Methotrexate treatment affects effector but not regulatory T cells in juvenile idiopathic arthritis

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Abstract

Objective. The balance between Treg and effector T cells (Teff) is crucial for immune regulation in JIA. How MTX, the cornerstone treatment in JIA, influences this balance in vivo is poorly elucidated. The aim of this study was to investigate quantitative and qualitative effects of MTX on Treg and Teff in JIA patients during MTX treatment.

Methods. Peripheral blood samples were obtained from JIA patients at the start of MTX and 3 and 6 months thereafter. Treg numbers and phenotypes were determined by flow cytometry and suppressive function in allogeneic suppression assays. Teff proliferation upon stimulation with anti-CD3, activation status and intracellular cytokine production were determined by flow cytometry. Effector cell responsiveness to suppression was investigated in autologous suppression assays. Effector cell cytokines in supernatants of proliferation and suppression assays and in plasma were measured by cytokine multiplex assay.

Results. MTX treatment in JIA did not affect Treg phenotype and function. Instead, MTX treatment enhanced, rather than diminished, CD4+ and CD8+ T cell proliferation of JIA patients after 6 months of therapy, independent of clinical response. Effector cells during MTX treatment were equally responsive to Treg-mediated suppression. MTX treatment did not attenuate Teff activation status and their capacity to produce IL-13, IL-17, TNF-α and IFN-γ. Similarly to Teff proliferation, plasma IFN-γ concentrations after 6 months were increased.

Conclusion. This study provides the novel insight that MTX treatment in JIA does not attenuate Teff function but, conversely, enhances T cell proliferation and IFN-γ plasma concentrations in JIA patients.

Key words: methotrexate, juvenile idiopathic arthritis, effector T cells, regulatory T cells, cytokines, suppression assays.

Rheumatology key messages

- MTX treatment in JIA does not target Treg number, phenotype and function.
- MTX treatment enhances T cell proliferation and IFN-γ plasma concentrations in JIA.
- Low-dose MTX treatment does not have immunosuppressive properties in JIA.

Introduction

In the past decade, intensive research has focused on FOXP3+ Treg in chronic autoimmune inflammation in rheumatic diseases [1, 2]. The question whether Treg number and function are altered in autoimmune inflammation is still a matter of debate [3]. In JIA, the most common childhood autoimmune disease, Treg are present in high numbers and are capable of suppressing CD4+ and CD8+ ...
T cells in vitro [4–6]. Nevertheless, the suppressive function of Treg can be hampered in vivo by the inflammatory environment in the joint and the resistance of CD4+ and CD8+ effector T cells (Teff) to suppression [2, 5, 6]. Therefore the balance between Treg and Teff is crucial for immune regulation in JIA.

The question arises of whether and how effective current treatments, such as MTX, influence this balance. MTX, the cornerstone DMARD in JIA, can induce disease remission in up to 70% of JIA patients [7–11]. Furthermore, 50% of patients remain in drug-free remission for >2 years upon MTX discontinuation [7]. In spite of its convincing efficacy, delineation of MTX’s effects on the balance between Treg and Teff is crucial for immune regulation in JIA.

In animal models, MTX’s effects have been attributed to MTX-induced anti-inflammatory adenosine, whose production is mediated by CD39 and CD73 ectoenzymes [12–16]. In humans, the in vitro binding of adenosine to receptors on Treg and adenosine production by CD39/CD73-expressing Treg leads to increased Treg numbers and suppressive function [17–20]. Furthermore, in vitro exposure to MTX has been shown to induce (sensitivity to) apoptosis of activated T cells [21–23]. This phenomenon is attributable to the inhibition of folate metabolism and de novo purine and pyrimidine synthesis [24], resulting in anti-proliferative effects, which is the most prominent feature of MTX. Although animal models and in vitro experiments offer clues about the effects of MTX on Treg and Teff, such systems are not representative of the clinical reality of JIA patients on MTX. In contrast to animal models, in which the effects of MTX are observed in a matter of days or weeks, and to cell culture systems, in which MTX effects are observed within hours or days, the full blown effects of MTX in patients can be reliably evaluated only after 3 or even 6 months of treatment [25]. Such delayed clinical effect is due in part to the time-dependent accumulation of long-chain MTX polyglutamates, MTX clinical efficacy mediators [26–28], whose accumulation does not occur during short exposure to MTX in vitro. Therefore ex vivo data from patients using MTX are required to clarify the effects of MTX on Treg and Teff.

