Reduction of peripheral blood T cells producing IFN-γ and IL-17 after therapy with abatacept for rheumatoid arthritis

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Abstract

Objective

Abatacept (ABA), a molecule used in the treatment of rheumatoid arthritis (RA), competes with the engagement of CD28, a T-cell receptor for co-stimulatory signals. CD28-mediated signalling regulates several T-cell functions, including inflammatory cytokine production and regulatory T cells (Treg) differentiation. Therefore, our objective was to evaluate the effects of ABA on peripheral blood T-lymphocyte cytokine production and on the number of circulating Treg.

Methods

In 24 RA patients treated with ABA for at least 6 months the proportions and absolute numbers of peripheral blood T cells producing interferon-gamma (IFN-γ) and interleukin-17 (IL-17) after in vitro stimulation, as well as those of Treg were longitudinally evaluated by flow cytometry.

Results

At baseline, compared with 16 healthy controls, RA patients had a higher percentage of $CD4^+$ and CD8+ T cells producing IL-17 (p=0.021, and p=0.006, respectively), as well as of circulating Treg (p=0.041). After 6 months of therapy with ABA, there was a decrease of the percentage of IFN-γ- and IL-17-producing $CD8^+$ T cells (p=0.033 and p=0.035, respectively), and of Treg (p=0.008), while that of IL-17-producing CD4+ T cells decreased after 12 months of treatment (p=0.005). The number of IL-17-producing T cells and of Treg, higher than in controls at baseline, normalised after ABA therapy. All these variations were statistically significant only in RA patients with EULAR good clinical response (n=17).

Conclusions

The blockade of CD28 signal caused by ABA induces the decrease in peripheral blood of IL-17- and IFN-γ-producing T cells.

Key words

abatacept, interferon-gamma (IFN-γ), interleukin-17, rheumatoid arthritis, T cells

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Received on May 29, 2013; accepted in revised form on November 12, 2013.

Funding: Bristol-Myers-Squibb Italy, provided unconditioned financial support for the study, but had no access to the conception and design of the research, acquisition, analysis, interpretation of data, and manuscript drafting.

Competing interests: none declared.

Introduction

Co-stimulation is a mandatory process for efficacious lymphocyte activation. The most important co-stimulatory signal for T cell is delivered by CD28, a surface molecule expressed in physiological conditions by almost all $CD4^+$ and more than 50% of $CD8^+$ T cells (1). After its interaction with CD80 and/or CD86, expressed on antigen presenting cells, different functional properties of T lymphocytes are enhanced, including production of cytokines, in particular interferon-gamma (IFN-γ) and interleukin-17 (IL-17) (2, 3). At the end of co-stimulatory process, the up-regulation of cytotoxic T lymphocytes antigen-4 (CTLA-4, CD152) on T-cell membrane, through its competition with CD28 for CD80-CD86 ligation, allows adaptive immune response switching off.

Abatacept (ABA) is a CTLA-4-Ig fusion molecule efficacious in rheumatoid arthritis (RA) treatment. Several possible mechanism of action of ABA have been proposed, including the modulation of B lymphocytes, dendritic cells, endothelial cells, and osteoclasts, but the extent of their contribution to the drug efficacy is still undetermined (4-6). The reduced production of pro-inflammatory cytokines, in particular of IFN-γ, by synovial macrophages is one of the most interesting effects on the immune response exerted by ABA (7-9). Accordingly, decreased serum levels of IFN-γ have been described in RA patients treated with ABA (6). Many of the biological actions of ABA are likely to be an indirect consequence of the blockade of the CD28 T-cell co-stimulatory signals necessary for the full activation of these cells. In fact, data from the RA synovium - SCID mouse model suggest that ABA does not act directly on the synovium, but more likely by preventing T-cell activation at a systemic level (10). However, not much is known on the modulatory effect of ABA on T-cell functions. In previous studies (11, 12), we demonstrated that, in RA patients, ABA may reduce the number of circulating $CD4^+$ and $CD8^+CD28^{neg}$ T cells, an effector and auto-reactive population (13-15), which number is related to worse prognosis and to the presence of extra-articular manifestations (15). The reduction of the number of the $CD28^{neg}$ T cells was also significantly correlated with an improvement of disease activity (11, 12).

