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Immunosuppressive Activity of Abatacept on Circulating T Helper Lymphocytes from Juvenile Idiopathic Arthritis Patients

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CD4+ T cells · Biological therapy · Cytokines

Abstract
Background: Abatacept is used in the treatment of juvenile idiopathic arthritis (JIA) patients, but the activity of the drug on T helper cell function is not yet fully known. ***Methods:*** The ability of abatacept to affect cytokine production in vitro and the proliferative response to both recall antigens and polyclonal stimulation was firstly assessed in healthy donors. Then, 10 JIA patients who were due to start abatacept treatment were recruited and longitudinally evaluated during the first 90 days of therapy. Both their clinical response to the treatment and in vitro analysis aimed to assess the proliferative response to recall antigens and the proportions of circulating T helper subsets. ***Results:*** Abatacept reduced the proliferative response to recall antigens and the production of proinflammatory cytokines such as IFN-γ and TNF-α in healthy donors in vitro. It was also efficient in improving symptoms and reducing parameters of inflammation in JIA patients. Abatacept reduced the proliferative response to recall antigens, and this effect was significant soon after drug infusion (2 days). Regarding the proportions of circulating CD4+ T lymphocytes, only a reduction in the frequencies of circulating Treg cells was observed. ***Conclusions:*** Abatacept in vitro inhibits proliferation and cytokine production in healthy donors, and reduces parameters of inflammation in vivo in JIA patients. The reduction of the proliferative response to recall antigens induced by abatacept was evident only soon after drug administration, suggesting that its immunosuppressive activity is maintained only for a short time.

Introduction

Juvenile idiopathic arthritis (JIA) is the most common form of persistent arthritis in children. The cause of the disease is unknown [1], but in its pathogenesis the adaptive immune response is certainly involved, as indicated by the presence of T and B lymphocytes infiltrating the synovial membrane of inflamed joints [2]. CD4+CD161+ T helper (Th) cells are enriched in the inflamed joints with respect to peripheral blood (PB), and they belong to either the Th1, Th17, or Th17/Th1 subset [3]. In particu-

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lar, the proportions of Th17/Th1 lymphocytes in the synovial fluid of inflamed joints positively correlated with parameters of disease activity, such as the erythrocyte sedimentation rate (ESR) and levels of C-reactive protein (CRP), in oligoarticular JIA [4].

Pharmacologic therapy in JIA is essentially based on the use of nonsteroidal anti-inflammatory drugs and disease-modifying antirheumatic drugs (DMARDs) [1]. Among both biologic and nonbiologic DMARDs, methotrexate, thanks to clinical benefits with an acceptable profile of toxic effects, is considered the standard first-line treatment [5]. Some patients, in particular those with the polyarticular subset of JIA, could have a suboptimal response to nonbiologic DMARDs, becoming candidates to receive biologic agents. The use of biologics has resulted in an improvement in the treatment of JIA, permitting disease control in patients refractory to the pharmacological agents previously available. Among biologics, etanercept, infliximab, and adalimumab act by blocking TNF-α (tumor necrosis factor-α), thereby preventing its proinflammatory actions. Other biologics used to treat JIA include interleukin-1 blockers (anakinra, canakinumab, and rilonacept), the interleukin-6 blocker tocilizumab, CD20/B cell-targeted rituximab and the T cell costimulatory signal blocker abatacept. Abatacept is a chimeric CTLA4 and IgG Fc fusion protein that inhibits the CD80/CD86-CD28 costimulatory signal required for full T cell activation [6]. Through its CTLA4 portion, abatacept binds to CD80 and CD86 on APCs (antigen-presenting cells), thereby inhibiting CD28 costimulation. CD28-mediated signals are relevant in the upregulation of CD154 (the ligand for CD40) on the T cell surface, a key process in the acquisition of the T cell 'helper' function. Consequently, in patients with rheumatoid arthritis (RA), abatacept has been shown to be able to reduce signs of polyclonal B cell activation, inducing a trend toward the normalization of serum levels of different classes of Ig, decreasing titers of anti-citrullinated protein antibodies and rheumatoid factor, and percentages of post-switch memory B cells [7]. The safety, tolerability, and clinical efficacy of abatacept in children and adolescents with JIA have been demonstrated in a double-blind, placebo-controlled, randomized withdrawal trial [8]. Recently, by comparing the efficacy of different biologics in the polyarticular course of JIA, it has been reported that the short-term efficacy of etanercept, adalimumab, and abatacept is similar [9]. In this study we evaluated the ability of abatacept to influence Th functions in JIA patients, both in vitro and ex vivo.

