

**Inhibition of IL-6 function corrects Th17/Treg imbalance
in rheumatoid arthritis patients**

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Authorship contributions

M. Samson and B. Bonnotte were the principal investigators and take primary responsibility for the paper. B. Bonnotte, S. Audia, P. Ornetti and J.F. Maillefert recruited the patients. M. Samson, S. Audia, M. Ciudad, M. Trad and J. Fraszczak performed the laboratory work for this study. M. Samson participated in the statistical analysis. M. Samson, J.F. Maillefert and B. Bonnotte coordinated the research. M. Samson, N. Janikashvili, Pierre Miossec and B. Bonnotte wrote the paper.

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ABSTRACT

Objective: From an immunological standpoint, the mechanisms by which Tocilizumab (TCZ), a humanized anti-interleukin-6 (IL-6) receptor antibody, improves Rheumatoid Arthritis (RA) patients are still not fully understood. In *vitro* studies and results from mouse models have emphasized the critical role of IL-6 in Th17 differentiation. Th17 lymphocytes have been demonstrated to be strongly involved in RA pathogenesis. The objective of the current study was to investigate the effect of IL-6 blockade on the Th17/Treg balance in patients with active RA.

Methods: Patients suffering from active RA for whom TCZ was indicated by their rheumatologist were enrolled in this study. Phenotypic analyses of T cell populations were performed and the disease activity score rated by the 28 joint count (DAS28). Serum cytokine levels and other inflammation parameters were measured before the first infusion and after 3 infusions of TCZ (8mg/Kg).

Results: Compared to controls, Th17 cells ($CD4^+IL-17^+$) were increased and Treg ($CD4^+CD25^{high}Foxp3^+$) decreased in the peripheral blood of active RA patients. The suppressive function of circulating Treg was not impaired in active RA patients. TCZ treatment induced a significant decrease in the DAS28 associated with a significant decrease in the percentage of Th17 cells (from 0.90 to 0.45%; $p=0.009$) and an increase in the percentage of Treg (3.05 to 3.94%; $p=0.0039$) in all the patients.

Conclusion: This study reports for the first time that the inhibition of IL-6 function by TCZ restores the Th17/Treg imbalance in RA patients.

Keywords: rheumatoid arthritis, T-cell, cytokines, tocilizumab

Introduction

The balance between Th17 cells and regulatory T cells (Treg) is of major importance in autoimmunity. From a general point of view, Th17 cells promote autoimmunity whereas Treg protect against the occurrence of autoimmune diseases (1). In rheumatoid arthritis (RA), Th17 cells have been shown to play a central role by secreting IL-17 which activates numerous cell types involved in the pathogenesis of RA, including synovial fibroblasts, monocytes, macrophages, chondrocytes and osteoblasts (2-3). In addition, RA is associated with the production of proinflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). Inhibition of IL-6 signalling by blocking the gp130 pathway or by knocking out the *IL-6* gene significantly improves autoimmune arthritis in experimental animal models (4-5). Moreover, tocilizumab (TCZ), a humanized anti-IL-6 receptor antibody, has been shown to be an efficient treatment for RA (6-7). However, why IL-6 blockade improves RA is still unclear since this cytokine may play a dual role. On one hand, IL-6 may trigger the hepatic acute-phase response and directly activate different cells such as B and T lymphocytes, macrophages and osteoclasts. On the other hand, IL-6 may act earlier in RA pathogenesis. Actually, IL-6 has been shown to be of particular importance in mouse Th17 differentiation. The addition of IL-6 to murine CD4⁺ T cells cultured in the presence of TGF- β skew their differentiation toward Th17 instead of immunosuppressive FoxP3⁺ Treg cells (1). In humans, the mechanisms of Th17 differentiation are still incompletely characterized. While IL-6 has also been shown to promote Th17 polarization *in vitro*, previous reports have demonstrated that other cytokines, such as IL-1 β and IL-23, are also involved in this process (8). Unlike to the mechanisms described in mice (1), TGF- β plays an indirect role in human by inhibiting Th1 generation and therefore enhancing Th17 polarization from precursor cells defined by a CD161⁺CD4⁺ phenotype (9-10).

