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Original article

Abatacept (CTLA-4Ig) treatment reduces T cell apoptosis and regulatory T cell suppression in patients with rheumatoid arthritis

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Abstract

Objective. Abatacept (CTLA-4Ig) blocks CD28-mediated T cell activation by binding to the costimulatory B7 ligands CD80/CD86 on antigen presenting cells. Costimulatory molecules, however, can also be expressed on T cells upon activation. Therefore, the aim of our study was to investigate direct effects of CTLA-4Ig on distinct T cell subsets in RA patients.

Methods. Phenotypic and functional analyses of $CD4^+$ T cells, including $CD4^+FoxP3^+CD25^+$ regulatory T cells (Treg), from RA patients were performed before and during CTLA-4Ig therapy. In addition T cells from healthy volunteers were analysed on *in vitro* culture with CTLA-4Ig or anti-CD80 and anti-CD86 antibodies. Apoptotic DNA fragmentation in $CD4^+$ and $CD4^+FoxP3^+$ T cells was measured by TUNEL staining.

Results. We observed an increase in T cells, including Treg cells, after initiation of CTLA-4Ig therapy, which was linked to a downregulation of activation-associated marker molecules and CD95 on $CD4^+$ T cells and Treg cells. CTLA-4Ig decreased CD95-mediated cell death *in vitro* in a dose-dependent manner. Functional analysis of isolated Treg cells from RA patients further revealed a diminished suppression of responder T cell proliferation. This was found to be due to CTLA-4Ig-mediated blocking of CD80 and CD86 on responder T cells that led to a diminished susceptibility for Treg cell suppression.

Conclusion. CTLA-4Ig therapy in RA patients exerts effects beyond the suppression of T cell activation, which has to be taken into account as an additional mechanism of CTLA-4Ig treatment.

Key words: abatacept, CTLA-4Ig, rheumatoid arthritis, regulatory T cells, Foxp3.

CLINICAL SCIENCE

Rheumatology key messages

- CTLA4-Ig therapy inhibits T cell activation in RA patients.
- CTLA4-Ig therapy prohibits CD95-mediated cell death in regulatory T cells in RA patients.
- CTLA-4Ig inhibits suppressive capacity of regulatory T cells in RA patients.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which is characterized by hyperplasia of the synovial membrane due to expansion of fibroblasts and infiltration of inflammatory cells. Among them activated T cells are thought to play a prominent role due to their capacity to activate monocytes, macrophages, synovial fibroblasts and B cells [1–3]. One of the strategies in the treatment of RA patients, therefore, aims at the inhibition of T cell activation.

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T cell activation in general requires at least two signals. The first is the engagement of the antigen-specific T cell receptor with peptide antigens that are presented by major histocompatibility complex class II molecules on the surface of antigen-presenting cells (APCs). The second signal is provided by the interaction of CD28 on T cells with costimulatory molecules CD80 (B7-1) and CD86 (B7-2) on APCs [4, 5]. Upon activation T cells start to express endogenous cytotoxic T-lymphocyte antigen-4 (CTLA-4) on the cell surface. CTLA-4 also binds to costimulatory molecules, but, in contrast to CD28, delivers anti-proliferative signals that downregulate T activation [6–8] (supplementary Fig. S1, available at *Rheumatology* Online).

In addition to APC, both CD80 and CD86 molecules can be expressed on conventional T cells [9–12] at later stages of T cell activation and the ligation of CTLA-4 via a T–T cell interaction has been shown to downregulate immune responses [13]. CD80 and CD86 molecules can also be expressed on Treg cells and seem to play a role in Treg function [14]. Beyond its costimulatory capacity, CD80 and CD86 signaling might be necessary to prevent uncontrolled T cell activation and proliferation; thus, CD80 and CD86 expression appear to have dual functions within the immune system by conveying activating as well as inhibiting signals (supplementary Fig. S1, available at *Rheumatology* Online).

CTLA-4Ig represents a soluble, recombinant fusion protein, comprising the extracellular domain of human CTLA-4 and the Fc portion of human IgG1, which has been modified to prevent complement fixation. This inhibition of the T cell activation has been shown to be an effective mechanism in the treatment of RA patients [15–18].

It has not been investigated so far whether CTLA-4Ig might also bind to costimulatory molecules on T cells, which can be expressed in particular under pathogenic [19–23] or inflammatory conditions [24–26] with chronic T cell activation. This binding might interfere with the interaction of CD28 or CTLA-4 on other T cells with potential downstream effects. Whether this results in a considerable positive or negative effect on the overall immune response has not been investigated so far.

The aim of our study was therefore to investigate direct effects of CTLA-4Ig therapy on phenotypic and functional characteristics of T cells in RA patients. We therefore investigated the effects of CTLA-4Ig therapy at earlier time points, including 2 and 4 weeks after initiation of therapy.

Methods

Patients

Patients with rheumatoid arthritis who fulfilled at least four of the revised criteria of the American College of Rheumatology [27] and receiving CTLA-4Ig treatment were randomly selected from our outpatient clinic (n = 15). This study was approved by the Ethics Committee of the Medical University of Vienna and informed consent from the patients was obtained. Heparinized whole blood samples were taken before (week 0), and after 2 and 4 weeks of treatment with CTLA-4Ig (Fig. 1A). In addition heparinized whole blood samples were taken from sex and age matched healthy volunteers (healthy controls, HCs). Detailed demographic and clinical characteristics of RA patients are provided in Table 1.