Table 1. Patients' demographic and clinical characteristics

Patient No.	Sex	Age, years	Diagnosis	Therapy (in addition to abatacept)
1	F	13	Polyarticular JIA	Steroids
2	F	14	Oligoarticular JIA	Methotrexate
3	F	12	Polyarticular JIA	Steroids
4	M	11	Oligoarticular JIA	Methotrexate
5	F	13	Oligoarticular JIA	Steroids, methotrexate
6	F	13	Polyarticular JIA	Steroids, methotrexate
7	F	14	Polyarticular JIA	Steroids
8	F	11	Oligoarticular JIA	Steroids
9	F	9	Oligoarticular JIA	Steroids, methotrexate
10	F	14	Polyarticular JIA	Methotrexate

Materials and Methods

Subjects

PB samples for the in vitro study were obtained from 7 healthy donors and from 10 JIA patients whose diagnosis was made in accordance with the International League of Associations for Rheumatology classification criteria for JIA [10]. The patients' demographic and clinical characteristics are presented in Table 1. The reason for introducing abatacept was mostly persistent arthritis, but the presence of refractory uveitis (especially in oligoarticular disease) was also a factor. Corticosteroids, when administered, were always given at low doses (prednisone <10 mg/day), which were maintained stable during the study period. Abatacept was administered intravenously with the usual protocol of 10 mg/kg at time zero (T0; half dose), 15 and 30 days, and monthly thereafter. At each administration a full general and articular examination was performed. The ESR (normal values up to 30 mm/h) and the CRP serum levels (normal values up to 0.3 mg/dL; assessed using the particle-enhanced turbidimetric immunoassay method) were evaluated by staff in the service laboratory of the Anna Meyer Pediatric Hospital in Florence, Italy.

The procedures followed in the study were in accordance with the ethics standards of the Regional Committee on Human Experimentation. Informed consent was obtained from parents or guardians.

PB samples from JIA patients were obtained before the beginning of abatacept treatment (T0) and at time points 2, 15, 17, 30, 32, 60, 62, 90, and 92 days after the beginning of abatacept treatment, representing each time of abatacept administration (before drug infusion), and 2 days later.

Reagents

The medium used for cultures was RPMI 1640 (Merck Millipore), supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 2×10^{-5} M 2-mercaptoethanol (all from Sigma), and 5% autologous serum for JIA patients or 5% AB serum (Lonza Ltd.) for healthy donors. Fluorochrome-conjugated monoclonal antibodies (mAbs) were from BD Biosciences (anti-CD3, CD4, CD8, CD161, IFN-γ, TNF-α, IL-4, IL-10) or from eBiosciences (anti-IL-17). Lymphoprep (Fresenius Kabi Norge)