Here we studied the quantitative and qualitative effects of MTX treatment on Treg and Teff of JIA patients at the start of MTX and while on MTX for 3 and 6 months. Our data indicate that MTX treatment does not alter Treg phenotype or suppressive function. Instead, MTX leads to enhanced T cell proliferation and higher IFN-γ levels in plasma, independent of clinical response. Taken together, low-dose MTX treatment does not target Treg; instead it enhances, rather than attenuates, the function of effector cells.

Patients and methods

Patients and study design

This prospective investigator-initiated clinical trial on MTX in JIA (ISRCTN13524271) was approved by the ethics committee of the University Medical Center Utrecht and was performed at the University Medical Center Utrecht between August 2007 and February 2013. Informed consent was obtained from all patients according to the Declaration of Helsinki.

Patients aged 2–18 years, with a confirmed JIA diagnosis [29] and starting MTX without concomitant biologic treatment were included (Table 1). Patients who stopped MTX for >6 months but restarted MTX due to a relapse were also included. At the start of MTX treatment and 3 and 6 months after MTX start, clinical data were collected and blood was sampled. In some patients with active disease, SF was acquired during therapeutic joint aspiration. Due to limited cell numbers, not all patients could be included in all experiments.

MTX clinical response was determined at 6 months after starting MTX, as this is a commonly used time point to establish MTX efficacy, using the ACR paediatric criteria [30]. MTX responders were defined as patients who satisfied at least ACR50 criteria (50% improvement in at least three of the six core criteria, with ≤30% worsening in more than one of the remaining criteria.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) at time points 0, 3 and 6 months or SF mononuclear cells were isolated using Ficoll-isopaque density gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and frozen in fetal calf serum (Invitrogen, Carlsbad, CA, USA) containing 10% dimethylsulphoxide (Sigma-Aldrich, St Louis, MO, USA) until further experimentation. Cells were cultured at 37°C and 5% CO2 in 10% human AB serum (Invitrogen) with RPMI 1640 medium (2 mM L-glutamine and 100 U/ml penicillin-streptomycin) and stimulated either with 1.5 μg/ml plate-bound anti-CD3 (clone OKT3; eBioscience, San Diego, CA, USA) or with anti-CD2/anti-CD3/anti-CD28 beads (Treg suppression inspector; Miltenyi Biotec, Bergisch Gladbach, Germany).

Suppression assays

Allogeneic

To study the suppressive capacity of Treg from time points 0, 3 and 6 months, an allogeneic assay was performed in which patient CD4+CD25+CD127low Treg were co-cultured with healthy donor CD4+CD25+ T cells, sorted by flow cytometry on an FACS Aria (BD Biosciences, San Jose, CA, USA) (supplementary Fig. S1, available at Rheumatology Online). Treg were co-cultured with 25 000 T cells at 1:8 and 1:4 ratios in 100 μl of culture volume and stimulated with anti-CD2/anti-CD3/anti-CD28 beads (Treg suppression inspector; Miltenyi Biotec). To control for cell density, CD4+CD25+ T cells instead of Treg were added at a 1:4 ratio.