Antibodies

The following monoclonal antibodies (mAb)/conjugates were used in this study: fluoresceïn isothiocyanate (FITC), R-phycoerythrin (PE), phycoerythrin–cyanin5 (PE-Cy5) and allophycocyanin (APC), PE-Cy7 and APC-Cy7-conjugated mAb against CD4 (SK3), CD8 (SK1), CD25 (2A3), CD69 (CH4), CD71 (M-A712), and human leucocyte antigens-DR (HLA-DR) (B8.12.2) were purchased from Becton Dickinson (San Jose, CA, USA); mAb against FoxP3 (236A/E7) was purchased from eBiosciences (San Diego, CA, USA); mAb against CD14 (RMO52), purified anti-Fas (CH-11, inducing) as well as conjugated anti-CD95 (UB2) were obtained from Beckman Coulter (Fullerton, CA, USA); mAb against CTLA-4 (48815) were purchased from R&D (Minneapolis, MN, USA).

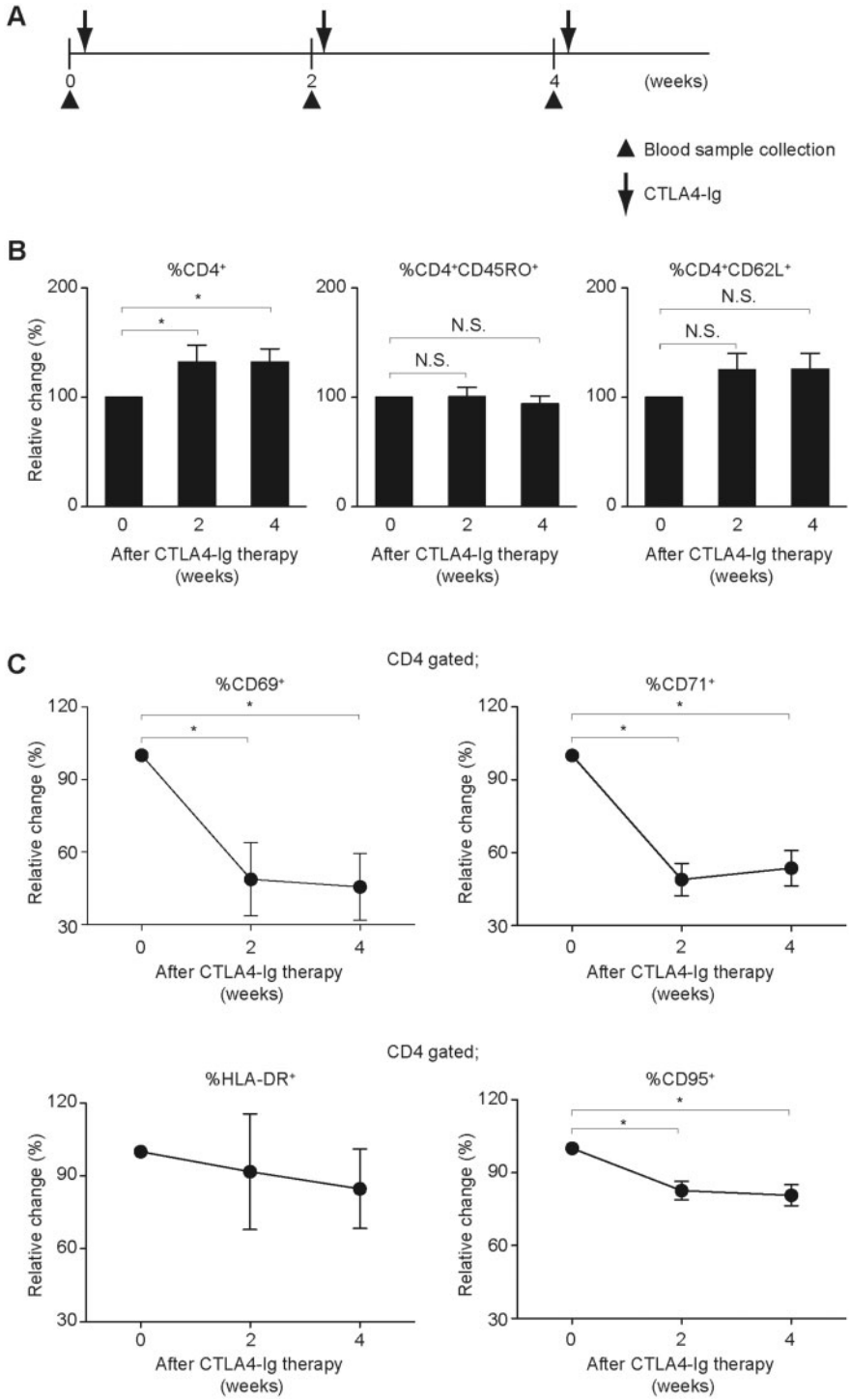
Phenotypic analyses

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation at 400 *g* of heparinized blood over LSM 1077 Lymphocyte Separation Medium (PAA laboratories, Pasching, Austria). PBMCs were resuspended in PBS/3% human Ig (Baxter International Inc., Vienna, Austria) in order to block Fc receptors and prevent non-specific antibody binding, incubated for 15 min at 4°C in the dark and stained with different combinations of FITC, PE, PE-Cy5, APC and PE-Cy7 and APC-Cy7-conjugated mAb and their appropriate isotype controls. Intracellular staining for FoxP3 was performed according to the instructions of the manufacturer (eBiosciences). The samples were analysed on a FACSCanto II (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) using FACSDiva software v.6.1.2 (BD Biosciences, San Jose, CA, USA) and FlowJo software v 7.1.2 (FlowJo/Tree Star, Ashland, OR, USA). All values obtained at week 0 were normalized to 100%. Values obtained after 2 and 4 weeks of CTLA-4Ig treatment were compared with values obtained before the start of CTLA-4Ig therapy (week 0) and are shown as change in relative percentage compared with the initial value.

In vitro activation of T cells

PBMCs from HCs were incubated with 0, 50 or 100 μg/ml CTLA-4Ig for 3 h in 24-well plates. Cells were then stimulated with 100 ng/ml anti-CD3 mAb (IOT3) overnight. The next day, cells were harvested and stained for CD4, CD25, CD14 and FoxP3. $CD4^+$ T cells and $CD4^+CD25^+FoxP3^+$ T cells were further analysed for the expression of CD69, CD71 and HLA-DR by flow cytometry.

