MEDITERRANEAN JOURNAL OF RHEUMATOLOGY
ΕΛΛΗΝΙΚΗ ΡΕΥΜΑΤΟΛΟΓΙΑ
27
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2016

ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ
ORIGINAL PAPER

Η τροποποίηση της συνδιέγερσης βελτιώνει τη ρευματοειδή αρθρίτιδα παρά τη μείωση της αναλογίας των $CD4^+CD25^{high}$ Τ ρυθμιστικών κυττάρων

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ΠΕΡΙΛΗΨΗ

Στόχος/Σκοπός: Το Αντιγόνο-4 των κυτταροτοξικών Τ λεμφοκυττάρων (Cytotoxic T Lymphocyte Antigen-4, CTLA-4) εκφράζεται σταθερά στην επιφάνεια των ρυθμιστικών Τ λεμφοκυττάρων (Regulatory T cells, Treg), αν και η λειτουργία το σε αυτό το πλαίσιο παραμένει ασαφής. Η αμπατασέπτη, ένα διαλυτό σύμπλοκο CTLA4-ανοσοσφαιρίνης, είναι ένας τροποποιητής της συνδιέγερσης που χρησιμοποιείται στη θεραπεία της ρευματοειδούς αρθρίτιδας (ΡΑ). Μελετήσαμε την επίδραση της αμπατασέπτης στον πληθυσμό των περιφερικών Treg κυττάρων ασθενών με ΡΑ που ξεκινούν θεραπεία με αμπατασέπτη.

Μέθοδοι: Συλλέχθηκε περιφερικό αίμα από 8 ασθενείς με ΡΑ πριν την πρώτη και μετά την πέμπτη έγχυση της αμπατασέπτης, καθώς και από 8 υγιείς εθελοντές. Το ποσοστό των Treg κυττάρων ($CD4^+CD25^{high}CD127^{-/low}$) μετρήθηκε με κυτταρομετρία ροής.

Αποτελέσματα: Αρχικά οι ασθενείς είχαν ένα μέσο ποσοστό Treg 2,7%, το οποίο ήταν παρόμοιο με των μαρτύρων (3,25%, p=0,495). Το αρχικό μέσο DAS28 ήταν 4,87, ενώ κατά την πέμπτη έγχυση είχε μειωθεί στο 3,3 (p=0,017). Κατά την πέμπτη έγχυση το μέσο ποσοστό των Treg κυττάρων είχε επίσης μειωθεί στο 1,15%, το οποίο ήταν χαμηλότερο από το αρχικό μέσο ποσοστό τους (p=0,012). Η διαφορά στο ποσοστό Treg μεταξύ των δύο χρονικών σημείων συσχετιζόταν θετικά με τη μεταβολή στον αριθμό των διογκωμένων αρθρώσεων (r=0,856, p=0,007).

Συμπέρασμα: Η αμπατασέπτη βελτίωσε σημαντικά τη δραστηριότητα της νόσου, αλλά επίσης ελάττωσε το ποσοστό των ρυθμιστικών Τ κυττάρων μεταξύ των περιφερικών Τ λεμφοκυττάρων.

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Λέξεις-Κλειδιά: Ρευματοειδής αρθρίτιδα, Ρυθμιστικά Τ λεμφοκύτταρα, Abatacept, CTLA-4.

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ORIGINAL PAPER

Co-stimulation modulation improves Rheumatoid Arthritis despite reducing the proportion of CD4^{+}CD25^{high} T regulatory cells

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ABSTRACT

Objective/Aims: Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) is constitutively expressed on the surface of regulatory T cells (Treg), although its function in this context remains unclear. Abatacept, a soluble CTLA-4·Ig construct is a co-stimulation modulator that is used for the treatment of rheumatoid arthritis (RA). We studied the effects of abatacept on peripheral blood Treg cell population in RA patients starting abatacept therapy.

Material and Methods: Peripheral blood was collected from 8 RA patients before the first and fifth abatacept infusion and from 8 healthy volunteers. The percentage of Treg cells (CD4^{+}CD25^{high}-CD127$^{-/low}$) was measured by flow cytometry.