Autologous

To study the responsiveness of effector cells at time points 0 and 6 to Treg-mediated suppression, autologous assays were performed. Total PBMCs were used as effector cells and co-cultured with sorted CD4+CD25+CD127low Treg from time points 0 and 6 and
TABLE 1 Patient characteristics

<table>
<thead>
<tr>
<th>Medication</th>
<th>MTX dose, median (IQR), mg/m²/week</th>
<th>Folic acid, n (%) 76 (100)</th>
<th>NSAIDs, n (%) 60 (78.9)</th>
<th>Local steroids, n (%) 12 (15.8)</th>
<th>Responder status (≥ACR50), n (%)</th>
</tr>
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<tr>
<td>3 months</td>
<td>47 (61.8)</td>
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<tr>
<td>6 months</td>
<td>51 (67.1)</td>
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aRF positive, n = 8 (22.9% of all polyarticular JIA patients).
Available in 74 (97.4%) patients. bAvailable in 72 (94.7%) patients. cAvailable in 71 (93.4%) patients. dAvailable in 72 (94.7%) patients. eTwo patients (3.6%) on parenteral MTX. One patient (polyarticular JIA) was on low-dose oral steroids (0.4 mg/kg/day) at the start of MTX. ACR50: 50% improvement in ACR criteria; CHAQ: Childhood Health Assessment Questionnaire; IQR: interquartile range.

vice versa (crossover assay). Effector cells (10 000 cells) were co-cultured with Treg at 1:8, 1:4 and 1:2 ratios and stimulated with plate-bound anti-CD3.

In both assays, at day 5, supernatants were collected to measure cytokine production. Subsequently, H was added during the last 16–19 h and its uptake was measured by liquid scintillation beta counting to quantify effector cell proliferation.

T cell proliferation and effector cell cytokine production

To measure T cell proliferation, PBMCs were labelled with 2 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) for 10 min at 37 °C and washed. CFSE-labelled PBMCs (60 000 cells) were plated into anti-CD3-coated wells. In some experiments, increasing concentrations of MTX (Emthexate, 2.5 mg/ml) were added in vitro in anti-CD3-coated plates (1.5 μg/ml) to either 200 000 PBMCs at time point 0 or to 200 000 SF mononuclear cells from the start of culture. At day 5, the proliferation of effector cells was analysed with flow cytometry by gating CFSE<sup>+</sup> cells. The proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was measured by gating CD3<sup>+</sup> cells, followed by gating CD4<sup>+</sup> and CD8<sup>+</sup> cells. Simultaneously, PBMCs (60 000 cells) not labelled with CFSE were plated into anti-CD3-coated wells in order to collect supernatants at day 4 to measure cytokine production. Furthermore, plasma was obtained by centrifugation of peripheral blood (PB) at 150 g for 10 min and then stored at −80 °C. Cytokine concentrations were measured with the Bio-Plex system combined with Bio-Plex Manager version 4.0 software (Bio-Rad Laboratories, Hercules, CA, USA), employing the Luminex technology, as previously described [31].

Flow cytometry

To determine the phenotype of Treg and T cells and to detect intracellular cytokine production, cells were stained ex vivo and measured with flow cytometry. To detect intracellular cytokine production, cells were stimulated with phorbol-12-myristate-13-acetate (PMA; 20 ng/ml; MP Biomedicals, Santa Ana, CA, USA) and ionomycin (1 μg/ml; Calbiochem, San Diego, CA, USA) for 5 h (+4.5 h of Golgistop (1/1500; BD Biosciences)). The staining protocol is described elsewhere [6]. Cells were acquired on FACScanto II and analysed using FACS Diva version 6.13 software (BD Biosciences). Flow cytometry antibodies are described in the supplementary data (section on antibodies used for flow cytometry, available at Rheumatology Online).

Statistical analysis

To analyse patient samples, t-test or Mann–Whitney U-test was used, as appropriate. To analyse paired patient samples, paired t-test or Wilcoxon matched pairs test was used, as appropriate. Statistical analysis was performed using GraphPad Prism version 5.03 (GraphPad Software, La Jolla, CA, USA) and SPSS version 20.0.0 (SPSS, Chicago, IL, USA).