FIG. 1 Increased proportions of CD4$^+$ T cells during CTLA-4Ig therapy



(**A**) Heparinized whole blood samples were taken before the patients received an i.v. infusion with CTLA-4Ig at week 0, 2 and 4. (**B**) T cells were analysed in RA patients (n = 15) by flow cytometry before and at different time point during CTLA-4Ig therapy. Proportions of CD4$^+$, CD4$^+$ CD62L$^+$ naïve and CD4$^+$ CD45RO$^+$ memory T cells were determined. The percentage of cells expressing the indicated marker molecules before the initiation of CTLA-4Ig treatment (week 0) was normalized to 100%. The relative change (mean % ± S.E.M.) is shown at week 2 and 4 after the initiation of CTLA-4Ig therapy. (**C**) CD4$^+$ T cells were analysed by flow cytometry for the expression of CD69, CD71, HLA-DR and CD95 surface marker molecules before and at different time points during CTLA-4Ig therapy. *$P < 0.05$, significant P value; N.S.: not significant. HLA: human leukocyte antigens.

Table 1 Baseline and patient characteristics

Baseline characteristic			
Age, mean, years	58		
Female, N	14		
Prednisolone, %	66		
Prednisolone, mean (S.D.), mg/dl	6 ± 6.5		
MTX, %	66		
Leflunomide, %	20		
Patient characteristic	Week 0	Week 2	Week 4
CRP, mean (S.D.) (range), mg/dl	0.95 (1.2) (0.02–4.5)	1.1 (1.5) (0.04–5.4)	0.7 (0.7) (0.02–2.3)
CDAI, mean (S.D.) (range)	25 (9.8) (8.6–40.4)	20.2 (7.8) (8–35.9)	20.2 (6.7) (9.1–35.4)
SDAI, mean (S.D.) (range)	24.4 (10.2) (9.1–40.8)	22.8 (7.3) (9.4–36.2)	22.5 (6.6) (13.4–35.8)
DAS28, mean (S.D.) (range)	4.1 (0.9) (2.5–5.6)	3.6 (0.6) (2.6–4.7)	3.7 (0.6) (2.7–4.8)

Demographic and clinical characteristics of RA patients are shown at baseline and during CTLA-4Ig treatment. CDAI: Clinical Disease Activity Index; N: number; SDAI: Simplified Disease Activity Index.

Analysis of apoptotic cells

PBMCs from HCs were stimulated with 100 ng/ml anti-CD3 mAb for 10 h and incubated with 0 μg/ml (medium), 10, 25 or 50 μg/ml CTLA-4Ig. After 10 h cells were washed twice and incubated with anti-Fas (CH-11) mAb for an additional 12 h. Cells were harvested, washed and stained for CD4 expression. Following fixation and permeabilization, according to the manufacturer's instructions, cells were stained for FoxP3. After incubation the proportion of cells undergoing programmed cell death was quantified by TdT-mediated dUTP nick/end labelling (TUNEL) using FLOWTACS, a cell death detection kit/FITC-dUTP from Roche (Mannheim, Germany).

T cell suppression assay

$CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells from RA patients were isolated before, and 2 and 4 weeks after the initiation of CTLA-4Ig therapy by FACS sorting on a BD FACSAria (BD Biosciences). Sort gates were set to obtain only $CD4^+CD25^+$ cells with the highest CD25 expression levels. Reanalyses of each single sorted cell population revealed purities of up to 98%. After cell isolation, $CD4^+CD25^-$ responder T cells were stimulated with soluble anti-CD3 mAb (100 ng/ml) in U-bottomed 96-well plates in the presence of $CD4^+CD25^+$ Treg cells (suppressor/responder cell ratios: 1:1) and irradiated PBMCs (6000 rad, ^{137}Cs source) in c-RPMI-1640/10 at 37°C in a humidified CO_2-containing atmosphere (5%). Proliferation of T cells was monitored by measuring 24 h cumulative methyl-[^{3}H]thymidine (Perkin Elmer, Boston, MA, USA) incorporation beginning at day 3 of culture. The proliferative response of $CD4^+CD25^-$ T cells in the absence of $CD4^+CD25^{high}$ T cells was normalized to 100 to calculate the percentage suppression resulting from the addition of $CD4^+CD25^{high}$ T cells at the defined ratio to the culture. Background proliferation was determined from cultures with $CD4^+CD25^-$ T cells and irradiated autologous PBMCs.

Statistical analysis

Values are shown throughout the manuscript as the mean ± standard error of the mean (S.E.M.) unless stated otherwise. Proportions of lymphocyte subpopulations were compared using Student's *t*-test for normally distributed populations. Relationships between separate groups of data were examined using Pearson's correlation coefficient or Spearman's rank correlation test, as appropriate. A P-value less than or equal to 0.05 was considered significant in all statistical tests. All statistical analyses were performed using Prism 4.0 (GraphPad Software, La Jolla, CA, USA) and SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Baseline demographics and clinical characteristics

Baseline demographics and clinical characteristics during the treatment of RA patients with CTLA-4Ig are shown in Table 1. After 2 and 4 weeks of treatment with CTLA-4Ig no significant changes in CRP, Simplified Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI) or DAS28 values were observed as compared with baseline values.