Signalling through CD28 may be important not only for the production of inflammatory cytokines, but also for several other T cell functions. In particular, it has been reported that CD28 may modulate the expansion and survival of regulatory T cells (Treg) (16).

The aim of the present study was to verify the hypothesis that ABA might reduce the production of pro-inflammatory cytokines (IFN-γ and IL-17) by T cells. Moreover, we have explored the effect of ABA therapy on the number of circulating Treg.

Materials and methods

Patients

Forty-four consecutive RA patients that previously failed to respond to at least one disease-modifying anti-rheumatic drug and were treated with ABA for at least 6 consecutive months were enrolled. The study was approved by local ethics committee. Clinical assessment of patients was performed using the Disease Activity Score 28 based on C-reactive protein (DAS28-CRP) (17) and clinical response was evaluated with the European League Against Rheumatism (EULAR) response criteria (18). Thirty-two and 16 patients of this cohort had participated in previous studies on the immune effect of ABA (12, 19).

Sixteen healthy donors (HD; 9 male, 7 female; median age: 49; 25–75th percentile: 39–53) were used as controls.

Methods

Blood samples were obtained at the start of ABA treatment (T0) and after 6 and 12 months of therapy (T6 and T12). Peripheral blood T-cell phenotypic characterisation was performed by flow cytometry (Cytomics FC-500, Beckman Coulter Inc., Fullerton, CA), as previously reported (12). Absolute cell count was determined by single-platform analysis using Flow-Count beads (Beckman Coulter). Treg were defined by the high expression of CD25 and the low expression/absence of CD127, using PE-conjugated anti-CD25 and

PC7-conjugated anti-CD127 (Beckman Coulter) (20). In addition, in a portion of patients (n=12), Treg were evaluated with FITC-conjugated anti-CD31 and ECD-conjugated CD45RA (Beckman Coulter), in order to divide them into a subset directly derived from the thymus (CD45RA+CD31+) and a subset induced in periphery (CD45RA neg).

Cytokine production by T cells was evaluated *in vitro* in 24 patients and in 16 HD. Peripheral blood mononuclear cells obtained by Ficoll-Hypaque gradient centrifugation were cultured at 37°C temperature and 5% CO_2 pressure, and stimulated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml) and ionomycin (500 ng/ml) for 5 hours in the presence of monensin (1μM; Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Since it is well-known that PMA induces a rapid down-regulation of CD4 molecule (21) that prevents direct evaluation of the $CD4^+$ populations, CD3+CD8- cells were considered as $CD4^+$ in the analysis of cytokine-producing cells. Cells were washed and stained with PC5-conjugated anti-CD3, ECD-conjugated anti-CD28 and PC7-conjugated anti-CD8 (Beckman Coulter) for 30 minutes at room temperature, fixed with paraformaldehyde (4%) for 5 minutes and permeabilised with saponin (0.2%; Sigma-Aldrich) for 15 minutes. Intra-cytoplasmic staining was performed for 30 minutes using PE-conjugated anti-IFN-γ and Alexa Fluor 488-conjugated anti-IL-17A (Becton Dickinson, San Jose, CA).

Statistical analysis

If not otherwise specified, data were expressed as the medians (25-75th percentile). Comparison between quantitative variables of different groups was performed with Mann-Whitney test; the variations of matched quantitative data were analysed with Wilcoxon test. Chi-Square test with Yates' correction was applied for comparison between qualitative variables. Simple regression test was used to evaluate correlation between quantitative variables.

Results

Main demographic and clinical features of 24 RA patients treated with ABA in which T-cell functional studies were performed are summarised in Table I. There was no significant difference with the total cohort of 44 consecutive patients who received ABA at our center (Table I). In the large majority of patients ABA was used after failure or adverse events to one or more other biological agents. After 6 months of treatment with ABA, 59% and 55% of the entire cohort achieved EULAR good clinical response and clinical remission, respectively, while these outcomes were reached in 71% and 62% of the 24 patients evaluated with T-cell functional studies. Five of 24 patients discontinued ABA treatment after 6 months for inefficacy, and none for severe adverse events.

Table I. Main clinical and demographic features of RA patients. Data are expressed as median (25th–75th percentile) if not otherwise specified.