Results: Initially, patients had a mean percentage of Treg cells 2.7%, which was similar to that of controls (3.25%, p=0.495). The baseline mean DAS28 was 4.87, whereas by the fifth infusion it had decreased to 3.3 (p=0.017). By the fifth infusion the mean percentage of Treg cells had also decreased to 1.15%, which was lower compared to baseline (p=0.012). The difference of Treg percentage between both time points positively correlated with the difference in the swollen joint count (r=0.856, p=0.007).

Conclusion: Abatacept significantly improved disease activity, but also decreased the percentage of Treg cells among the peripheral CD4^{+} T cells.

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Mediterr J Rheumatol 2016;27(1):15-9

https://doi.org/10.31138/mjr.27.1.15

Keywords: Rheumatoid arthritis, Regulatory T cells, Abatacept, CTLA-4.

INTRODUCTION

Rheumatoid arthritis (RA) is a T cell-driven autoimmune disease, wherein T cells are activated against one or more as yet elusive (auto)antigens.

The precise role of regulatory T cells (Treg) in controlling this aberrant chronic immune process is still debated. In the peripheral blood of RA patients, $CD4^+CD25^+$ Treg cells have been found in higher, lower or similar proportions compared to controls.[1-4] Peripheral Tregs from active RA have impaired regulatory activity, which could, though, be reversed with tumor necrosis factor α (TNFα) blockade[5]. RA synovial fluid is enriched in Treg cells, which are more potent effector cell suppressors than their peripheral blood counterparts, although RA synovial fluid effector cells may also be less responsive to suppression[1].

The peripheral pool of Treg cells consists of both natural Tregs and peripherally induced Treg cells that act synergistically.[6] Induced Tregs differentiate from naïve $CD4^+$ T cells in a process that depends on the cytokine milieu: in the presence of transforming growth factor-β (TGF-β) differentiation toward Treg cells is favored, but in the combined presence of TGF-β, interleukin-6 (IL-6) and IL-23 the pro-inflammatory subset T_H17 is promoted instead.[7] Consistent with this, IL-6 blockade with tocilizumab in RA patients has been reported to increase the proportion of peripheral Treg cells, although the T_H17 subset was not significantly affected[8].

CD80/86-mediated co-stimulation is crucial for natural Treg generation in the thymus[9], although its role in the peripheral conversion of naïve T cells to Tregs is less clear.[10-11] Moreover, CTLA-4 is constitutively expressed on Treg cells and possibly participates in their function.[12] Mice with selective deletion of *CTLA-4* on Treg cells are prone to autoimmunity[13], while disease triggered by transfer of *CTLA-4*$^{-/-}$ T cells to lymphopenic mice could be prevented by co-transfer of CTLA-4 sufficient Tregs.[14] Abatacept (CTLA-4·Ig), a soluble form of CTLA-4, aims to prevent T effector cell activation by blocking CD80/86:CD28 co-stimulation. However, abatacept may also affect Treg co-stimulation or antagonize with natural CTLA-4 occurring on the surface of Treg cells for binding to CD80/86 on other cells, e.g. dendritic cells. We studied the effects of co-stimulation blockade on the proportion of peripheral blood Tregs of RA patients initiating abatacept treatment.

MATERIALS AND METHODS

We recruited patients with active RA starting treatment with abatacept. Abatacept was infused intravenously at a dose of ~10 mg/kg on weeks 0, 2, 4 and every 4 weeks thereafter. Peripheral blood was collected before the first and fifth infusion (week 12). Age- and sex-matched healthy subjects were sampled as controls.

The study was performed in accordance with the declaration of Helsinki.

Informed consent form all study subjects was obtained.