CTLA-4Ig treatment increases proportions of $CD4^+$ T cell and inhibits $CD4^+$ T cell activation in RA patients

In order to determine the effect of CTLA-4Ig treatment on T cells, we analysed PBMCs from RA patients for proportion of $CD4^+$ cells by flow cytometry. We normalized each patient's percentage of $CD4^+$ T cells as assessed before the initiation of CTLA-4Ig therapy to 100% and determined the relative change (mean % ± S.E.M.) in $CD4^+$ cells 2 and 4 weeks after the initiation of CTLA-4Ig therapy. We observed a significant increase in $CD4^+$ T cells 2 weeks (132 ± 15%; P = 0.04) and 4 weeks (132 ± 12%; P = 0.002) after the initiation of CTLA-4Ig therapy. No significant changes were observed for proportions of $CD4^+CD62L^+$ naïve and $CD4^+CD45RO^+$ memory T cells (Fig. 1B and supplementary Table S1, available at *Rheumatology* Online). We further analysed $CD4^+$ cells for the expression of activation-associated marker molecules. We found a significant reduction in $CD69^+$ and $CD71^+$ among $CD4^+$ T cells after 2 and 4 weeks of

CTLA-4Ig treatment. No significant differences were observed for proportions of HLA-DR$^+$ T cells. Interestingly, also the percentage of cells expressing the Fas receptor CD95, which is required for Fas-induced apoptosis, was significantly decreased after 2 and 4 weeks (Fig. 1C). No significant difference was observed for the proportions of CD8$^+$ cells 2 and 4 weeks after the initiation of CTLA-4Ig therapy. In contrast to CD4$^+$ T cells no difference was observed for the expression of CD69, CD71, HLA-DR and CD95 (supplementary Fig. S2, available at *Rheumatology* Online).

CTLA-4Ig treatment increases proportions of CD4$^+$CD25$^+$FoxP3$^+$ cells and inhibits activation of Treg cells in RA patients

For the detection of Treg cells, we stained PBMCs from RA patients who were treated with CTLA-4Ig for the expression of CD4, CD25 and FoxP3. We normalized the percentage of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells before the initiation of CTLA-4Ig therapy to 100% and determined the relative change (mean % ± S.E.M.) in CD4$^+$CD25$^+$FoxP3$^+$ cells 2 and 4 weeks after the initiation of CTLA-4Ig therapy. We observed a significant increase in proportions of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells 2 weeks (131.4 ± 14%; $P = 0.04$) and 4 weeks (134 ± 7.8%; $P = 0.001$) after the initiation of CTLA-4Ig treatment (Fig. 2A). Similar to the CD4$^+$ T cell population, we found that proportions of cells with the expression of activation-associated marker molecules were significantly decreased among Treg cells after 2 and 4 weeks of CTLA-4Ig treatment. Expression of Treg-associated marker molecules, such as CTLA-4, did not change after treatment with CTLA-4Ig (supplementary Fig. S3, available at *Rheumatology* Online). In addition the percentage of CD95$^+$ expressed on Treg cells was also significantly decreased 2 and 4 weeks after CTLA-4Ig treatment (Fig. 2B). Collectively, these data indicate that CTLA-4Ig affects numbers of CD4$^+$ T cells, including Treg cells. In addition, T cell activation was diminished after treatment with CTLA-4Ig.

CTLA-4Ig prevents the activation of CD4$^+$ T cells and Treg cells *in vitro*

In order to see whether CTLA-4Ig can also prevent the activation of T cells and Treg cells *in vitro*, we cultured PBMCs from HCs with different concentrations of CTLA-4Ig for 3 h and stimulated the cells afterwards with anti-CD3 mAb overnight. The percentages of cells expressing the indicated marker molecules, which were cultured in the absence of CTLA-4Ig before anti-CD3 stimulation, were normalized to 100%. We determined the relative change (mean % ± S.E.M.) for cells that were incubated with 50 μg/ml and with 100 μg/ml CTLA-4Ig. As depicted in Fig. 3A and supplementary Table S1, available at *Rheumatology* Online, we observed a significant and dose-dependent reduction in proportions of CD4$^+$CD69$^+$ and CD4$^+$CD71$^+$ cells upon incubation with 50 and 100 μg/ml CTLA-4Ig as compared with cells cultured in the absence of CTLA-4Ig. No significant difference was observed for proportions of HLA-DR$^+$ cells. Likewise among CD4$^+$CD25$^+$FoxP3$^+$ cells, we observed a significant and dose-dependent decrease in the percentage of CD69$^+$ cells after incubation with 50 and 100 μg/ml CTLA-4Ig. Proportions of CD71$^+$ cells were found to be decreased only after incubation with 100 μg/ml CTLA-4Ig. Again no significant differences were found for proportions of cells expressing HLA-DR (Fig. 3B). These data show that CTLA-4Ig also inhibits T cell activation *in vitro*.

CTLA-4Ig prevents CD95-mediated apoptosis of CD4$^+$ T cells and Treg cells *in vitro*