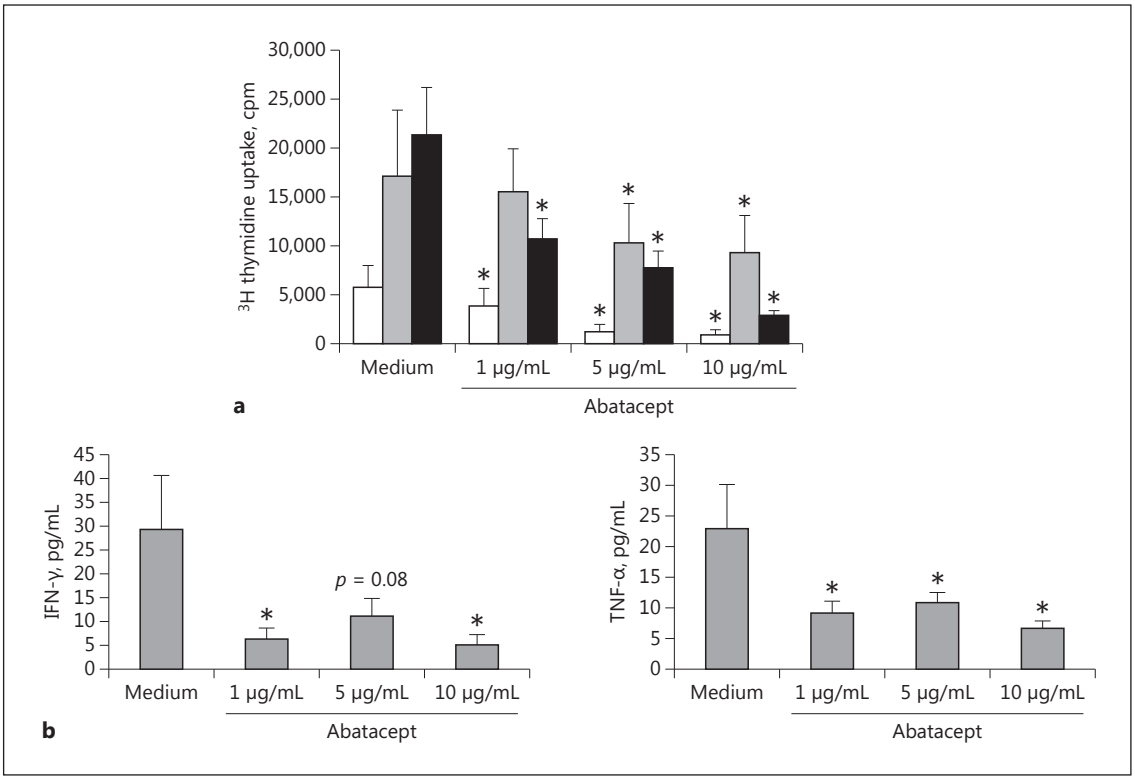

Fig. 1. Abatacept in vitro inhibits T cell proliferation and TNF-α production in PBMCs from healthy donors. **a** Columns represent mean values (±SE) of proliferative response to TT (white bars), SK (gray bars), and polyclonal stimulation with anti-CD3 plus IL-2 (black bars), in the absence (medium) or presence of different abatacept doses in PBMCs obtained from 7 healthy donors. **b** Columns represent mean values (±SE) of cytokine levels in culture supernatants of the anti-CD3 plus IL-2 cultures in the same donors, in the absence or presence of abatacept. * $p < 0.05$, presence of abatacept versus medium of each culture condition.

was used to obtain MNCs (mononuclear cells) by density gradient stratification from the PB of healthy donors and JIA patients.

Abatacept for the in vitro part of the study was provided by Bristol-Myers Squibb. Drug powder was dissolved in deionized water at a concentration of 250 mg/mL and then dialyzed in an appropriate tube (dialysis/Visking tubing, diameter 6.3 mm; Medicell International Ltd) versus deionized water. The final formulation was aliquoted and stored at –20°C.

Proliferation Assay

10^5 MNCs from the PB of healthy donors and JIA patients were stimulated in triplicate on 96 U-bottomed plates (Nunc, Sigma Aldrich) with anti-CD3 plus anti-CD28 mAbs (BD Biosciences, 5 μg/mL each) and IL-2 (25 U/mL; Proleukin, Novartis), or anti-CD3 and IL-2 (25 U/mL), or tetanus toxoid (TT; 10 μg/mL), or streptokinase (SK; 5,000 U/mL), in the presence or absence of abatacept (ranging from 1 to 10 μg/mL). On day 5, T cell culture supernatants were recovered in order to evaluate IL-2, IL-4, IL-10, IL-17, TNF-α, and IFN-γ levels by using BD Biosciences Cytometric Bead Array (CBA), whereas cells were pulsed for 16 h with 0.5 μCi of ^{3}H-TdR (Perkin Elmer) and harvested. Then, radionuclide uptake was measured by a β-counter in the presence of scintillation buffer.

Intracellular Cytokines Detection

PBMCs from patients with JIA were analyzed by flow cytometry, using a BD Biosciences LSR II instrument and Diva software, to assess the production of intracellular cytokines and expression of surface molecules, as previously described [4]. Briefly, freshly isolated PBMCs were stimulated with phorbol myristate acetate (PMA) plus ionomycin (Sigma) for 6 h, with the last 4 h in the presence of Brefeldin A (Sigma) as a Golgi inhibitor. The cells were then fixed in 2% formaldehyde (Sigma), permeabilized with 0.5% saponin (Sigma) and stained with the following fluorochrome-conjugated mAbs: anti-CD3, CD4, CD8, CD161, IFN-γ, TNF-α, IL-4, IL-10, and IL-17. The gating strategy was as follows. PBMCs were first analyzed for their distribution in a FSC/SSC dot plot and the lymphocyte region was selected, excluding monocytes. The lymphocytes were then analyzed for CD3 expression (in a dot plot FSC/CD3) and CD3+ cells were gated in a CD4/CD8 dot plot to select CD3+CD4+ T cells. Then, this latter population was analyzed for intracellular markers in association with CD161.