Flow cytometry of T cell populations was performed using the following markers: anti-CD4 PerCP, anti-CD25 FITC (*BD Biosciences)* and anti-CD127 PE (*Beckman coulter)* on a FACSCalibur *(Becton Dickinson)* and data were analysed using FCS Express *(De Novo Software)*. Viable lymphocytes were gated according to their forward/side scatter profile and cells were subsequently gated for CD4 and CD25 expression. The CD127 marker was used to select $CD4^+CD25^{high}CD127^{-/low}$ T cells, as this subset has been shown to contain a highly enriched $FoxP3^+$ Treg population in humans (**Figure 1**).[15-16] Statistical comparisons were performed using Mann-Whitney U test and Wilcoxon's signed-rank test, while correlation analysis was performed

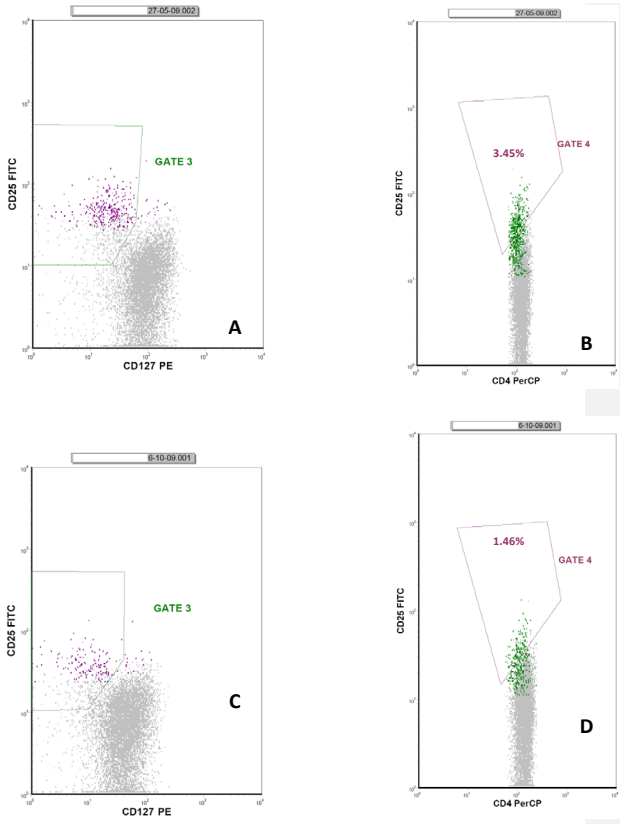


Figure 1. Dot plots of peripheral $CD4^+CD25^{high}CD^{127-/low}$ T cells in a patient before (A, B) and after (C, D) treatment with abatacept. CD4+ T lymphocytes were selected using combined gating based on forward scatter and side scatter properties and CD4 expression. $CD25^+CD127^{-/low}$ cells (GATE 3) were selected (green events) and $CD4^+CD25^{high}CD127^{-/low}$ (GATE 4) were discriminated as CD25 highly and CD4 slightly lower expressing cells. In retrospect this population was back gated as purple events in A and C.

Table 1. Parameters of disease activity at baseline and after 12 weeks of treatment with abatacept

	Baseline	Week 12	p*
TJC (28)	6.9±4.6	2.3±3.8	0.025
SJC (28)	1.8±1.6	0.3±0.5	0.039
PGA (0-100 mm)	56.9±19.8	25.6±21.9	0.011
ESR (mm/h)	41.3±34.7	30.4±16.3	0.528
CRP (mg/L)	6.6±5.2	5.4±3.5	0.348
DAS28	4.87±1.06	3.3±1.08	0.017
$CD4^+CD25^{high}CD127^{-/low}$ T cells (%)	2.7±0.98	1.15±0.41	0.012

Values are means (SD)

* Wilcoxon's test

TJC(28): tender joint count-28 joints, SJC(28): swollen joint count-28 joints, PGA: patient's global assessment, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, DAS28: disease activity score-28 joints

with Spearman's order correlation test. The level of statistical significance was set at p=0.05.