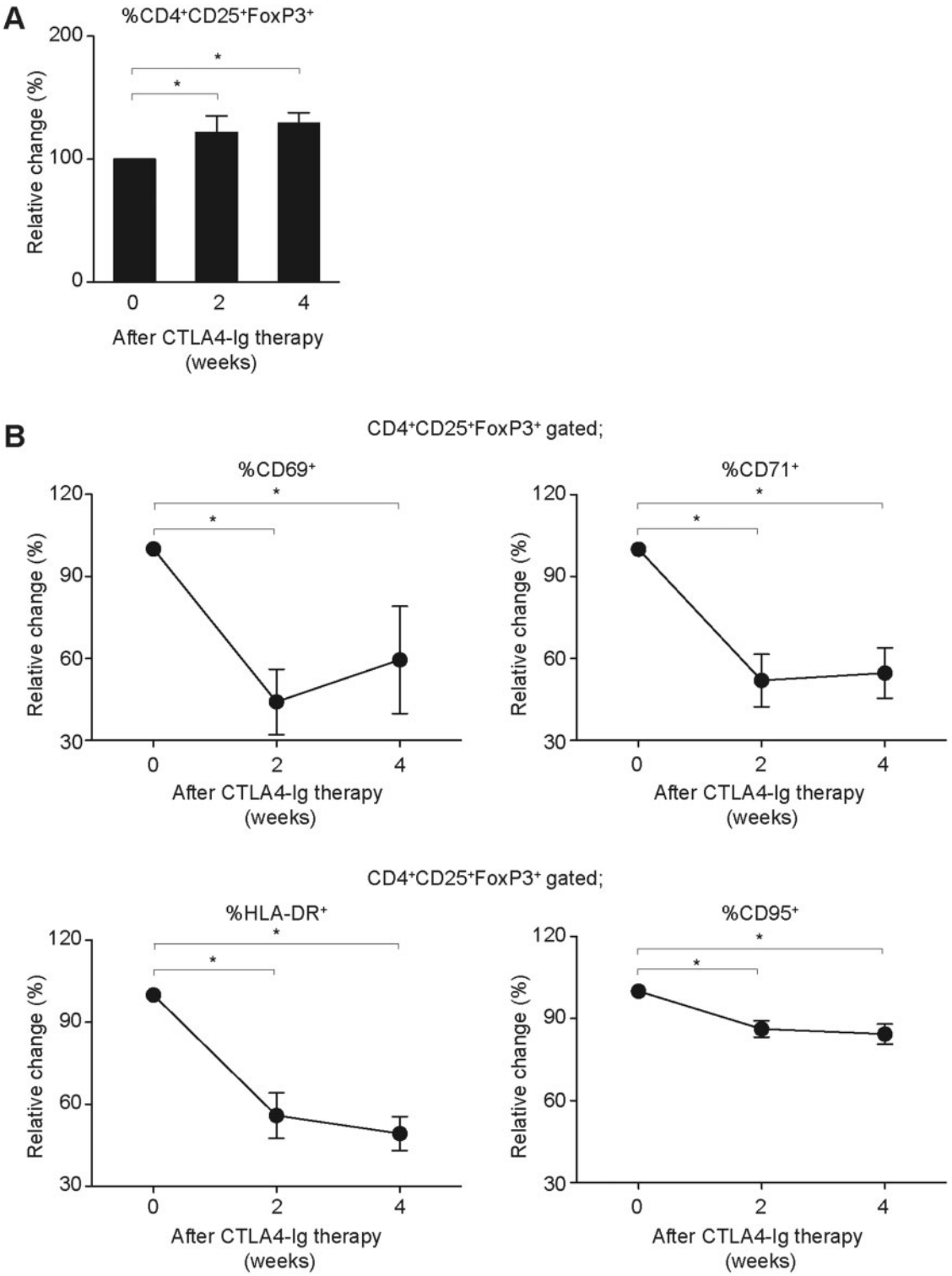
Treg cells can express the death receptor CD95 on the cell surface and are highly sensitive to apoptosis induced by anti-CD95 antibody *in vitro* [28]. In addition Treg cells are sensitive to CD95-mediated cell death *in vivo* in a non-disease setting [29] as well as in tumours and in the intestine during colitis [30–33]. The observed increase in proportions of CD4$^+$ cells and Treg cells, which was paralleled by a decrease in CD95 expressing cells, both *in vivo* and *in vitro*, suggested that CTLA-4Ig treatment might affect CD95-mediated apoptosis. In order to address this we analysed the effect of CTLA-4Ig on CD95-mediated cell death *in vitro*. We stimulated PBMCs from HCs with anti-CD3 mAb overnight. Afterwards we cultured the cells with different concentrations of CTLA-4Ig and induced apoptosis with anti-Fas antibody. DNA fragmentation was measured by TUNEL staining. Cells were stained for CD4 and FoxP3. CD4$^+$FoxP3$^+$ and CD4$^+$FoxP3$^-$ cells (Fig. 4A) were analysed for the expression of TUNEL. As shown in Fig. 4B, we observed a dose-dependent decrease in percentage apoptotic cells after CTLA-4Ig incubation. Already upon the addition of 10 μg/ml CTLA-4Ig we observed an over 50% reduction in TUNEL$^+$ cells among CD4$^+$FoxP3$^-$ T cells and CD4$^+$FoxP3$^+$ Treg cells (supplementary Table S2, available at *Rheumatology* Online). Within these data we could demonstrate that CTLA-4Ig can inhibit CD95-mediated cell death in CD4$^+$ T cells, including FoxP3$^+$ Treg cells *in vitro*.

CTLA-4Ig treatment leads to a diminished susceptibility of responder T cells for Treg cell-mediated suppression

Next we addressed the question of whether CTLA-4Ig treatment of RA patients affects the capacity of Treg cells to suppress T cell proliferation. For this purpose we isolated CD4$^+$CD25^{high} Treg cells from RA patients before and 2 and 4 weeks after the initiation of CTLA-4Ig therapy and analysed the cells for their capacity to suppress the proliferation of CD4$^+$CD25$^-$ responder T cells *in vitro*. To our surprise we observed a significantly ($P = 0.028$) diminished suppression of T cell proliferation with CD4$^+$CD25$^+$ Treg cells that were isolated from RA patients after 4 weeks of CTLA-4Ig treatment (Fig. 5A).