In mice, it has been shown that the blockade of the IL-6 pathway results in a decrease in Th17 immune response (5). It has been demonstrated that TCZ improves the condition of RA patients and decreases inflammatory parameters. However whether TCZ efficacy may be related to its ability to negatively affect Th17 differentiation *in vivo* has not been investigated (6-7). In the current study, we used the ability of TCZ to inhibit IL-6 function in order to decipher the role of IL-6 on the immune balance between Th17 and Treg cells in patients suffering from active RA.

Materials and Methods

Study population

Fifteen patients (11 women and 4 men) fulfilling the American College of Rheumatology/European League Against Rheumatism revised criteria for RA (11), with a median [IQ range] DAS28 of 5.53 [4.32-6.31], for whom TCZ was prescribed by their rheumatologist, were enrolled in this study after their informed consent was obtained. The study was approved by the local ethics committee (2010-018696-21). All patients suffered from seropositive RA with a median duration of 11 years [4-13]. They had all received methotrexate (MTX) in their past history and six of them (40%) were still receiving MTX when TCZ was started. Patients had received a median of 2 [1-3] courses of biotherapy before TCZ: infliximab (n=5), etanercept (n=12), adalimumab (n=7), certolizumab (n=1), rituximab (n=5), anakinra (n=1) and abatacept (n=3). Eleven patients (73.33%) were also treated with steroids because of the disease activity at a median daily dose of 20 mg of prednisone [10-40]. TCZ was prescribed monthly at the dose of 8 mg/kg for all patients. Blood samples were collected just before the first and the 4th infusion of TCZ. These 15 active RA patients were compared to 17 healthy volunteers. Healthy volunteers had no inflammatory syndrome (C-reactive protein (CRP) < 0.5 mg/dL), no recent steroid therapy or immunosuppressive drugs,

no history of cancer, no recent acute or chronic infectious diseases, no autoimmune or autoinflammatory diseases. TCZ treatment was stopped in 2 patients because of urticaria and in one because of diarrhea with fever. Paired data from 6 women and 3 men with a median age of 56 years [37-63], a median disease duration of 10 years [3.5-12.5 years] and a median DAS28 of 5.53 [4.37-6.16] were analyzed before and after TCZ treatment.

Cell preparation, Culture, and Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll gradient centrifugation. CD4⁺ T cells were then purified by magnetic cell sorting (Miltenyi Biotec, Paris, France) and stimulated with 0.1 µg/mL of phorbol 12-myristate 23-acetate (PMA) plus 1 µg/mL of ionomycin (Sigma-Aldrich, Saint Quentin Fallavier, France) for 8 hours; 1 µL/mL of Brefeldin A (BD Golgi Plug, BD Bioscience, Le Pont de Claix, France) was added for the last 4 hours. The cells were stained with anti-IL-17A (PE), anti-IFN-γ (APC) monoclonal antibodies (eBioscience, Paris, France). CD4⁺ lymphocytes were isolated before cytokine staining, because stimulation with PMA triggers the internalization and degradation of CD4, which disturbs the identification of Th1 (CD4⁺IFN-γ⁺) and Th17 (CD4⁺IL-17⁺) cells (12). Treg (CD4⁺CD25^{high}Foxp3⁺) were stained with anti-CD4 (PECy5.5), anti-CD25 (PE) and anti-Foxp3 (Alexa488) (Human Treg Flow™ Kit, Biolegend, Ozyme, Saint Quentin Yvelines, France) following the manufacturer's instructions. Flow cytometry staining was performed and analyzed at the IFR 100 Santé STIC Cytometry platform, at the University of Burgundy, on a BD Bioscience LSRII cytometer and analysed with FlowJo® software.

Proliferation assays

CD4⁺CD25^{high} (Treg) and CD4⁺CD25⁻ (T effectors) cells were isolated from PBMCs using a human Treg isolation kit (Miltenyi Biotec), following the manufacturer's instructions. Effector T cells were stained with cell trace violet cell proliferation kit (Invitrogene) and

cultured with or without anti-CD2/CD3/CD28 microbeads (Treg suppression inspector, Miltenyi Biotec) and Treg, as indicated. After 4 days of culture, cell trace dilution was analyzed by flow cytometry and the proliferation index was calculated using the ModFit LTTM 3.0 software.

Cytokine assays

IL-17A, IL-1 β and IL-6 were quantified in the serum by ELISA (eBioscience).