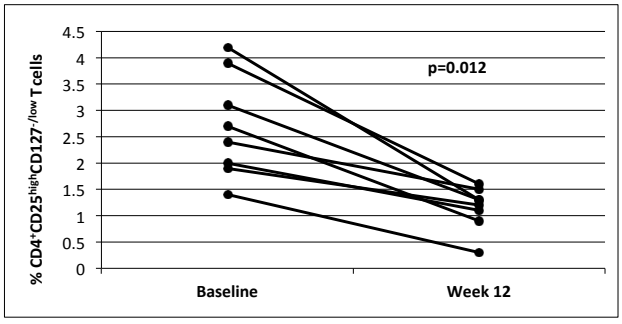
RESULTS

Eight patients were included (5 females, 3 males) with mean age (SD) 63.1 (15.5) years and median disease duration 21 years (range 3–42). Six had previously been treated with TNFα antagonists, 3 were on concomitant methotrexate, 4 on leflunomide and one patient received no other disease-modifying anti-rheumatic drugs (DMARD). Six patients received prednisone at a median dose 10 mg/day.

At baseline patients had active disease with mean disease activity score-28 joints (DAS28) 4.87 (**Table 1**). Patients retained stable DMARD treatment throughout the study, except for one patient who discontinued leflunomide due to adverse event. In 3 patients prednisone was tapered. DAS28 improved in 7 patients and remained roughly unchanged in one patient, so that by week 12, the mean (SD) DAS28 was 3.3 (1.08), which was significantly lower compared to baseline (p=0.017).

Regarding Treg cells, at baseline, the mean percentage of Tregs among the whole peripheral blood $CD4^+$ T cells of RA patients was 2.7% (0.98) which was comparable to controls [3.25% (1.53), p=0.495]. The proportion of peripheral Tregs declined with treatment in all patients (**Figure 2a**). By week 12, the mean (SD) percentage of Tregs was 1.15% (0.41), significantly lower than baseline (p=0.012). At baseline, the percentage of Treg cells correlated significantly with DAS28 (Spearman's r=0.786, p=0.021). The decrease of Tregs by week 12 correlated significantly with the decrease in the number of swollen (28) joints (r=0.856, p=0.007, **Figure 2b**).

2a



2b

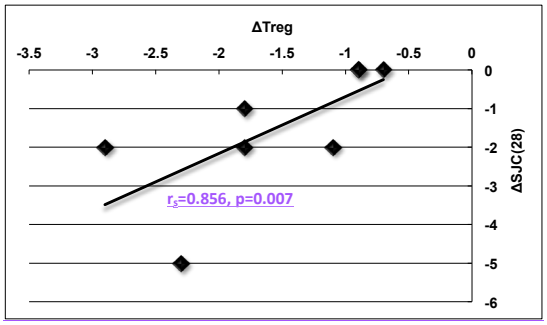


Figure 2. a. Proportion of peripheral $CD4^+CD25^{high}CD127^{-/low}$ T cells in patients with rheumatoid arthritis prior and 12 weeks after abatacept treatment. Comparison made by Wilcoxon's test. b. A scatter plot with a trendline depicting the correlation between the improvement of swollen joint count [ΔSJC(28)] and the decline in the proportion of peripheral Treg cells (ΔTreg). r_s: Spearman's correlation co-efficient, SJC(28): Swollen joint count-28 joints.

DISCUSSION

This small prospective study showed that the proportion of peripheral blood Treg cells in patients with active RA on synthetic DMARDs and/or glucocorticoids was similar to that observed in healthy controls. Further, treatment with abatacept improved disease activity and reduced peripheral Treg cells by almost half. This decrease was observed in all patients, including the one patient with no change in DAS28 score.

Our results corroborate a previous cross-sectional study, showing that the proportion of peripheral $CD4^+CD25^+$ Treg cells of RA patients receiving abatacept was lower than those not receiving abatacept, although the suppressive function of the Tregs of the abatacept-exposed patients was actually enhanced.[17] Recently, abatacept was also associated with a reduction of Treg cells in salivary gland biopsies of patients with Sjögren's syndrome.[18] However, another study on the effects of abatacept on the peripheral Treg cell compartment of RA patients who had previously failed TNFα inhibition, showed that there was no difference in the Treg proportions before and after 6 months of abatacept treatment. Further, these investigators observed that the suppressive function of Treg cells from RA patients *ex vivo*, which had been impaired before therapy relative to healthy donors, was restored following 6 months of abatacept treatment.[19] Conversely, a recent study seems to challenge the above results, by showing that in RA patients the Treg compartment expands along the whole compartment of the $CD4^+$ T cells following 4 weeks of treatment with abatacept, which appears to result from suppression of apoptosis. Interestingly, both total $CD4^+$ and Treg cells downregulate various markers of activation, which is reasonable taking into account the mode of action of abatacept. Most interesting, though, is their observation that abatacept reduced Treg suppressive function on T effector cells *ex vivo*. In an effort to clarify whether abatacept actually suppressed Treg function or rendered T effector cells less responsive to suppression, the investigators performed *in vitro* co-cultures using peripheral mononuclear cells form healthy donors and concluded that the apparent attenuation of the Treg regulatory activity was due to the CD80/CD86 blockade by CTLA-4·Ig on the surface of T effector cells, highlighting the significance of these molecules not only for co-stimulation, but also regulation of those cells.[20]