	Total cohort of patients (n=44) (%)	Cohort evaluated with T cell functional studies (n=24) (%)	*p*-value
Sex (male/female)	6/38	4/20	1.00
Age (years)	54 (47-60)	52 (42-61)	0.21
Disease duration (years)	8.5 (4-13)	6.5 (1-11.5)	0.06
Smokers [%]	14 [31.8%]	10 [41.7%]	0.58
Rheumatoid factor positivity [%]	33/42 [78.6%]	18/20 [90%]	0.46
Anti-CCP antibodies positivity [%]	32/38 [84.2%]	15/18 [83.3%]	1.00
Number of previous DMARDs	3 (2-5)	3 (2-4)	0.10
Number of previous TNF-alpha blocking agents	2 (1-2)	2 (1-2)	0.20
Patients with previous Rituximab treatment [%]	8 [18.2%]	3 [12.5%]	0.79
Causes of interruption of previous biologic treatments:			
Inefficacy [%]	32 [75%]	16 [66.7%]	0.81
Others [%]	7 [16%]	5 [20.8%]	0.86
Number of patients treated with ABA as first line biological treatment [%]	6 [13.6%]	3 [12.5%]	1.00
DAS28 (CRP) at baseline	5.17 (4.46-6.11)	5.08 (4.63-5.67)	0.19
Concomitant use of DMARDs:			
Methotrexate (MTX) [%]	33 [75%]	19 [79.2%]	0.93
MTX + other DMARD	5 [11.4%]	4 [16.7%]	0.23
Others [%]	4 [9.1%]	3 [12.5%]	0.28
None [%]	7 [15.9%]	2 [8.4%]	0.61
Dosage of MTX at baseline (mg/week)	12.5 (6-15)	13.7 (9.3-15)	0.30
Concomitant use of vitamin D at baseline [%]	29 [65.9%]	15 [62.5%]	0.99
Vitamin D dosage at baseline (IU of colecalcipherol/day)	800 (0-833)	800 (0-808)	0.83

CCP: cyclic citrullinated peptide; DMARDs: disease-modifying anti-rheumatic drugs; ABA: Abatacept; DAS: Disease Activity Score.

At baseline (T0) the percentages of $CD4^+$ and $CD8^+$ T cells producing IL-17 were higher in RA patients than in HD (p=0.021 and p=0.006; Fig. 1). There was no significant difference between the two groups in the proportion of $CD8^+$ T cells producing IFN-γ, whereas the number of $CD4^+$ T cells producing IFN-γ was lower in RA than in HD (p=0.049; Fig. 1).

When the production of IFN-γ was evaluated in T-cell subsets identified according CD28 expression, no differences between HD and RA patients at T0 were found. Figure 2 demonstrates data on the $CD28^{neg}$ subpopulations; as expected, these were a better source of IFN-γ than the $CD28^+$ counterparts.

To calculate the absolute number of potential cytokine-producing T cells in RA patients, the proportion found after *in vitro* stimulation was multiplied by the absolute number of circulating cells. At T0 the absolute number of $CD4^+$ IL-17-producing T cells was significantly higher in RA patients than in HD (12 cells/microliter (4-19) *vs*. 4 (3-5); p=0.005), while the other T-cell subtypes did not differ between the two groups.

No difference in cytokine production

was observed at T0 when patients were separated according to their treatment with drugs that potentially modulate T-cell functions, such as methotrexate and vitamin D (data not shown).

The percentages of IFN-γ- and IL-17 producing cell populations were not different between RA patients who achieved a good EULAR clinical response after therapy with ABA and those who did not (data not shown).

At T0, the proportion of circulating Treg ($CD4^+CD25^{high}CD127^{low/absent}$) was higher in RA patients than in HD (6.4% (4.8–7.5) *vs*. 4.7% (4.0–4.8); p=0.041; Fig. 3), whereas Treg absolute number did not differ (51/microliter (28–63) *vs*. 40 (36–46); p=0.73).

The variations of cytokine production upon *in vitro* stimulation after 6 (T6) and 12 months (T12) of ABA therapy in RA patients are reported in Figure 1. The percentage of IFN-γ- and IL-17-producing $CD8^+$ T cells significantly decreased at T6 (p=0.033 and p=0.035, respectively), whereas the reduction of IL-17 producing $CD4^+$ T cells reached statistical significance only at T12 (p=0.005). The percentages of IL-17 producing T cells, that were higher than in HD at T0, normalised after ABA therapy. When T cells were analysed according the expression of CD28, the percentages of IFN-γ-producing cells did not significantly change in any subset (Fig. 2).

