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SHORT COMMUNICATION

The in vitro addition of methotrexate and/or methylprednisolone determines peripheral reduction in Th17 and expansion of conventional Treg and of IL-10 producing Th17 lymphocytes in patients with early rheumatoid arthritis

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Abstract The aim of our study was to evaluate methotrexate (MTX) and methylprednisolone (MP) effect on peripheral Th17 and Treg subsets in patients with rheumatoid arthritis (RA). We enrolled 15 patients (10 early RA and 5 long-standing disease) with active RA and 10 age-matched healthy donors as controls. Frequencies of Th17 and Treg were quantified using flow cytometry before and after in vitro addition of MTX, MP or both drugs. Our results showed a reduction in the overall Th17 population followed by an increase in Th17 IL-10$^+$ and Treg, after in vitro treatment of PBMCs with the drugs in patients with early RA. Long-standing disease patients showed a less evident increase in Treg cells and less enhancement of IL-10 Th17 cells. We suggest that the treatment with MTX and MP could ameliorate RA disease activity by normalizing the distribution/imbalance of Th17/Treg and indicate a new regulatory role of IL-17$^+$ cells in RA patients.

Keywords Rheumatoid arthritis · Methotrexate · Steroid · IL-17 · Treg · Th17

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Introduction

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune diseases in which an imbalance between pro-inflammatory and anti-inflammatory subsets of T lymphocytes seems to occur [1].

The chronic synovial damage and the resulting progressive destruction of cartilage and joints are mainly mediated by pro-inflammatory cytokines released by Th1 and Th17 subsets of T effector cells [2].

A defect in Treg functions has been also described in RA [3]. Treg cells inhibit proliferation and cytokine production of conventional T cells in physiologic conditions, and their deficiency seems to be associated with autoimmunity [4]. Treg cells control the occurrence of abnormal immune responses not only through the down-regulation of co-stimulatory molecules but also through the secretion of suppressive cytokines such as TGF-β, IL-10 and IL-35. Recently, it has been also demonstrated that Treg may potentially produce pro-inflammatory cytokines, such as IL-17, particularly when in vivo exposed to an inflammatory environment [5, 6]. Conventional Treg are usually identified as CD4$^+$/Foxp3$^+$/CD25$^+$/IL-10$^+$ cells. Recently, however, a new subset of T cells producing both IL-17 and IL-10 and displaying regulatory function (Th17$^+$IL-10$^+$ cells) has been described [5]. Data on Th17 IL10$^+$ in RA are, until now, lacking.

It is widely accepted that the therapeutic goal for patients with RA is the achievement of clinical remission and several evidences suggest that T cell subsets may be influenced by anti-RA agents. Studies evaluating the changes in T cell subset distribution in response to conventional disease modifying anti-rheumatic drugs (DMARD) are, however, limited at present.

The aim of the present study was to evaluate the in vitro effect of methotrexate (MTX) and/or methylprednisolone

Table 1 Baseline characteristics of RA patients and control subjects

	Controls ($N = 10$)	ERA patients ($N = 10$)	LRA patients ($N = 5$)
Age (years) (SD)	48 (3)	44 (3)	49 (2)
Female (%)	100	80	100
Disease duration (years), median (SD)	NA	<1 (0.6)	7.7 (2.3)
DAS-28 CRP, median (SD)	NA	6 (1.6)	5.9 (1.4)
HAQ, median (SD)	NA	2 (0.6)	3 (0.7)
CRP (mg/l), median (SD)	NA	5 (1.9)	5 (1.9)
ESR (mm/h), median (SD)	NA	57 (22)	47 (18)
RF, frequency, conc. (UI/l) (range)	NA	72 [87 (32–210)]	65 [80 (30–222)]
Anti-CCP, frequency, conc. (UI/l) (range)	NA	86 [130 (54–355)]	80 [135 (50–398)]

(MP) on the percentage of Th17 and Treg cell subsets. Here, we demonstrate that MTX and/or MP are capable to modify in vitro the imbalance of Th17/Treg in the peripheral blood of patient with early RA and suggest a possible regulatory function of Th17 cells through the expression of IL-10.