Results

Treg numbers are not increased during MTX treatment

Previous studies showed that Treg frequency in RA increased during treatment with anti-TNF-α drugs [32, 33]. We investigated whether treatment with MTX also leads to increased Treg numbers. The frequency of CD4<sup>+</sup>FOXP3<sup>+</sup> Treg in the PB of JIA patients at 3 [mean 4.1% (S.E.M. 0.5)] and at 6 months [mean 3.5% (S.E.M. 0.3)] did not increase compared with their frequency at the start of MTX [mean 4.1% (S.E.M. 0.3%) (Fig. 1A)]. Instead, Treg frequency at 6 months was lower than at the start of MTX (P < 0.05), which was the case in MTX responders and non-responders (not statistically significant) (Fig. 1B). Taken together, Treg numbers do not increase during MTX treatment in JIA and do not correlate with clinical efficacy of MTX.
MTX treatment does not alter Treg phenotype and function

Since MTX treatment did not increase Treg numbers, we investigated Treg phenotype and suppressive capacity. We examined the expression of CTLA-4 and GITR as well as of ectonucleases CD39 and CD73, which have important roles in the Treg suppressive function [17–20, 34–37]. These markers were not altered during MTX treatment (Fig. 1C) in either responders or non-responders (data not shown).

To investigate Treg suppressive capacity during MTX treatment, allogeneic suppression assays were performed.

(A) Percentage of CD4<sup>+</sup>FOXP3<sup>+</sup> cells in PBMCs at the start of MTX (white bars), at 3 months (grey bars) and at 6 months (black bars) (n = 42). (B) Percentage of CD4<sup>+</sup>FOXP3<sup>+</sup> cells at the start of MTX in MTX responders (R) (n = 16) and non-responders (NR) at 6 months (n = 14). (C) Percentage of GITR<sup>+</sup>, CTLA-4<sup>+</sup>, CD39<sup>+</sup> and CD73<sup>+</sup> cells (within FOXP3<sup>+</sup>) at time points 0, 3 and 6 months. (D) Allogeneic suppression assay: healthy donor Teff proliferation in the presence of JIA Treg at a Treg:Teff ratio of 1:8 or 1:4. Percentage of proliferation in the presence of Treg relative to Teff proliferation (set at 100%). Bars and ranges: mean ± s.e.m., n = 3. *P < 0.05 compared with time point 0. PBMCs: peripheral blood mononuclear cells; Teff: effector T cells.
in which sorted CD4⁺CD25⁺CD127low Treg from JIA patients were cultured with sorted CD4⁺CD25⁺ Teff from a healthy donor. As depicted in Fig. 1D, the suppressive capacity of Treg was not altered upon MTX treatment. Moreover, Treg from all three time points were equally capable of suppressing the Teff production of IL-13, IFN-γ and TNF-α (supplementary Fig. S2, available at Rheumatology Online). Taken together, MTX treatment does not affect Treg phenotype and function.

MTX treatment leads to increased T cell proliferation in JIA patients after 6 months of therapy

Since MTX has anti-proliferative properties [24], we hypothesized that MTX has inhibitory effects on T cell proliferation in JIA. First, we asked whether in vitro exposure to MTX inhibited T cell proliferation at the start of MTX. We demonstrated that 1 and 10 nM concentrations, corresponding to low-dose MTX treatment in patients [38, 39], did not inhibit the proliferation of either PB CD4⁺ and CD8⁺ T cells (Fig. 2A, left panels) or of the highly activated SF T cells [6] (Fig. 2A, right panels). However, higher MTX concentrations (50 and 100 nM), corresponding to high-dose MTX used for malignancies, did inhibit the proliferation of both PB and SF T cells (Fig. 2A).