Treg Cells Evaluation

To determinate the frequency of Treg cells, a human Foxp3 staining set was used (eBioscience) following the manufacturer's

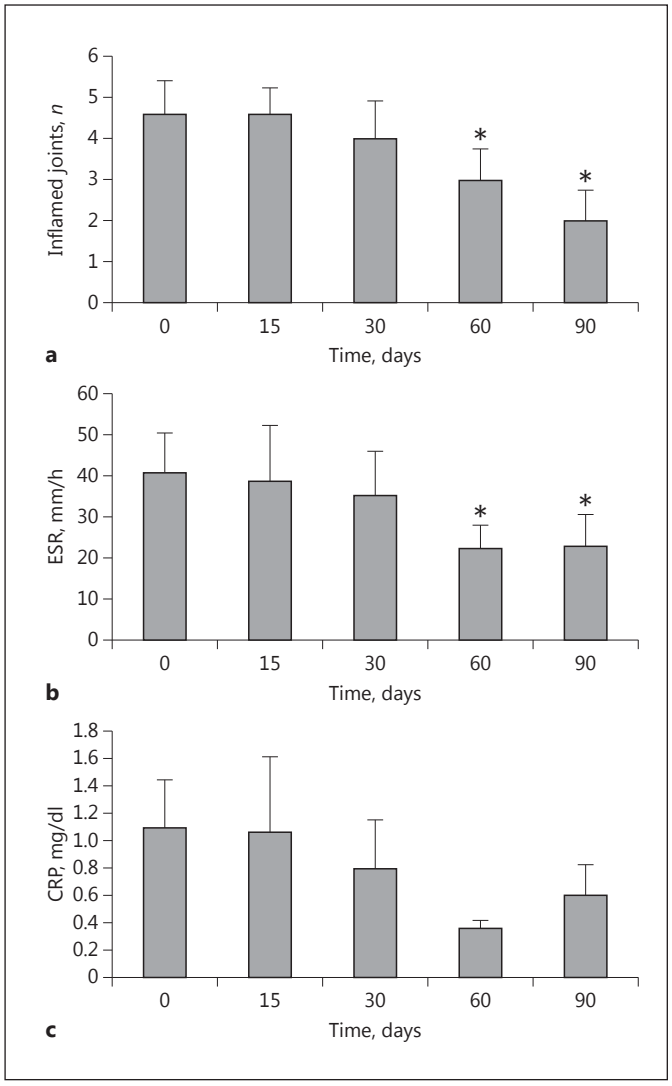

Fig. 2. Treatment with abatacept reduces the number of inflamed joints and serological parameters of inflammation. Columns represent the mean values (±SE) of the number of inflamed joints (**a**), of the ESR (**b**), and of the CRP serum levels (**c**) in 10 JIA patients at different time points after commencing abatacept treatment. * $p < 0.05$, each time point versus T0.

instructions. Briefly, PBMCs from JIA patients were fixed with fixation/permeabilization buffer diluted 1:4, then they were permeabilized in the presence of 1× permeabilization buffer and stained with the following fluorochrome-conjugated mAbs: anti-CD3, CD4, CD25, and anti-Foxp3 (PCH101). Finally, the cells were analyzed by flow cytometry LSR II and the gating strategy was as follows. PBMCs were first analyzed for their distribution in a FSC/SSC dot plot and the lymphocyte region was selected, excluding monocytes. Then lymphocytes were analyzed for CD3 and CD4 expression (in a dot plot CD3/CD4) and CD3+CD4+ cells were selected and evaluated in a CD25/Fopx3 dot plot.

Statistical Analysis

A standard 2-tailed paired *t* test was used for statistical analysis. *p* values ≤0.05 were considered significant.

Results

Abatacept Inhibits Recall Antigen-Induced T Cell Proliferation and the Production of Proinflammatory Cytokines in vitro

PBMCs from 7 healthy donors were evaluated in vitro for their proliferative response to both polyclonal and recall antigen stimulation in the absence or presence of abatacept. The inhibition of the proliferative response in the TT-induced proliferation was significant, starting from the lowest dose of abatacept (1 μg/mL), whereas in the SK-induced proliferation it became evident at a concentration of 5 μg/mL. The proliferative response to CD3 stimulation was clearly inhibited from the lowest dose (Fig. 1a). Of note, CD3/CD28-stimulated samples were only slightly inhibited at the highest dose (10 μg/mL) of abatacept (data not shown).

Cytokines were detected in the supernatants of proliferation wells. The levels of IL-2, IL-4, IL-17, and IL-10 were low or undetectable in all of the samples (data not shown), whereas IFN-γ and TNF-α, even if undetectable in the TT cultures, were appreciable in both SK (data not shown) and CD3-stimulated cultures. In these latter, we found a significant reduction of these cytokines in the presence of abatacept (Fig. 1b).

