients after TCZ therapy (11). Indeed we observed, only a transient early increase in Treg expression *in vitro* experiments and it is not possible to exclude that an initial expansion of Treg could also occur in the early phase of *in vivo* TCZ treatment contributing to limiting the inflammation in RA. This effect, however, accordingly to our results, could be not relevant in the late phases of treatment.

Interestingly, we also demonstrated that the apoptotic-dependent reduction of Th1/Th17/Treg cells was accompanied by an increase of the percentage of Th2 cells and an enhanced expression of IL-4, an important Th2-related cytokine. This different immunologic behaviour could be explained by the different response of activated Th1 and Th2 cells to the Fas-mediated apoptosis, since that this form of apoptosis may suppress Th1 but not Th2 cells (18, 19). In addition, since that Th1 cells inhibit the proliferation, and therefore the development of Th2 cells, we cannot exclude that the reduction of Th1 response by TCZ may directly favour a Th2 polarisation.

In conclusion our study supports previous results on the protective mechanisms of IL-6 blockade in RA that indicates a role of TCZ in the down-regulation of Th1/Th17 helper responses and suggests that the expansion of Th2 cells could represent a relevant immunologic mechanism contributing to the resolution of inflammation in TCZ-treated RA patients.

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