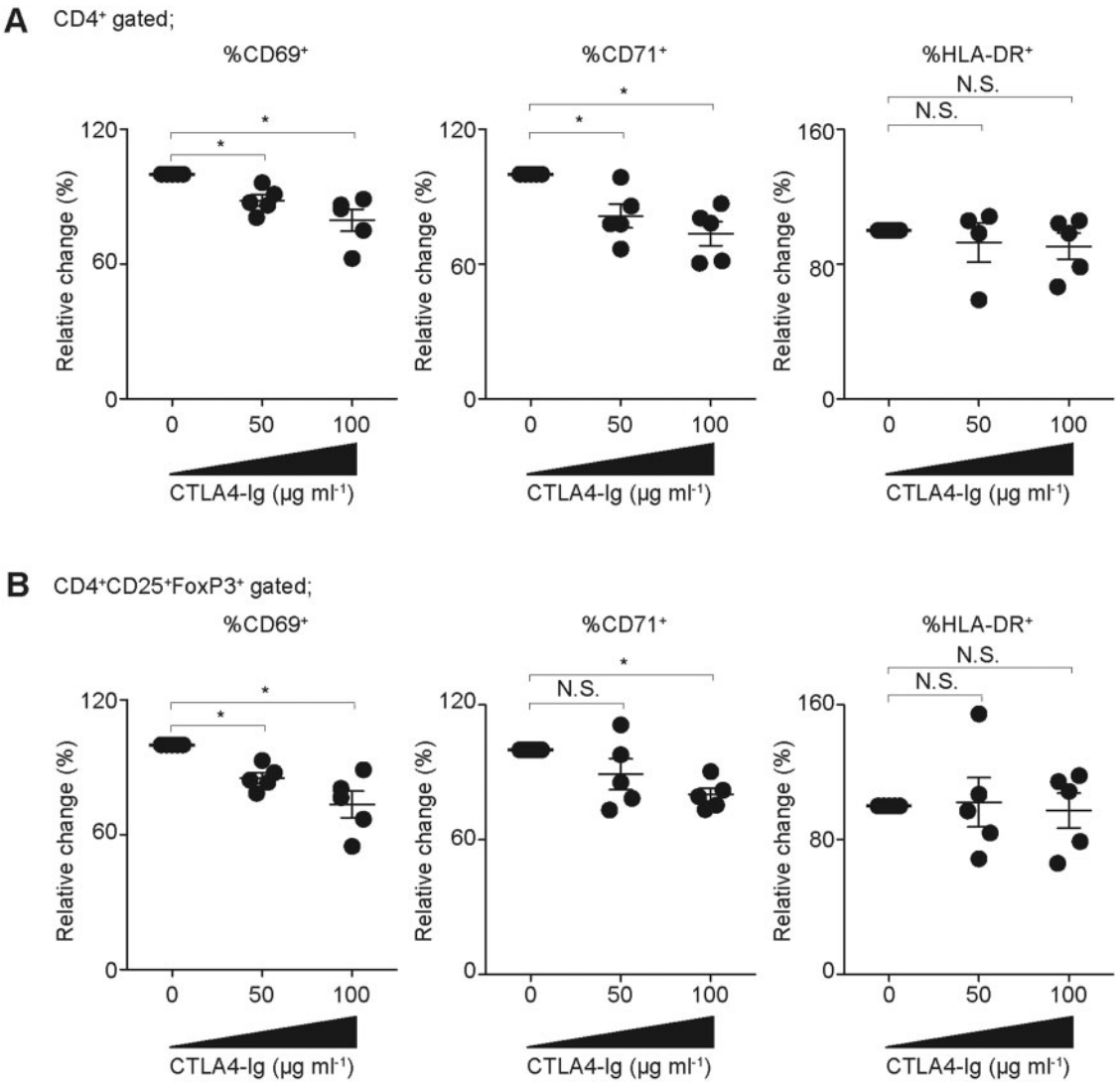
This observation, however, might be due to an effect of CTLA-4Ig on Treg cells, or alternatively on the CD4$^+$CD25$^-$ responder T cell population. To address this we isolated CD4$^+$CD25^{high} Treg cells and

FIG. 2 Increased proportions of Treg cells during CTLA-4Ig therapy



(**A**) Proportions of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells were determined in RA patients (n = 15) by flow cytometry before and at different time points during CTLA-4Ig therapy. The percentage of positive cells before the initiation of CTLA-4Ig treatment (week 0) was normalized to 100%. The relative change (mean % ± S.E.M.) in positive cells is shown at week 2 and at week 4 after the initiation of CTLA-4Ig therapy. (**B**) $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells were analysed for the expression of CD69, CD71, HLA-DR and CD95 surface marker molecules before and at different time points during CTLA-4Ig therapy.
*$P < 0.05$, significant P value; N.S.: not significant. HLA: human leukocyte antigens.

FIG. 3 CTLA-4Ig inhibits the activation of $CD4^+$ and $CD4^+CD25^+FoxP3^+$ T cells *in vitro*