Calculating the absolute number of cytokine-producing T cells at T6, we observed a significant decrease of IL-17 producing $CD4^+$ and $CD8^+$ T cells (from 12 cells/μl (4–19) to 8 (4–14); p=0.044; and from 4 (2–5) to 2 (1–4); p=0.028), as well as of IFN-γ producing $CD8^+$ T cells (from 80 cells/μl; (47-119) to 54 (35-81); p=0.023). The reduction of the absolute number of IFN-γ producing CD8+ cells was accounted for by the reduction of the IFN-γ^+ $CD28^{neg}$ population of $CD8^+$ T cells (p=0.033; Fig. 2), while IFN-γ^+CD28^+ cells did not significantly change (data not shown). Moreover, also the IFN-γ^+ $CD28^{neg}$ population of $CD4^+$ cells decreased after 6 months of ABA therapy (p=0.049; Fig. 2).

When RA patients were divided into two groups according to their clinical response, the reduction at T6 of the percentage of IFN-γ producing $CD8^+$ was statistically significant only in patients with good clinical response (n:17; p=0.019), whereas the variation was milder and not significant in the other group of patients (n=7). Similarly, only in patients with good clinical response to ABA the percentage of IL-17 producing $CD4^+$ and CD8+ T cells decreased

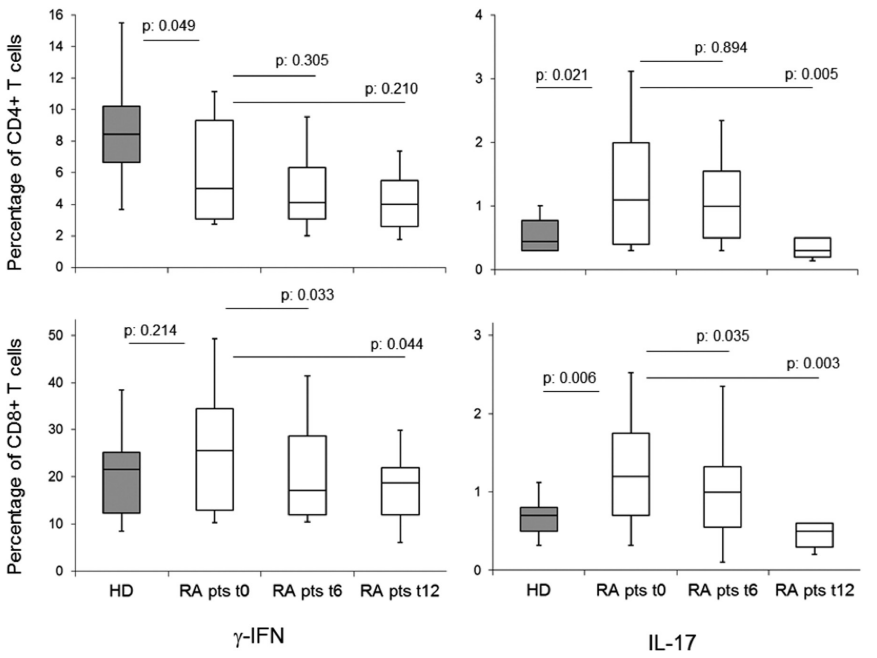

Fig. 1. Percentages of IFN-γ- and IL-17-producing T cells after *in vitro* stimulation. Data are expressed as box plots: median (horizontal bar), 25^{th}-75^{th} percentile (box) and 10^{th}-90^{th} (error bars). Grey: healthy donors (HD); white: RA patients before (T0) and after 6 (T6) and 12 months (T12) of treatment with Abatacept.