Materials and methods

Subjects

Blood from 15 RA patients and 10 healthy donors was obtained for this study. Gender and clinical data of all subjects are shown in Table 1. Patients fulfill the 1987 criteria of the American College of Rheumatology for RA. Ten of the patients, classified as having an early RA (ERA), were DMARDs naïve and did not assume prednisone or equivalents since last 4 weeks before blood collection. Five patients had a long-standing disease (LRA). All of them exhibited insufficient response to leflunomide or salazopyrin in monotherapy and were MTX intolerants and were taking glucocorticoids <10 mg per day. Patients and healthy donors signed the informed consent, as suggested by the bioethical committee of Paolo Giaccone University Hospital, Palermo.

Isolation, cell culture and intracellular staining

Peripheral blood mononuclear cells (PBMCs) were obtained as previously described [7]. Part of recovered cells (1×10^6/ml) were in vitro cultured with PMA (50 ng/ml) and ionomycin (1 μg/ml) (Sigma St. Louis, MO) for 24 h; the remaining cells were incubated with MTX (10 μM), MP alone (1 μg/ml) or MTX + MP at 37 °C in 5 % CO_2. After 2 h of incubation, Brefeldin A (10 μg/ml; Sigma St. Louis, MO) was added, and after 24 h of incubation, cells were collected and stained with the following monoclonal antibodies (mAb): anti-human CD4-PE-Cy5.5 (BD Biosciences, San Josè, CA), anti-human IL-17-APC (R&D system, Minneapolis, MN), anti-human CD25-FITC (BD Biosciences, San Josè, CA) and anti-human IL-10-PE (BD Biosciences, San Josè, CA).

Isotype-matched irrelevant antibodies were used as a negative control. Cells were incubated with mAbs for 30 min on ice and washed twice in PBS, containing 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 permeabilized with a permeabilization solution (BD Biosciences) for 10 min at room temperature and stained with antibodies for intracellular antigens for 30 min at 4 °C. Four color 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.

Statistical analysis

Statistical analysis of quantitative variables was performed using the Mann–Whitney rank-sum test. Data are expressed as mean ± SEM. p values < 0.05 were considered significant.

Results

At baseline, the percentage of Th17 effector T cells was found to be significantly higher in the peripheral blood of both ERA (3.7 ± 0.4) and LRA (4 ± 0.5) patients when compared to those of control subjects (1 ± 0.07), no difference being present between ERA and LRA (Fig. 1a, f). As shown in Fig. 1d, the expression of RORγt did not significantly differ between ERA (2.5 ± 0.4) and LRA (3 ± 0.5). The percentages of the conventional Treg identified as $CD4^+/CD25^+/IL\text{-}10^+/IL\text{-}17^-$ T cells were significantly reduced in RA patients (ERA 1 ± 0.03; LRA 0.8 ± 0.02) compared to controls (2.3 ± 0.6) (Fig. 1b, g). The same reduction was found among $FoxP3^+$ cells in ERA (0.8 ± 0.02) and LRA (0.5 ± 0.05). $CD4^+IL\text{-}17^+IL\text{-}10^+$ T