Abatacept in JIA Patients Reduces Parameters of Disease Activity

Once the capacity of abatacept to suppress the in vitro proliferative response and the production of cytokines in PBMCs from healthy donors was established, we evaluated the effect of the drug in a group of 10 JIA patients, both before and during abatacept treatment. These patients were unresponsive to conventional treatment and their main features are reported in Table 1.

The clinical response was evaluated in these patients by looking at the number of involved joints after 15, 30, 60, and 90 days of abatacept treatment. Moreover, serum parameters of inflammation, such as ESR and CRP, were also evaluated. Both the number of inflamed joints (Fig. 2a) and ESR (Fig. 2b) were significantly reduced after 60 days of abatacept treatment, with this effect being maintained at least until day 90, whereas only a trend to reduction without significance was observed in the CRP levels (Fig. 2c). Flares of uveitis tended to decrease, but

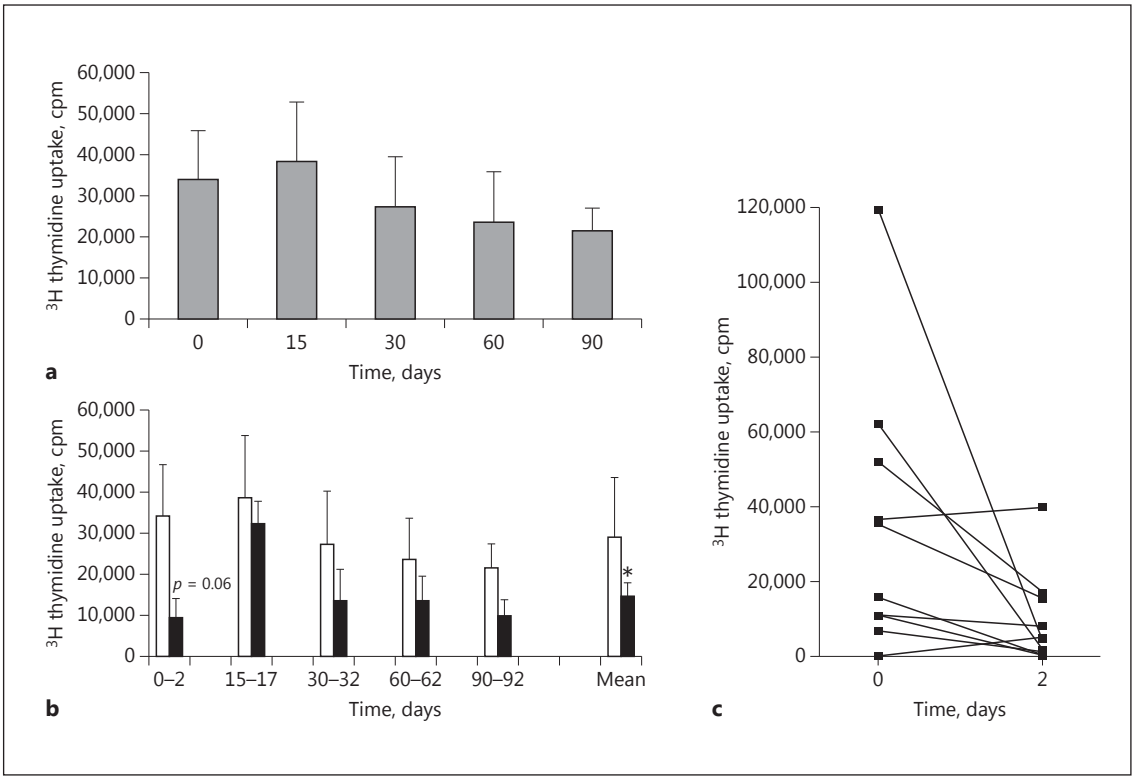

Fig. 3. Treatment with abatacept reduces the in vitro proliferative response to TT soon after drug administration. **a** Columns represent mean values (±SE) of proliferative response to TT at different time points after commencing abatacept treatment. **b** Columns represent mean values (±SE) of proliferative response to TT at different times after commencing abatacept treatment in PBMCs obtained from the 10 JIA patients. White bars indicate the value obtained just before drug infusion and black bars the value obtained 2 days later. * $p < 0.05$. **c** Proliferative response to TT of PBMCs in each patient at T0 and after 2 days of abatacept treatment.

the relative short follow-up and small numbers precluded statistical comparisons (data not shown).