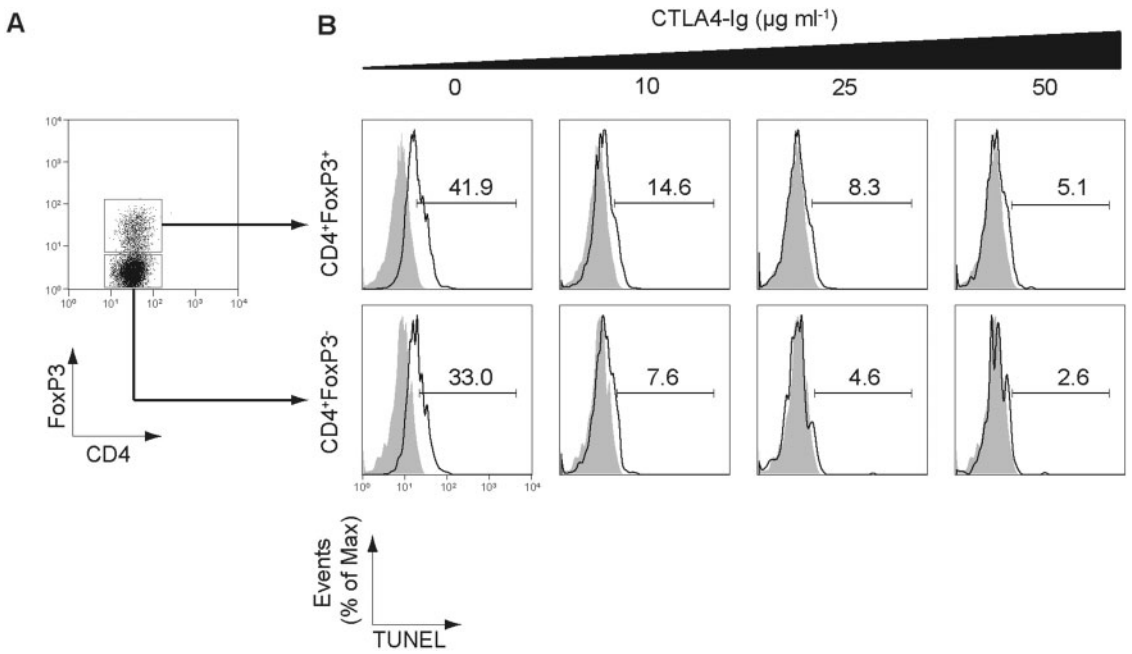


PBMCs from HCs (n = 5) were incubated without or with 50 or 100 μg/ml CTLA-4Ig for 3 h and afterwards stimulated with anti-CD3 overnight. (**A**) $CD4^+$ T cells and (**B**) $CD4^+CD25^+FoxP3^+$ Treg cells were analysed for the expression of CD69, CD71, HLA-DR and CD95 surface marker molecules. The percentages of cells expressing the indicated marker molecules, which were incubated without CTLA-4Ig before anti-CD3 stimulation, was normalized to 100%. The relative change (mean % ± S.E.M.) in positive cells is shown for cells that were incubated with 50 μg/ml and with 100 μg/ml CTLA-4Ig. *$P < 0.05$, significant P value; N.S.: not significant. HLA: human leukocyte antigens; PBMC: peripheral blood mononuclear cells.

$CD4^+CD25^-$ responder T cells from HCs and pre-incubated them separately with 100 μg/ml CTLA-4Ig overnight. Afterwards the cells were co-cultured in criss-cross experiments for the assessment of T cell suppression as described above (Fig. 5B). As depicted in Fig. 5C, only the pre-incubation of the $CD4^+CD25^-$ responder T cell population with CTLA-4Ig led to a significant reduction of T cell suppression ($P = 0.006$). On the other hand we detected no effect of CTLA-4Ig on the functional capacity of Treg cells. In summary this suggests that CTLA-4Ig exerts an effect on $CD4^+CD25^-$ responder T cells that results in a reduced susceptibility to Treg cell-mediated suppression.

Finally we addressed the question of whether the reduced susceptibility to Treg cell-mediated suppression can be attributed to the binding of CTLA-4Ig to C80/CD86 molecules and, therefore, blocking of CD80/CD86 signalling in $CD4^+CD25^-$ responder T cells. For this purpose we isolated $CD4^+CD25^{high}$ Treg cells and $CD4^+CD25^-$ responder T cells from HCs and pre-incubated the $CD4^+CD25^-$ responder T cell population with anti-CD80, anti-CD86, or a combination of anti-CD80 and anti-CD86

FIG. 4 CTLA-4Ig reduces CD95-mediated cell death in a dose dependent manner



PBMCs from HCs were stimulated with anti-CD3 for 12 h and afterwards without or with 10, 25 or 50 μg/ml CTLA-4Ig. Cells were incubated with anti-Fas mAbs for an additional 8 h. (**A**) $CD4^+$ $FoxP3^-$ T cells and $CD4^+$ $FoxP3^+$ Treg cells were gated and apoptotic DNA fragmentation was measured by TUNEL staining using the FLOWTACS kit. One representative dot plot out of five is shown. (**B**) Open bars show % of $TUNEL^+$ cells among $CD4^+$ $FoxP3^-$ T cells and $CD4^+$ $FoxP3^+$ Treg cells and filled bars show the appropriate isotype controls. One representative example out of five is shown. PBMC: peripheral blood mononuclear cells; TUNEL: TdT-mediated dUTP nick-end labelling.

mAbs or the appropriate negative control mAbs. The suppression of proliferation of $CD4^+$ $CD25^-$ responder T cells that were incubated with medium alone was 72.1 ± 5.4%. We observed a significantly reduced suppression upon pre-incubation with anti-CD86 mAbs (52.8 ± 5.6%; P = 0.005) and even more pronounced with anti-$CD80^+$ anti-CD86 mAbs (36.8 ± 6.8%; P = 0.006). The pre-incubation of $CD4^+$ $CD25^-$ responder T cells with mAbs against CD80 did not result in a significantly reduced suppression of T cell proliferation (64 ± 5.7%; P = 0.14) as shown in Fig. 5D. In summary these data suggest that the observed reduction in Treg cell-mediated T cell suppression upon CTLA-4Ig treatment is due to a diminished susceptibility of the responder $CD4^+$ $CD25^-$ T cell population based on the blocking of CD80/CD86 molecules.