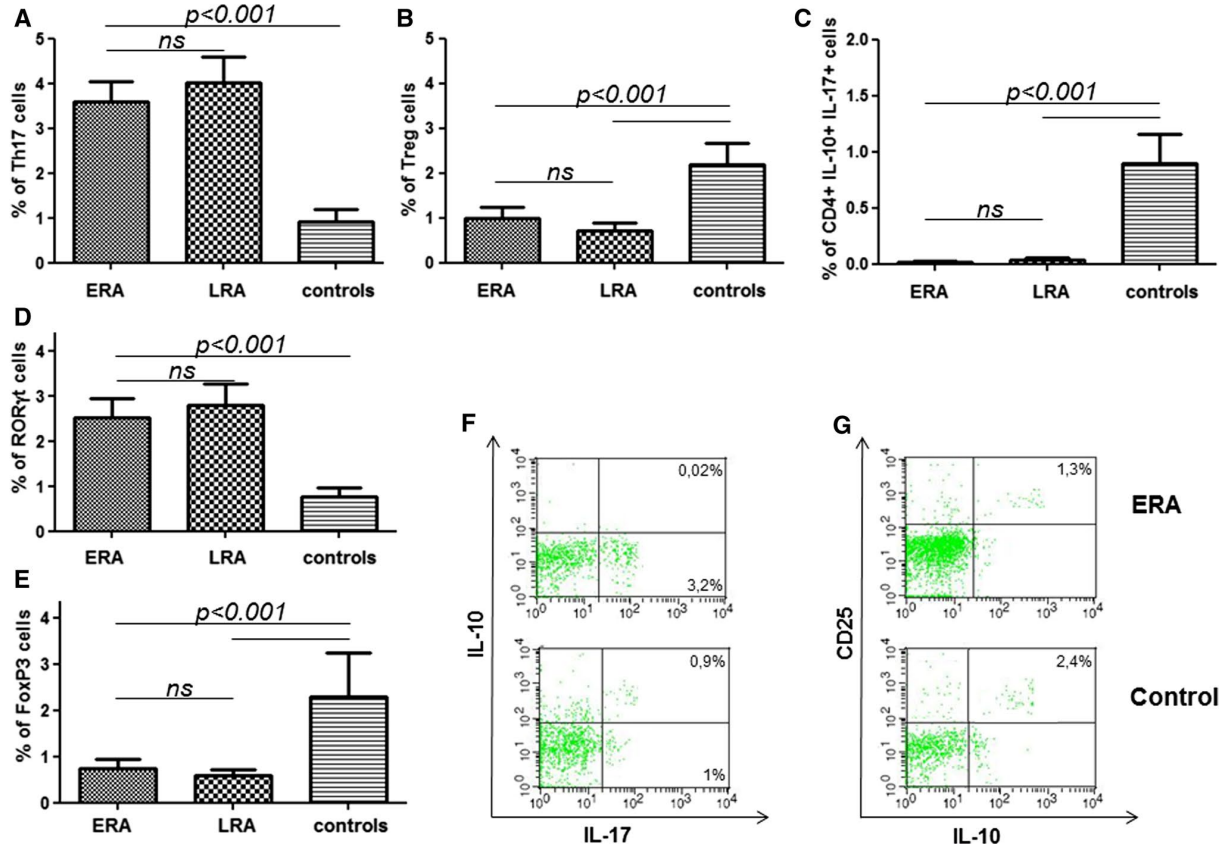

Fig. 1 Ex vivo percentages of Th17, Treg and Th17-IL-10$^+$ subsets. **a** Mean percentage of Th17 cell subset in peripheral blood of ERA and LRA patients and controls. **b** Mean percentage of Treg cell subset in peripheral blood of ERA and LRA patients and controls. **c** Mean percentage of Th17-IL-10$^+$ cell subset in peripheral blood of ERA and LRA patients and controls. **d** Mean percentage of RORγt$^+$ cells in peripheral blood of ERA and LRA patients and controls. **e** Mean percentage of FoxP3$^+$ cells in peripheral blood of ERA and LRA patients and controls. **f**, **g** Dot-plot analysis of Th17, Treg and Th17-IL-10$^+$ from a representative ERA patients and a healthy subject

cells were not detected in the PBMC from patients but were present in healthy subjects (1 ± 0.05) (Fig. 1c, f).

After incubation with MTX, a significant reduction in the percentage of Th17 effector cells (1.9 ± 0.03) was observed in the PBMC from ERA patients (Fig. 2a, d) together with a significant increase in Treg cells (2.27 ± 0.15) (Fig. 2b, e). Moreover, the percentage of RORγt positive cells was also significantly reduced in ERA (1.7 ± 0.2) compared to LRA patients (2.7 ± 0.3). A massive increase in CD4$^+$IL-17$^+$IL-10$^+$ T cells (1.1 ± 0.02) after incubation with MTX was also observed (Fig. 2c, d). Interestingly, these cells expressed both RORγt and FoxP3 differently from conventional Tregs that do not express RORγt (CD4$^+$IL-17$^+$IL-10$^+$ RORγt$^+$ FoxP3$^+$ 0.96 ± 0.04).