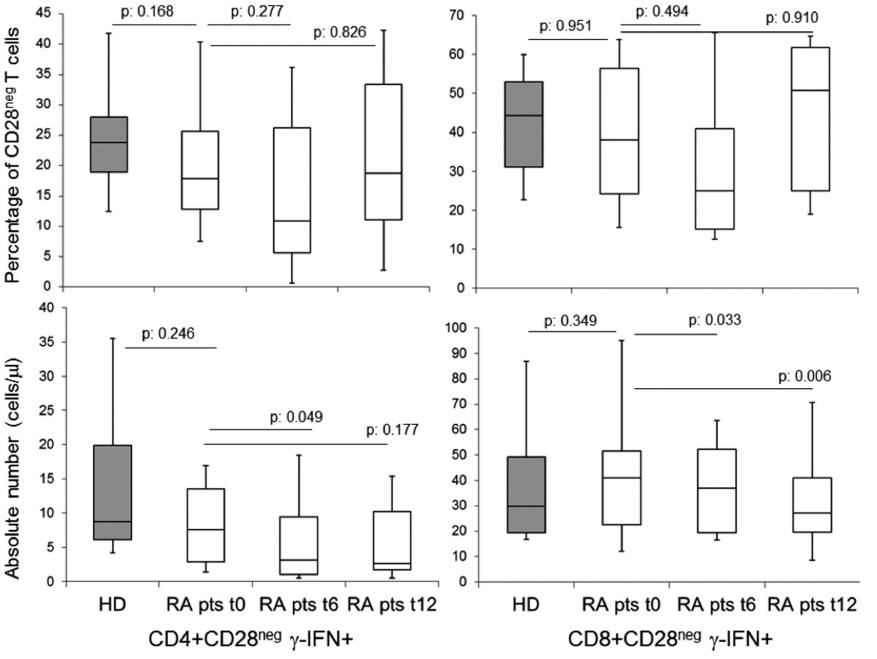

Fig. 2. Percentages and absolute number of IFN-γ-producing T cells within the $CD28^{neg}$ subsets after *in vitro* stimulation. Data are expressed as box plots: median (horizontal bar), 25^{th}-75^{th} percentile (box) and 10^{th}-90^{th} (error bars). Grey: healthy donors (HD); white: RA patients before (T0) and after 6 (T6) and 12 months (T12) of treatment with Abatacept.

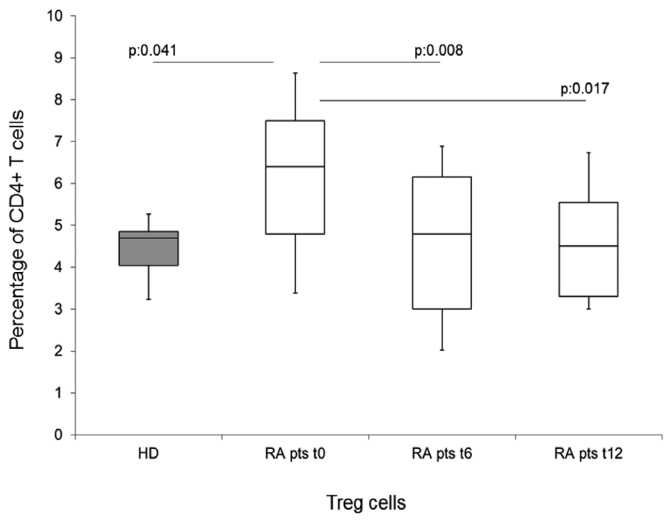

Fig. 3.
Percentages of Treg ($CD4^+CD25^{high}$ $CD127^{low}$) within the peripheral blood $CD4^+$ T cell population. Data are expressed as box plots: median (horizontal bar), 25^{th}-75^{th} percentile (box) and 10^{th}-90^{th} (error bars).
Grey: healthy donors (HD);
White: RA patients before (T0) and after 6 (T6) and 12 months (T12) of treatment with Abatacept.

at T6 (p=0.019 and p=0.014, respectively). No difference in the variations of cytokine production was instead observed separating patients according demographic and clinical features (including autoantibody seropositivity).
The percentage of Treg, that was higher than in HD at T0, normalised after 6 months of ABA therapy, with a significant reduction (from 6.8% (4.8–7.5) to 4.6 (3.0–6.2) of $CD4^+$ T cells; p=0.008; Fig. 3). However, the reduction of the absolute number of Treg did not reach statistical significance (from 51 cells/μl; (28–63) to 39 (27–56); p=0.23). In 12 RA patients evaluated for this purpose, no variation was observed in the percentage of recent thymic emigrants (CD45RA+CD31+) and peripheral memory induced (CD45RAneg) subsets within the population of total Treg (data not shown).
There was no significant correlation between Treg changes after ABA therapy and clinical response.