Abatacept Inhibits TT-Induced Proliferation Soon after Its Administration

Once the efficacy of abatacept to improve symptoms and serum parameters of inflammation in JIA was established, we proceeded to assess the ability of the drug to inhibit the in vitro proliferative response to TT. We compared the in vitro proliferative response to TT stimulation before the beginning of the treatment (T0) with the responses after 15, 30, 60, and 90 days of treatment, finding only a nonsignificant trend of reduction (Fig. 3a). We then wondered if this lack of appreciable effects was due to the fact that we checked proliferative responses at a long time interval after drug administration. To answer this question, we compared the proliferative responses to TT stimulation on days 15, 30, 60, and 90 (15 or 30 days after abatacept administration) with those on days 2, 17, 32, 62, and 92 (2 days after abatacept administration). As shown in Figure 3b and c, TT-specific proliferation was constantly reduced soon after abatacept administration. Indeed, the mean of the proliferative response obtained 2 days after drug administration (i.e. time 2, 17, 32, 62, and 92) was significantly lower than that obtained at a longer interval from drug administration (i.e. time 0, 15, 30, 60, and 90 days; Fig. 3b).

Abatacept Activity on the Proportions of Circulating CD4+ T Helper Subsets

Since we recently showed the ability of etanercept to reduce the proportions of CD4+CD161+ (nonclassic) Th1 lymphocytes in the PB of JIA patients [11], we wondered if abatacept had a similar behavior. However, we did not find any variation in the frequency of circulating Th1 (classic and nonclassic), Th17, and Th17/Th1 subsets (Fig. 4a), or of Th2 lymphocytes (data not shown). Similarly, we assessed the ability of PBMC-derived CD4+