Incubation of PBMC from patients with LRA determined a less evident increase in Treg cells (1.76 ± 0.1) (Fig. 2b), a less consistent expansion of CD4$^+$IL-17$^+$IL-10$^+$ T cells (0.25 ± 0.09) (Fig. 2c) and minor significant reduction in Th17 cells (3 ± 0.1) (Fig. 2a). Similar results were obtained by adding MP to PBMC culture both in ERA (Th17 1.8 ± 0.16, Treg 2.5 ± 0.4, CD4$^+$IL-17$^+$IL-10$^+$ T cells 0.6 ± 0.23) and LRA (Th17 3.2 ± 0.8, Treg 2.1 ± 0.07, CD4$^+$IL-17$^+$IL-10$^+$ T cells 0.4 ± 0.06) and in controls (Th17 0.6 ± 0.03, Treg 3.2 ± 0.05, CD4$^+$IL-17$^+$IL-10$^+$ T cells 0.9 ± 0.07) (Fig. 2). The addition of both MTX and MP determined a significant enhancement of Treg and CD4$^+$IL-17$^+$IL-10$^+$ T cells in ERA patients (Treg 3 ± 0.2, CD4$^+$IL-17$^+$IL-10$^+$ T cells 1 ± 0.08) but not in LRA (Treg 3.3 ± 0.5, CD4$^+$IL-17$^+$IL-10$^+$ T cells 1 ± 0.06) (Fig. 2b, c).

Discussion

Our results show that the in vitro addition of MTX and/or MP in PBMC cultures from patients with ERA is capable to modify the imbalance of Th17/Treg and suggest a possible regulatory function of Th17 cells trough the expression of IL-10, being the differences observed in LRA probably