To determine whether MTX treatment exerted anti-proliferative effects on T cells ex vivo, proliferation of CD4⁺ and CD8⁺ T cells was determined at time points 0, 3 and 6 months. CD4⁺ and CD8⁺ T cell proliferation at 3 months was comparable to that at the start of MTX, whereas CD4⁺ and CD8⁺ T cell proliferation at 6 months [mean 75.3% (s.e.m. 4.1) and 76.6% (s.e.m. 4.1), respectively] was significantly higher than at the start of MTX [58.5% (s.e.m. 5.0) and 58.1% (s.e.m. 5.1), respectively] (P < 0.05) (Fig. 2B and C, right and left panel). T cell proliferation in JIA patients was similar to that of healthy controls [64.0% (s.e.m. 13.8) for CD4⁺ and 59.6% (s.e.m. 14.9) for CD8⁺ T cells, n = 4].

The observed increase in proliferation was independent of response to MTX (Fig. 2D). At the start of MTX, however, future responders showed higher CD4⁺ (mean 67.7%) and CD8⁺ (67.7%) T cell proliferation compared with future non-responders (CD4⁺ and CD8⁺ 47.7%; P < 0.05). All together, these data demonstrate that low-dose MTX treatment does not lead to inhibition, but rather to enhancement, of T cell proliferation in JIA patients.

MTX treatment does not diminish Teff activation status or cytokine production in JIA patients

We hypothesized that T cells during MTX treatment showed reduced activation status and lower cytokine production. However, ex vivo measured T cell proliferation marker Ki-67 and activation markers CD25, CD69 and HLA-DR did not decrease after 6 months (supplementary Fig. S3, available at Rheumatology Online). Furthermore, ex vivo measured CD4⁺ production of IL-10, IL-17, IFN-γ and TNF-α and CD8⁺ production of IFN-γ and TNF-α were not lower at 3 or 6 months (supplementary Fig. S4, available at Rheumatology Online). In addition, upon anti-CD3 stimulation, effector cells did not produce less IL-10, IL-13, IL-17, IFN-γ and TNF-α in culture supernatants at time points 3 and 6 months (Fig. 3). There were no differences between MTX responders and non-responders (data not shown). Moreover, exposure to 1 and 10 nM of MTX did not inhibit production of IL-13, IL-17, IFN-γ and TNF-α by effector cells from PB and SF (supplementary Fig. S5, available at Rheumatology Online). Taken together, MTX treatment does not attenuate activation status and cytokine production of Teff.

Effectors at 6 months are equally responsive to Treg-mediated suppression as effector cells at the start of MTX

Since T cell activation and cytokine production were not affected by MTX treatment, we investigated whether the responsiveness of effector cells to Treg-mediated suppression differed between time points 0 and 6 months. As T cells at time point 6 months showed heightened responsiveness to anti-CD3 upon MTX treatment, we hypothesized that these effector cells would also be more responsive to Treg-mediated suppression than effector cells at the start of MTX. Therefore we performed crossover autologous suppression assays, in which PBMCs (effector cells) from time point 6 months were co-cultured with sorted CD4⁺ CD25⁺CD127low Treg from time point 0, and vice versa (Fig. 4A, white and black striped bars). In addition, effector cells were also co-cultured with Treg from the corresponding time points (Fig. 4A, white and black bars). Contrary to our hypothesis, time point 6 effector cells were equally responsive to suppression of proliferation by Treg from both time points, compared with time point 0 effector cells.

In RA, it has been shown that despite proficient suppression of proliferation, Treg-mediated suppression of T cell cytokine production was compromised [32]. We therefore asked whether time point 6 effector cells were more responsive to Treg-mediated suppression of cytokine production compared with effector cells at the start of MTX. Time point 6 effector cells were equally responsive to suppression of cytokine production (IL-13, TNF-α and IFN-γ) as effector cells from time point 0, as shown both in own and crossover experiments (Fig. 4B). IL-17 was resistant to Treg-mediated suppression at both time points (Fig. 4B), which was observed before [33, 40]. Taken together, in spite of their enhanced proliferation, effector cells 6 months after the start of MTX were equally responsive to Treg-mediated suppression of proliferation and cytokine production compared with effector cells at the start of MTX.