Statistical analysis

The Wilcoxon signed rank test was used to compare paired patients before and after treatment. The Mann Whitney U test was used to compare patients before treatment (active RA) with the control group. Results were considered statistically significant when $p < 0.05$. All the tests were two tailed. Data were presented as median [IQ range]. Analyses were performed using GraphPad Prism[®].

Results

Th17 are increased and Treg decreased in active RA patients

The percentage of circulating Th17 cells, defined as CD4⁺IL-17⁺ lymphocytes (figure 1A), was significantly increased in RA patients (n=15) compared to controls (n=17): 0.88 vs. 0.36 ($p=0.0008$) (figure 1B). Phenotypic analyses confirmed that these cells exhibit a phenotype consistent to that of Th17 lymphocytes (CCR6⁺CD161⁺CD45RA⁻) in both groups (data not shown). In contrast, no difference in the percentage of Th1 cells, defined as CD4⁺IL-17⁻IFN- γ ⁺ cells, was observed between RA patients and healthy subjects: 12.3 vs. 12.42% ($p=0.8799$) (figure 1A, 1B). The percentage of Treg, defined as CD4⁺CD25^{high}Foxp3⁺ cells, was reduced in active RA patients: 3.05 vs. 4.31% ($p=0.0396$) (figure 1B). However, the

immunosuppressive function of Treg ($CD4^+CD25^{\text{high}}$) was not different between RA patients and healthy volunteers (figure 1C). RA patients exhibited an increase in the Th17 to Treg ratio: 0.25 vs. 0.09 ($p<0.0001$) (figure 1D). IL-6 was heterogeneously increased in the serum of active RA patients, whereas it was not detected in the serum of healthy controls ($p=0.006$) (not shown). The serum levels of IL-17 and IL-1 β were very low and not different between RA patients and healthy controls (not shown).

TCZ reduces the Th17 to Treg ratio

All 9 patients responded to TCZ treatment as they achieved a significant decrease in the DAS 28 (table I, figure 2B). No significant difference was seen in the evolution of neutrophils, CD4 and CD8 T lymphocytes after TCZ treatment (Table I). Blockade of IL-6 effects with TCZ did not significantly modify Th1 lymphocytes (Figure 2A). Interestingly, a significant decrease in the percentage of Th17 cells was detected after TCZ treatment, from 0.9 to 0.45% in total $CD4^+$ cells ($p=0.009$) (figure 2A). In contrast, TCZ administration resulted in an increase in the percentage of Treg, from 3.05 to 3.94% in total $CD4^+$ cells ($p=0.0039$) (figure 2A). Importantly, Treg from TCZ-treated patients were still capable of suppressing the proliferation of autologous effector T cells (figure 1C). Thus, TCZ induced a clinical improvement associated with a correction of the Th17/Treg ratio, from 0.3 to 0.11 ($p=0.009$) (figure 2C). The serum level of IL-6 was not significantly modified by TCZ (from 13.14 to 8.43, $p=0.6406$, data not shown). Interestingly, we observed that Th17 cells begin to decrease and Treg to increase before the complete remission of the disease ($DAS<2.6$) (figure 2D).

Discussion

This study confirmed the major imbalance between Th17 cells and Treg in active RA patients as it has already been reported in the literature (13-14). Furthermore, we have demonstrated that the Th17/Treg imbalance occurring in RA patients can be corrected by TCZ-mediated

blockade of IL-6 signals, which is associated with improved clinical outcome. These results emphasize the benefit of therapeutic approaches based on Th17 inhibition and/or Treg promotion in RA. By blocking the IL-6 pathway, TCZ may either decrease IL-6-induced inflammation and/or affect Treg and Th17 differentiation. Interestingly, IL-6 was not elevated in the serum of all RA patients. Furthermore, TCZ treatment did not induce a significant decrease in IL-6 serum concentration, whereas clinical improvement associated with a decrease in the Th17/Treg ratio was observed in all these patients. These results therefore suggest that decrease in IL-6 induced inflammation may not be the primary mode of action of TCZ. Since TCZ corrects the Th17/Treg imbalance in RA patient before complete clinical remission, the modulation of these two antagonistic lymphocyte subsets may represent a likely mechanism of action of TCZ underlying its clinical efficacy. Evidence has been provided that helper T lymphocytes can be redirected to other lineages depending on the cytokine environment (15). In fact, the cytokine milieu can differentially activate master regulator genes (T-bet, Gata3, and ROR γ -t), which, associated with Signal Transducer and Activator of Transcription proteins (STATs) such as STAT3, -4, -5 or -6 or Socs3, change T helper polarization (15). Although additional studies are needed to identify the molecular bases controlling TCZ-mediated Treg increase and Th17 reduction, the present report demonstrates for the first time that *in vivo* inhibition of IL-6 function with TCZ can restore a physiologic Th17/Treg balance in RA patients. This study opens a new field in the treatment and the monitoring of anti-cytokine therapies in RA and other autoimmune diseases in which IL-6, Treg and/or Th17 cells may be involved.