Discussion

Even if the blockade of co-stimulation signals with ABA represents a valuable option for RA treatment, the mechanisms by which this therapy is successful in RA are not yet fully elucidated (4). At this regard, mouse model data suggest that ABA acts by preventing T-cell activation at a systemic level, and not directly on the synovium (10).
Phenotypic effects of ABA therapy on peripheral T cells were reported in our previous studies; in particular, we demonstrated a decrease of the number of circulating $CD28^{neg}$ T cells, and we suggested that ABA, by competing with CD28 repetitive engagement, avoids down-modulation of this molecule and the consequent expansion of the $CD28^{neg}$ T cells (11, 12), a population that have peculiar functional aspects, including a high production of IFN-γ (13).
According with these premises, we demonstrated a reduction of the peripheral blood T cells capable to produce IFN-γ after *in vitro* stimulation in patients treated with ABA. The reduction was statistically significant as far as the percentage and absolute number of $CD8^+$ T cells and the absolute number of $CD4^+$ T-cells. A reduction of $CD4^+$ IFN-γ-producing cells was very recently reported by another group in a smaller number of patients treated with ABA (22). In this study, contrary to our findings, this reduction was significant only in ACPA positive patients. However, these authors used a protocol of *in vitro* stimulation (with CD3 only) that lead to the activation of a much smaller number of $CD4^+$ T-cells, and it is possible that the use of different techniques may account for this discrepancy.
In our experience, the reduction of IFN-γ production was associated with a good clinical response to ABA. Analogously, when a reduction of IFN-γ production was observed in RA patients treated with TNF-blocking agents, this was correlated with disease improvement (23). These findings may suggest an association between this functional feature and RA disease activity, indicating that this might be a common pathogenic pathway targeted by different therapies through different mechanisms of action (24). However, while some authors reported a decrease of IFN-γ producing cells after treatment with anti-TNF drugs (23, 24), others on the contrary described an increase of IFN-γ production in patients treated in this way (25, 26). The decrease in IFN-γ producing T cells observed after ABA therapy cannot therefore be clearly interpreted as a non-specific sign of disease activity reduction. Since we did not find significant variations in the proportion of IFN-γ-producing cells within the subpopulations defined according CD28 expression, our data suggest that the reduction of the circulating $CD28^{neg}$ subpopulations (which are a better source of IFN-γ production than the $CD28^+$ counterparts), can also explain the decrease of IFN-γ-producing cells observed after ABA therapy. A reduction of $CD28^{neg}$ cells, was also found in some patients treated with anti-TNF (28, 29), but in those studies the number of IFN-γ-producing cells was not evaluated. We suggest that the possible effect of previous therapies, including more than one TNF-blocking agent in the majority of our patients, might explain the observations that before ABA treatment, despite an active state of RA, there was no difference in the number of IFN-γ-producing cells between patients and HD, and the percentages of IFN-γ^+ $CD4^+$cells were even lower in patients.
Others have described a reduction of IFN-γ serum levels (6, 22) and of its expression in synovial cells (7) after ABA therapy. Accordingly, synovial T cells decrease their expression of IFN-γ after treatment *in vitro* with ABA (30) The synovial compartment of RA is usually enriched by memory/activated T cells able to produce IFN-γ (7, 30, 31). The decrease of IFN-γ synovial expression observed after ABA therapy was correlated with magnetic resonance imaging improvement (7), and this prompted to the suggestion that T cell "deactivation", with reduced IFN-γ production, rather than synovium depopulation, is important in mediating the effects of ABA (7). It is therefore conceivable

that the co-stimulation blockade by ABA can reduce the production of cells with these functional characteristics in secondary lymphoid organs and that this situation is reflected in the peripheral blood. Indeed, migration from secondary lymphoid organs to peripheral tissues, like the synovium, is facilitated by down-modulation of CCR7 (32), and we have previously demonstrated a reduction of the $CCR7^{neg}$ T-cell populations after ABA therapy (12). Not many data, however, are so far available to substantiate this hypothesis. While decrease in the infiltrate of lymphoid B cells after 4 months of therapy (7), and also of T cells after longer treatment (33), has been described in the synovial tissue of ABA treated patients, less is known on the effect on $CD28^{neg}$ T cells in the rheumatoid synovium. The only available information is that these cells are rare in the synovial membrane and synovial fluid, despite significant frequencies in the circulation (34, 35).