Enhanced IFN-γ concentrations in plasma of JIA patients after 6 months of therapy

Cytokine levels in plasma during MTX treatment were quantified. While TNF-α concentrations were not affected by MTX treatment, concentrations of another pro-inflammatory cytokine, IL-6, decreased at 3 and 6 months (Fig. 5C), independently of clinical response. Conversely, IFN-γ, a T cell-derived cytokine associated with T cell proliferation, increased at 6 months but not at 3 months after the start of MTX (Fig. 5A). Similarly to T cell proliferation,
MTX treatment leads to increased T cell proliferation in JIA patients after 6 months of therapy

(A–D) CFSE-labelled PBMCs or SFMCs at time points 0, 3 and 6 months, cultured in the presence of anti-CD3. At day 5, PBMCs were stained for CD4 and CD8 and proliferation was measured. Bars and ranges represent mean ± S.E.M. (A) Proliferation of CD4+ (left upper panel) and CD8+ (right upper panel) from PB (n = 9) and SF (lower panels, n = 3) at the start of MTX during *in vitro* exposure to MTX. (B and C) Proliferation of CD4+ and CD8+ T cells at time points 0 (white bars/circles) and 3 and 6 months (black bars/circles) (n = 13). (D) Proliferation of CD4+ and CD8+ T cells of responders (R) (n = 7) and non-responders (NR) (n = 5) at 6 months. *P < 0.05 compared with time point 0. CFSE: carb-oxyfluorescein succinimidyl ester; PBMCs: peripheral blood mononuclear cells; SFMCs: synovial fluid mononuclear cells.
IFN-γ was increased in both responders and non-responders, although the increase in non-responders was not statistically significant (Fig. 5B). Therefore MTX treatment also enhances effector cell function with respect to IFN-γ levels in plasma.

**Discussion**

About three decades ago, MTX revolutionized the treatment of rheumatic diseases [25]. In JIA treatment, MTX became an anchor drug because of its safety and efficacy [11]. Although the clinical effect of MTX on inflammation has been firmly established in numerous clinical trials in JIA [7–10], investigation of MTX’s effects on Treg and Teff which control and drive the inflammation, has been lacking. Here we studied these compartments in JIA patients before and after the start of MTX therapy. We showed that Treg phenotype and function were not affected by MTX treatment. Conversely, CD4+ and CD8+ T cell proliferation was enhanced, independent of clinical response to MTX, and plasma IFN-γ levels were increased after 6 months of MTX treatment. Taken together, MTX treatment does not attenuate, but rather enhances, effector cell function.

Previously, no difference in Treg numbers was shown in MTX-treated RA patients [32]. Treg numbers in JIA and RA [33, 41] were also not altered upon treatment with an anti-TNF-α agent, etanercept, although an increase in Treg was demonstrated in RA after treatment with other anti-TNF-α agents (infliximab and adalimumab) [32, 33]. These biologics also restored the compromised Treg-mediated suppression of TNF-α, IFN-γ [32] and IL-17 in RA [33], whereas we found that Treg-mediated suppression of proliferation and cytokine production in JIA was not increased by MTX treatment. Nevertheless, Treg failed to suppress IL-17. In fact, co-cultures of Treg and effector cells produced more IL-17 than effector cells alone, suggesting that Treg may produce IL-17, which has been observed recently [42, 43]. Recently a shift (increase) in the Treg:Th17 ratio was reported as a possible mechanism by which immunomodulatory treatment, such as MTX, could exert its effects [44]. In our cohort, we did not observe significant changes in this ratio after MTX treatment compared with time point 0, although interpretation of these data was hampered as we were able to determine Treg:Th17 ratios only in a limited number of patients (data not shown).
Because of its known folate, purine and pyrimidine antagonism, we expected MTX to exhibit anti-proliferative effects on T cells. However, neither in vitro exposure to low nanomolar concentrations of MTX, corresponding to low-dose MTX treatment [14, 38, 39], nor the low-dose MTX treatment itself inhibited proliferation of T cells ex vivo and upon T cell receptor stimulation. Instead, we observed enhanced proliferation of CD4+ and CD8+ T cells at 6 months compared with T cell proliferation at the start of MTX in both responders and non-responders.