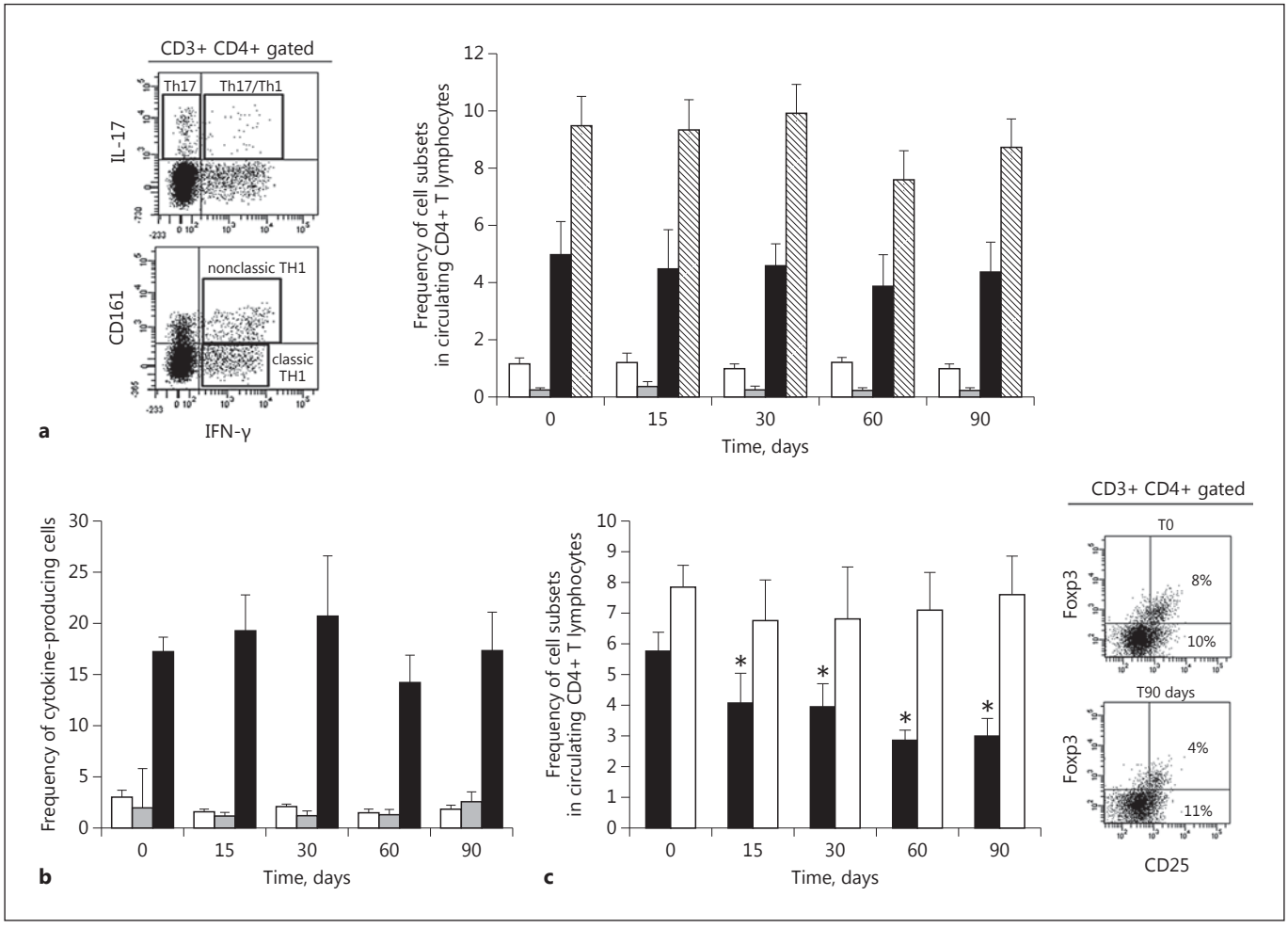

Fig. 4. Treatment with abatacept reduces the frequencies of circulating CD4+CD25+Foxp3+ in JIA patients. **a** One example of the gating strategy for distinguishing such Th subsets is depicted. Columns represent mean values (±SE) of the frequencies of Th17 (white bars), Th17/Th1 (grey bars), nonclassic Th1 (black bars), and classic Th1 (dashed bars), subsets, based on their IL-17, IFN-γ, and CD161 expression after in vitro polyclonal stimulation with PMA plus ionomycin, in PB CD4+ T cells of 10 JIA patients, at different time points after commencing abatacept treatment. **b** Columns represent mean values (±SE) of the frequencies of IL-4- (white bars), IL-10- (grey bars), and TNF-α- (black bars) producing Th cells after in vitro polyclonal stimulation with PMA plus ionomycin, in PB CD4+ T cells of the same 10 JIA patients at different time points after commencing abatacept treatment. **c** Columns represent mean values (±SE) of the frequencies of the Tregs (CD4+CD25+Foxp3+, black bars) and the T effector (CD4+CD25+Foxp3–, white bars) subsets in PB of the same patients. Plots (right) are of 1 representative patient at T0 and 90 days. * $p < 0.05$, each time point versus T0.

T lymphocytes to produce a panel of several cytokines (IL-4, IL-10, TNF-α) after in vitro stimulation with PMA plus ionomycin, and again did not find any variation during the period of treatment (fig. 4b). Moreover, we found a decrease, at least in terms of frequency, of the regulatory CD4+CD25+Foxp3+ subset, which was reduced from 15 days after starting the treatment until day 90 of observation (fig. 4c).

Discussion

The activation of T cells involves 2 major steps, the presentation of a peptide by an APC to the T cell receptor, and the signal derived from costimulatory molecules, the most important being the interaction between CD80/CD86 and CD28. The molecules CD80/CD86 were expressed by APCs, whereas CD28 were expressed by T cells. In particular, virtually all T cells (both naïve and

memory) express CD28, even if in the elderly a subset of memory T cells may become CD28 negative as a result of repetitive cell divisions, the influence of TNF-α, or infections [12]. CTLA-4 (CD152) is a member of the CD28 immunoglobulin superfamily expressed on the T cell membrane. It has a structural resemblance to CD28, but its affinity to CD80/CD86 is much higher. CD28 transmits a stimulatory effect, enhancing the activation and upregulation of downstream pathways, whereas CTLA-4 transmits an inhibitory signal to T cells, inducing anergy [13]. The absence of the ITIM motif in CTLA-4 has suggested the hypothesis that the function of CTLA-4 cytoplasmic domain is not to transmit inhibitory signals but to precisely control the turnover, cellular location, and membrane delivery of CTLA-4 to facilitate its central function, i.e. regulating the access of CD28 to their shared ligands [14].