We wished to evaluate whether co-stimulation blockade modify T-cell functional activities not only by decreasing the production of $CD28^{neg}$ populations. Therefore, since it has been shown that the T-cell differentiation *in vitro* toward IL-17 production may be increased by CD28 mediated signalling (3), we measured the production of this cytokine. Like others (36, 37), we found an increase of circulating IL-17 producing T cells in RA patients, but, here, we first demonstrated that these cells significantly decrease after treatment with ABA, returning to normal levels. In a study on a slightly smaller number of patients, a reduction of Il-17 producing $CD4^+$ T cells, although not statistically significant, was also recently described (22). Furthermore, we found that the decrease of circulating IL-17 producing T cells after ABA therapy was significantly associated with improvement of disease activity. We analysed the possible effect of concomitant therapies that might affect T-cell functions (38, 39), but we did not observe any clue for a possible effect of concomitant methotrexate, or vitamin D. We propose, therefore, that CD28 co-stimulation blockade might be responsible also for the decrease of IL-17 production observed in patients treated with ABA. Considering the capability of IL-17 to amplify the pro-inflammatory cytokine cascade, the effect of the reduction of IL-17 producing cells might be one of the mechanisms by which ABA performs its favourable action in RA. This effect, however, is not a specific feature of ABA, since it was already described after therapy with TNF- or IL-6- blocking-agents, and not only in peripheral blood, but also in inflamed tissues (36, 37). These results suggest that IL-17 production might be another pathogenic mechanism which participates to a possible common final pathway leading to RA (24), which may be targeted by various and differently acting therapeutic agents.

Finally, since it has been suggested that CD28 signals are important for the expansion and survival of Treg (16), we analysed the possible effect of the CD28 blockade performed by ABA on peripheral blood Treg. To quantitate Treg we counted $CD4^+CD25^{high}CD127^{low/absent}$ T lymphocytes, since CD127 has been demonstrated as a good biomarker for human Treg cells (20). We observed a significant decrease of the percentage of circulating Treg, confirming previous results obtained evaluating the expression of Foxp3, CTLA4, Helios or CD39, other biomarkers of Treg, on $CD4^+CD25^{high}$ cells (22, 40). On the contrary, an increased percentage of circulating Treg was observed in RA patients after treatment with TNF or IL-6 blocking therapy (29, 41, 42), which can be explained by the down modulating action of these cytokines on the generation of Treg (43). To interpret our results, we evaluated whether ABA favour the proportional expansion of the subset of Treg induced in periphery upon the effect of anti-inflammatory cytokines. For this reason, we tried to dissect the effect of ABA treatment on different phenotypic subsets of Treg (thymic, and periphery induced), but our results, which are in accordance with other reports (22), did not allow further conclusions. It should be also mentioned that, like others (40), we did not observe a correlation between Treg number changes and clinical response to ABA. Although concordantly described (22, 40), the clinical significance of the decreased peripheral blood Treg number after ABA therapy, is therefore still unclear. Moreover, conflicting results on their functional activity have been found. An increased functionality of peripheral blood Treg was reported, and it was speculated that the engagement of CD80/86 on dendritic cells might induce a tolerogenic behaviour (40), but, on the other hand, no enhancement of synovial fluid Treg suppressive function was described by others (22). The complex action of ABA on Treg in RA deserves therefore further studies.

There are some limitations in our study, including the relatively small number of patients evaluated and their heterogeneity. These limitations are similar to those of other studies concerning the effects of biological drugs which were mainly used a second line. However, data presented here show that several phenotypic and functional features of circulating T cells are modified after therapy with ABA. Although we cannot exclude that some of the reported changes, in particular the reduction of IL-17-producing cells, are related to a reduction in disease activity rather than to a specific effect of the drug, other findings appear to be more directly related to the blockade of CD28 signal. These more specific effects of ABA might include the reduction of IFN-γ-producing cells, which is likely mediated through the decrease of the $CD28^{neg}$ T-cell subpopulation (a highly experienced cell subtype that can produce IFN-γ independently of CTLA-4 control), and the reduced percentage of (phenotipically) Tregs.

Altogether, these findings suggest multiple ways of action of ABA, confirming its role in blocking T-cell differentiation before antigen presentation, as suggested by animal models (10, 44), and underlie the relevance of the signal mediated by CD28 as pharmacological target for RA.

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