**Fig. 4** Effector cells at 6 months are equally responsive to suppression as effector cells at the start of MTX.

(A and B) Autologous suppression assays: Treg from time points 0 and 6 months were co-cultured with PBMCs (Eff) (white and black bars) at 1:8, 1:4 and 1:2 ratios. In crossover experiments, Effs from time point 0 were co-cultured with Treg from time point 6 (white striped bars) and vice versa (black striped bars). At day 5, culture supernatants were harvested to measure cytokine production. (A) Proliferation in the presence of Treg relative to proliferation of Effs alone (set at 100%). Bars: mean ± S.E.M., n = 4. (B) IL-13, IFN-γ, TNF-α and IL-17 levels in the absence (Eff) or presence of Treg at 1:8, 1:4 and 1:2 ratio. Mean cytokine levels in picograms per millilitre ± S.E.M., n = 4. PBMCs: peripheral blood mononuclear cells.
This suggests that T cell proliferation could be directly affected by MTX, rather than by clinical improvement. The mechanism by which MTX enhances T cell proliferation remains elusive; nonetheless, this concept is interesting in the light of earlier findings that SF T cells in RA patients with active disease were hyporesponsive to antigen or mitogen stimulation compared with healthy controls [45, 46]. In the present study, T cell proliferation during active disease was similar to that of healthy controls. This suggests that T cells in JIA, in contrast to RA, were not hyporesponsive, although their responsiveness could still be enhanced by MTX treatment.

IFN-γ levels in plasma can vary considerably, as reported earlier in a cross-sectional study of JIA patients and healthy controls [31]. Nevertheless, we observed significantly increased plasma levels of IFN-γ after 6 months of MTX treatment, whereas in short stimulation assays the numbers of IFN-γ-expressing CD4+ and CD8+ T cells were similar between the time points. A possible explanation could be that plasma IFN-γ is also derived from other immune cells, such as NK cells and NK T cells. However, IFN-γ is a known T cell cytokine, and its increased plasma concentration could reflect increased T cell proliferation in vivo during MTX treatment.

Increased plasma levels of IFN-γ, in concert with increased T cell proliferation, suggest that MTX enhances Teff function in JIA patients during MTX treatment. The fact that MTX therapy in JIA patients is associated with a slightly increased risk of infectious adverse events [47] is not contradictory to our observations, since augmented overall T cell responsiveness cannot be directly translated into improved antigen-specific immune responses to infectious agents in JIA patients. The question remains whether Teff are targeted directly by MTX or perhaps through other immune cell compartments. Since total mononuclear cells were used in our assays to mimic the in vivo situation as closely as possible, MTX may have mediated the observed effects on Teff through antigen-presenting cells.

In conclusion, the present study provides evidence that MTX treatment in JIA does not target Treg but does target Teff. Our results provide the novel insight that low-dose
MTX treatment does not attenuate Teff function but, conversely, enhances T cell proliferation and plasma concentrations of IFN-γ in JIA patients. These immunological data are contrary to the common belief that low-dose MTX treatment in rheumatic diseases has immunosuppressive properties.

Acknowledgements

We wish to acknowledge A. Blaauw and M. J. W. van Opdorp for valuable assistance during patient inclusion, case report form completion and investigator site file maintenance; J. Meerdig for experimental assistance; and all members of the Center for Molecular and Celluar Intervention for blood sample processing. M.B.C. was supported with the Mosaic grant and F.v.W. with the Veni grant by the Dutch Organization for Scientific Research (NWO). B.J.P. was supported by the Dutch Rheumatology Foundation.

Funding: No specific funding was received from any funding bodies in the public, commercial or not-for-profit sectors to carry out the work described in this article.

Disclosure statement: The authors have declared no conflicts of interest.

Supplementary data

Supplementary data are available at Rheumatology Online.

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