In this study we evaluated the capacity of abatacept, a soluble chimeric CTLA4 protein, which binds with high affinity to the B7 molecules CD80 and CD86 limiting the activation and reactivation of CD4+ T cells via CD28, to suppress Th functions such as antigen-specific proliferation and cytokine production, both in vitro in healthy subjects and ex vivo in a group of abatacept-treated JIA patients. First of all, we checked the ability of abatacept to inhibit T cell proliferation in vitro in response to antigen-specific and polyclonal stimulation. Abatacept was able to inhibit the TT-specific and the SK-specific proliferation, as well as the proliferative response induced by CD3-specific mAb. As expected, when the blocking activity of the drug was bypassed by the CD3/CD28 stimulation, the inhibitory effect was not evident. These data substantially reflect the mechanism of action of the drug. In fact, through binding to CD80/CD86, abatacept selectively impairs the costimulation of T cells exerted by APCs. In the same model, we also checked the levels of cytokines in the culture supernatants. Among the cytokines evaluated IFN-γ and TNF-α were detectable in both SK and polyclonal stimulations. Both these proinflammatory cytokines were significantly reduced in the presence of abatacept only in the CD3-stimulated samples. These proinflammatory cytokines, and particularly TNF-α, certainly play a crucial role in the triggering and in the maintenance of joint inflammation. Collectively, these in vitro data confirm the capacity of abatacept to impair the in vitro T cell proliferation [15] and TNF-α production, the role of which in joint inflammation in JIA is well established [16].

In the second part of the study, we longitudinally evaluated 10 JIA patients undergoing abatacept treatment. Patients were checked for clinical parameters, such as the number of involved joints and serum parameters of inflammation, and for in vitro functional features, such as the ability to proliferate and to produce cytokines in response to different stimuli, and the distribution of different Th subsets in PB. The evaluations were performed at T0, 2, 15, 17, 30, 32, 60, 62, 90, and 92 days, with the aim of assessing if the in vitro functions were affected soon after drug administration or if the effects were maintained until the following infusion.

Despite the relatively low number of recruited patients, the clinical efficacy of abatacept was evident after just 60 days, when we started to observe a reduction in the number of involved joints and of ESR. This was maintained at least until 90 days of treatment, in agreement with previous data obtained in RA patients [17]. Concerning the proliferative response to TT stimulation, PBMCs from JIA patients showed no differences between T0 and 90 days. These data are in contrast to those reported by Pieper et al. [18], and also with our observation that the exogenously added abatacept in vitro is able to inhibit both the proliferation and cytokine production in PBMCs of healthy donors. Interestingly, when we looked at TT-induced proliferation soon after abatacept administration we found a significant reduction. Of note, this reduction reflects the data obtained in the in vitro model of exogenously added abatacept. This finding led us to speculate that the activity of abatacept could progressively decrease after its administration.

Regarding the ex vivo assessment, we found no modification in the frequencies of circulating Th1, both classic and nonclassic, Th17, and Th17/Th1 subsets during the first 90 days of treatment. Of note, we recently reported that etanercept in JIA patients was able to reduce the frequencies of circulating nonclassic Th1 cells and to enhance the frequencies of Th17 cells by inhibiting the TNF-α-driven shift of Th17 lymphocytes toward a nonclassic Th1 phenotype [11]. Also, the frequencies of cells able to produce other cytokines (IL-4, IL-10, and TNF-α) were unaltered during the first 90 days of treatment. Moreover, the analysis of JIA patients subgrouped on the basis of diagnosis (5 oligoarticular and 5 polyarticular JIA) did not reveal any difference in Th cell phenotype during this time. Interestingly, the only subset that was modified by the therapy was the regulatory one. In particular, we found a significant reduction in the frequencies of the CD4+CD25+Foxp3+ subset, in agreement with Pieper et al. [18]. A possible explanation for this reduction could be that abatacept, by binding with CD80/CD86, is able to prevent the interaction not only with

CD28, but also with CTLA-4, which has been shown to be highly expressed on CD4+CD25+ Tregs [19]. Moreover, other papers demonstrated that the reduction of Treg cells after CTLA4-Ig treatment could depend on the lack of a CD28 costimulation signal [20] as well as on the reduction of IL-2, which is necessary for Treg expansion [21]. However, it has been clearly shown in RA that this reduction of the frequencies of Treg is not associated with a decrease, but rather with an enhancement of their activity [22–23].

Collectively, our data confirm that abatacept is useful in ameliorating JIA in subjects with an inadequate response to other DMARDs, including biologics [24–25]. Moreover, we provided evidence that abatacept impaired some Th functions, such as the in vitro proliferative response to recall antigens, and the ability to produce cytokines. To our knowledge, this is the first report indicating that such effects are evident only when abatacept levels are expected to be highest (soon after its administration). Finally, further studies are needed to investigate if the reduction of the intervals among drug administration could be of help for optimizing the immunosuppressive activity exerted by abatacept.

Acknowledgments

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Disclosure Statement

Bristol-Myers Squibb Italy did not interfere with the conception and design of the study, the acquisition, analysis and interpretation of data, or drafting of the manuscript

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