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TABLES:
Table I: Characteristics of RA patients before and after TCZ

	Patients treated by TCZ (8 mg/Kg)		
	Before treatment	After treatment	<i>p</i> value
	median [IQ range]	median [IQ range]	
Leucocytes (G/L)	10,400 [7,800-11,055]	8,000 [6,150-10,380]	<i>0.4409</i>
Neutrophils (G/L)	4,990 [4,040-7,677]	3,580 [2,372-6,660]	<i>0.4258</i>
Lymphocytes (G/L)	2,461 [1,300-3,793]	2,930 [2,440-3,352]	<i>0.3594</i>
T cells (G/L)	1,936 [877-2,715]	2,250 [2,085-2,780]	<i>0.4258</i>
CD4 T cells (G/L)	1,477 [577-1,842]	1,536 [1,080-1,823]	<i>0.25</i>
CD8 T cells (G/L)	605 [277-873]	576 [501-922]	<i>0.6523</i>
B cells (G/L)	194 [92-375]	248 [104-363]	<i>0.7344</i>
DAS 28	5.61 [4.37-6.16]	2.00 [1.34-2.73]	<i>0.0039</i>
Tender joint count (n)	4 [2.5-12]	2 [0-3.5]	<i>0.0249</i>
Swollen joint count	8 [2-19]	1 [0-3.5]	<i>0.0156</i>
ESR (mm/hr)	28 [13.8-60.8]	2 [2-4.5]	<i>0.0078</i>
VAS general health patient (mm) (%)	70 [53-73]	20 [20-38]	<i>0.009</i>
C-Reactive Protein (mg/L)	13 [4-70.4]	2 [0.5-2.5]	<i>0.0078</i>
Prednisone (mg/day)	10 [4.5-50]	9 [0-17.5]	<i>0.125</i>

IQ range: interquartile range

FIGURE LEGENDS:

Figure 1: Circulating Th17 are increased in RA patients whereas Treg are decreased but functional. A, B: Flow cytometry analysis of Th17 (CD4⁺IL-17⁺), Th1 (CD4⁺IL-17⁻IFN- γ ⁺) and Treg (CD4⁺CD25^{high}Foxp3⁺) cells in RA patients (n=15) and healthy subjects (n=17). **C:** Functional analysis of Treg (CD4⁺CD25^{high}). The proliferation index was measured using the ModFit LTTM 3.0 software. The percentage of inhibition was calculated using the proliferation index of stimulated T effectors without Treg as reference. Percentages of inhibition were compared ratio to ratio. Active RA patients (n=6), TCZ treated RA (n=3) and healthy controls (n=6). **D:** Th17/Treg ratio. RA patients (n=15), healthy subjects (n=17). The Mann Whitney U test (Kruskall-Wallis test for 1C) was used for statistical analysis. NS: not statistically significant. Whiskers are as follow: the median (horizontal bar), the IQ range (box) and the extreme values (error bars).

Figure 2: Correction of the Th17/Treg ratio is linked to the clinical response. A, C: Percentages of Treg, Th1 and Th17 cells (A) and Th17/Treg ratio (C) from RA patients before and after TCZ treatment (n=9). **B:** DAS28 before and after TCZ treatment in RA patients (n=9). **D:** Evolution of the DAS28 and the percentages of Treg and Th17 cells during the treatment of a RA patient with TCZ. Wilcoxon signed rank test was used. NS: not statistically significant. Error bars are the IQ range.