The diversity of the results on the peripheral Treg proportions and functions reported by various investigators probably reflects the complexity of the functions of the CD28/CTLA4/CD80/CD86 co-stimulatory system, which may act differently on Treg, T effector, antigen-presenting cells or other cell types. This variation may be even greater in disease states, such as RA, and may change over time. Therefore, CD80/86 blockade by CTLA4·Ig may have multiple ramifications concerning Treg cell conversion, proliferation, survival, function and impact on their target cells, which may differ in different states of RA activity or different disease stages. However, other aspects concerning the above studies and possibly responsible for the conflicting results may be the small numbers of patients, differences in time points the experiments were performed and, finally, differences in the methodologies employed for identifying the Treg cells.

For this study, we did not use FoxP3 as a Treg marker, because human naïve $CD4^+$ T cells transiently express FoxP3 upon activation, without necessarily acquiring a regulatory phenotype.[21-22] Since abatacept principally inhibits activation of naïve $CD4^+$ T cells, changes in the proportion of FoxP3 expressing cells might reflect alterations not only in Treg, but also T effector cell kinetics. Therefore, instead of intracellular FoxP3, we chose low expression of CD127 on the surface of $CD4^+CD25^{high}$ T cells as a marker of Treg cells, as has already been used for RA patients by other investigators.[4,8,19] Besides, low CD127 expression as a Treg marker has been evaluated in humans with type 1 diabetes,[15] systemic lupus erythematosus,[23] and is increasingly used for defining Tregs in other autoimmune diseases.[24]

The contraction of the peripheral Treg subset with abatacept might simply reflect a slowdown in peripheral Treg conversion, which possibly depends on CD80/86:CD28 interaction.[10] Alternatively, since the proportion of peripheral Tregs at baseline correlated with DAS28, a relatively expanded baseline Treg population may represent an -unsuccessful- homeostatic immunological response to counter the heightened T effector activity. Hence, as disease activity abates with treatment, the Treg subset may contract again and this phenomenon may be a marker of treatment response rather than an abatacept-specific effect. However, the Treg proportion in RA patients at baseline was not higher than in healthy controls, contesting the assumption of reactive Treg expansion. Perhaps, the decline in the proportion of peripheral Treg cells in parallel to the reduction of the swollen joint count may represent, after all, two equally parallel but distinct effects of abatacept on both Treg and T effector cell function and kinetics, including peripheral conversion and tissue homing.

Besides, apart from the Treg-to-T effector ratio, equally important is the potency of the regulatory activity of the former and the responsiveness to such an activity of the latter. However, our study is limited by that we did not perform functional analyses, e.g. by investigating the production of suppressive cytokines from Tregs or their ability to suppress T effector cell proliferation before and after institution of abatacept.

Overall, Tregs are a dynamic cell subset which may

be a collateral target of modern biological therapies. The exploration of the effects of such treatments on this subset has only recently begun and will possibly shed light on the importance of these cells for RA pathophysiology and better clarify the modes of actions of those treatments.[25]

CONCLUSIONS

In conclusion, abatacept treatment is associated with a relative decrease of peripheral Treg cells which correlates with clinical improvement. Whether this observation is related to the drug's mechanism of action and which are the drug effects on qualitative traits of T regulatory cells in patients with RA remains to be explored.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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