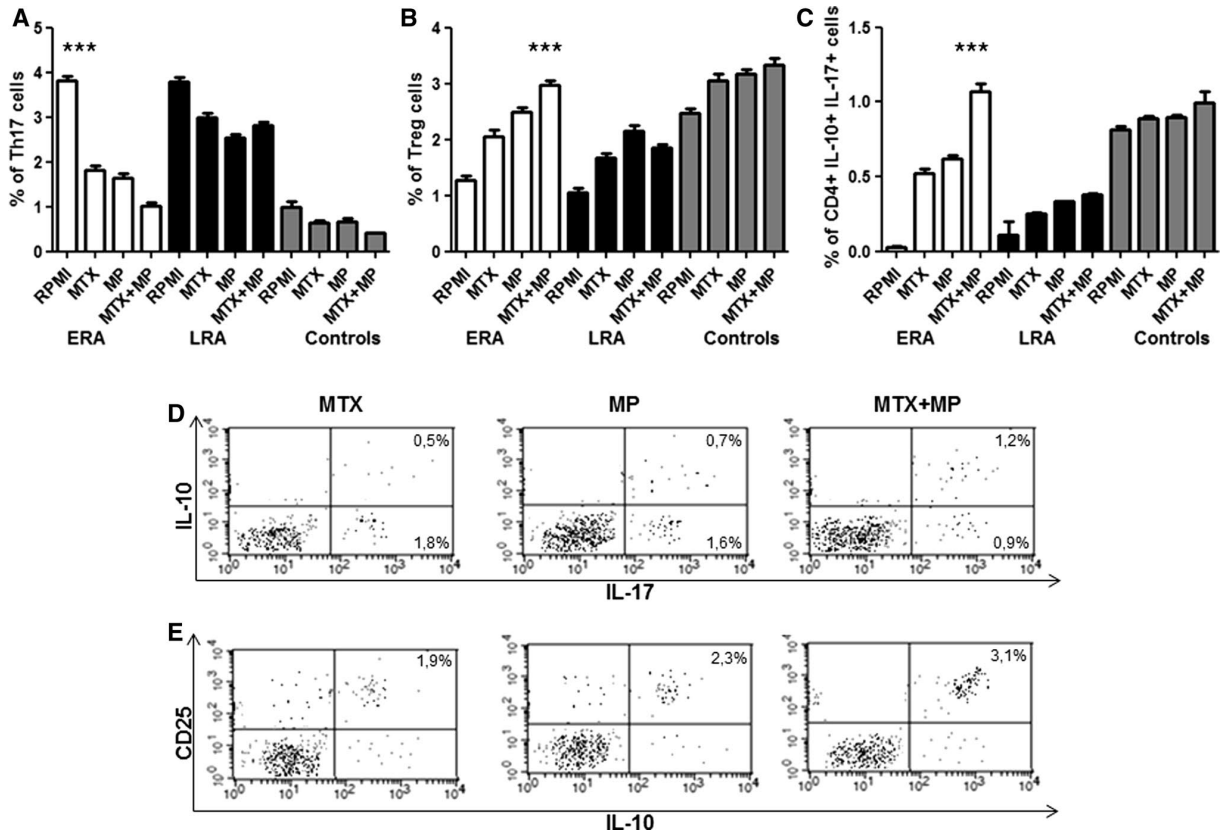

Fig. 2 In vitro effect of MTX, MP and MTX + MP treatment on PBMCs obtained from ERA and LRA patients and controls. **a** Mean percentage of Th17 cell subset after in vitro treatment with MTX, MP and MTX + MP of PBMCs from ERA and LRA patients and controls **b** Mean percentage of Treg cell subset after in vitro treatment with MTX, MP and MTX + MP of PBMCs from ERA and LRA patients and controls. **c** Mean percentage of Th17-IL-10$^+$ cell subset after in vitro treatment with MTX, MP and MTX + MP of PBMCs from ERA and LRA patients and controls. **d**, **e** Dot-plot analysis of Th17, Treg and Th17-IL-10$^+$ from a representative ERA patients after pBMCs incubation with several drugs. *$p < 0.05$

due to the difference stage of disease and/or lower number of patients studied and/or prior therapy and/or indicating an unresponsive status to MTX.

The paradigm of a Th1/Th2 imbalance in RA was refined recently by the identification of Th17 and Treg lymphocyte subsets [4, 8, 9].

It is well known that Th17 cells contribute to tissue damage in RA [8]. Th17 cells act promoting tissue inflammation by the secretion of IL-17 that stimulates synovial fibroblast to release several mediators of joint inflammation including IL-6, IL-8, GM-CSF and PGE2 [10]. Treg function is known to be reduced or absent during RA flares and restored by several targeted treatments such as TNF-α antagonists, anti-IL6R antibodies and rituximab [3, 11]. No data are present in the literature concerning the effect of MTX alone or in combination with MP on Th17, on conventional Treg cells and in particular on the recently described subset of IL-17$^+$ IL-10$^+$ CD4 T lymphocytes.

Recently, Scottà et al. [6] described a subpopulation of conventional Treg producing both IL-10 and IL-17 and demonstrated that in vitro pre-treatment with rapamycin or rapamycin plus retinoic acid completely prevented IL-17 and IFN-γ production, while untreated or retinoic acid-treated Tregs produced both cytokines.

The IL-10 expression among Th17 cell population was also evaluated. Brunsing and Prossnitz [12] demonstrated that an agonist of the membrane-bound G-protein-coupled estrogen receptor GPER, namely G-1, is capable to elicit IL-10 expression in Th17-polarized cells, increasing the number of IL-10$^+$ and IL-10$^+$ IL-17A$^+$ cells via de novo induction of IL-10. G-1 appeared to act directly on CD4$^+$ T cells, providing the first example of a synthetic small molecule capable of eliciting IL-10 expression in Th17 or hybrid T cell populations [12]. In this case, this subset of lymphocytes could be considered as a conventional Treg subpopulation able to produce effector cytokines such as

IL-17 or a Th17 subset showing regulatory functions producing IL-10.

According to the results of our study, the addition of MTX and MP on PBMCs from patients with early RA was able of favoring the secretion of both cytokines in $CD4^+$ cells as well as the retinoic acid or promoting the differentiation of CD4 T cells in the regulatory subsets as well as G-1.

In this preliminary study, we demonstrated that, at least in vitro, MTX and MP are capable to regulate the Th17 effector functions and speculate that these molecules are capable to induce proliferation of $CD4^+$/IL-17^+/IL-10^+ lymphocytes. A recent paper published by Szalay et al. [13] provides a longitudinal follow-up of the prevalence of $CD4^+$ cell subsets during different RA treatment protocols demonstrating, in according with our results, that the pro-inflammatory immune phenotype Th17 is normalized under steroid and MTX treatment in early RA. The imbalance between Th17/Treg is a key mechanism in the pathogenesis and progression of the disease. In this regard, it appears to be necessary to better characterize the role of therapy on this subset in in vivo studies.

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Conflict of interest The authors disclaimed no financial conflict of interest.

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