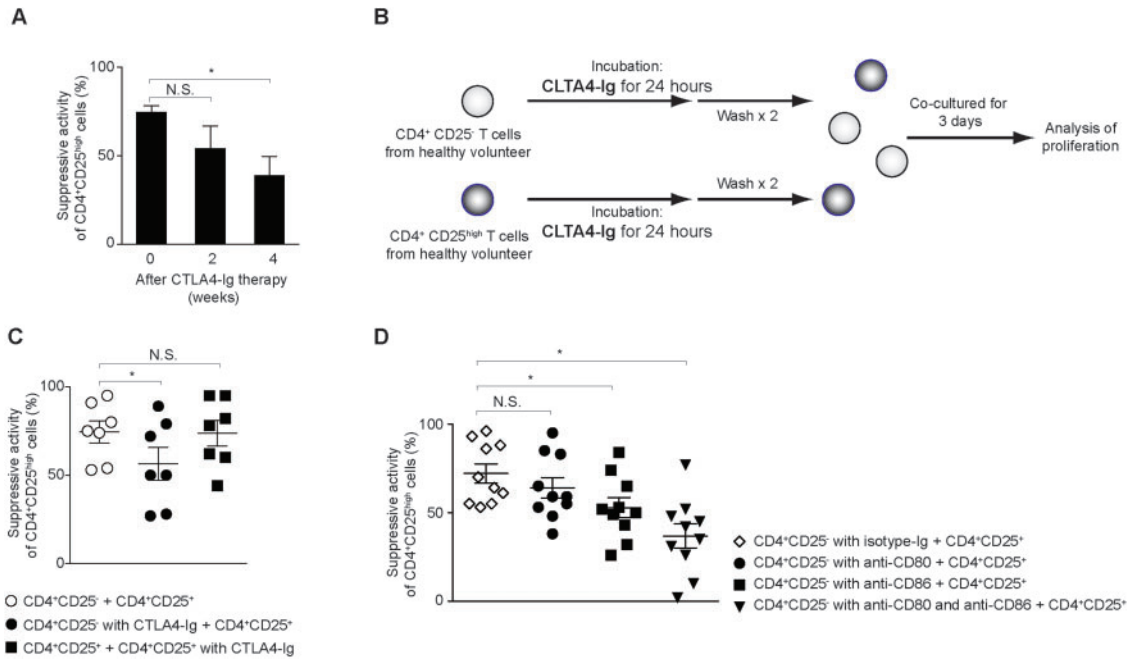
Discussion

In this study we show that CTLA-4Ig treatment of RA patients leads to increased proportions of $CD4^+$ T cells and Treg cells, which display phenotypic characteristics of diminished activation. Increased proportions of T cells, including Treg cells, came with a downregulation of CD95 and a reduction in CD95-mediated apoptosis. Functional analyses further suggested a diminished suppressive capacity of Treg cells *in vitro* at first sight. This, however, was found to be due to an effect of CTLA-4Ig on responder T cells leading to a diminished susceptibility for Treg cell-mediated suppression after blocking of CD80/CD86 molecules.

The role of CTLA-4Ig treatment on T cell subsets has been investigated in different studies [34–36]. The data within these studies for Treg cell frequency are conflicting, which is partly due to different time points when Treg cell frequencies were analysed and partly due to different patient cohorts. The purpose of this study was to address the mechanism of CTLA-4Ig therapy. In particular we were interested in the short term effect of CTLA-4Ig treatment on T cell subsets. We observed on average a 1.3-fold increase in proportions of $CD4^+$ T cells, and interestingly also in $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells. Under normal conditions, Treg cells specialize in the suppression of effector T cell proliferation and play an important role in the maintenance of peripheral immunological self-tolerance [37]. Therefore, quantitative and/or qualitative deficiencies of Treg cells have been suggested to be involved in the pathogenesis of a variety of autoimmune diseases. In regard to RA, however, conflicting data concerning Treg cell numbers as well as their functional capacity have been published so far [38].

Phenotypic analysis also revealed a downregulation of CD95 expression upon CTLA-4Ig treatment, which might result in increased cell numbers due to a diminished

FIG. 5 CTLA-4Ig affects Treg cell-mediated suppression through interaction with responder T cells



(**A**) $CD4^+ CD25^{high}$ and $CD4^+ CD25^-$ T cells were isolated by FACS from PBMCs from RA patients before and at different time points during CTLA-4Ig therapy. $CD4^+ CD25^{high}$ Treg cells were cocultured with $CD4^+ CD25^-$ T responder cells (ratio: 1:1) and stimulated with soluble anti-CD3 mAb and irradiated PBMCs. Proliferation of T cells was monitored by measuring methyl-[^{3}H]thymidine incorporation on day 4 of culture. A significant reduction was observed in the extent of T cell suppression after 2 and 4 weeks of CTLA-4Ig treatment. *$P < 0.05$, significant P value ($n = 7$). (**B**) $CD4^+ CD25^{high}$ Treg cells and $CD4^+ CD25^-$ responder T cells were isolated by FACS from HCs and were pre-incubated with 100 μg/ml CTLA-4Ig overnight before the start of the suppression assay ($n = 7$). (**C**) Pre-incubation of $CD4^+ CD25^-$ responder T cells but not of $CD4^+ CD25^{high}$ Treg cells led to a significant reduction in the extent of T cell suppression. *$P < 0.05$, significant P value. (**D**) $CD4^+ CD25^-$ T cells were isolated by FACS from HCs and were pre-incubated with anti-CD80, anti-CD86, or anti-CD80 + anti-CD86 Abs or the appropriate isotype-matched negative control Abs overnight. Pre-incubation of $CD4^+ CD25^-$ responder T cells with anti-CD86 mAbs and anti-CD80 + anti-CD86 mAbs led to a significant reduction of Treg cell-mediated T cell suppression. *$P < 0.05$, significant P value ($n = 10$); N.S.: not significant. PBMC: peripheral blood mononuclear cells.

apoptosis of T cells. In line with this it has been shown that CTLA-4 signalling inhibits expression of CD95 as well as CD95 ligand (L) on T cells and enhances the expression of the anti-apoptotic molecule Bcl-2. Thereby CTLA-4 promotes survival of antigen-specific T helper cells by maintaining the resistance of these cells against CD95/CD95L (FAS/FAS ligand)-induced apoptosis [39]. Likewise, at least in the murine system, Treg cells were found to be sensitive to CD95-mediated apoptosis [28].

As for the human system, however, the role of CTLA-4Ig on CD95-mediated apoptosis of T cells is unknown so far. We therefore analysed the influence of CTLA-4Ig on the Fas-induced apoptosis in $CD4^+$ T cells and Treg cells *in vitro*. We detected a dose-dependent decrease of apoptotic cells in the presence of CTLA-4Ig, suggesting that increased proportions of $CD4^+$ T cells and Treg cells after CTLA-4Ig treatment can be explained by the down-regulation of CD95 expression and a reduction in CD95-mediated cell death in T cells.

Data for the effects of CTLA-4Ig on Treg cell function are very limited and conflicting. While Alvarez-Quiroga *et al.* [34] described an enhanced suppressive capacity of Treg cells, isolated from the periphery after abatacept therapy, Pieper *et al.* could not detect an increased suppressive capacity of synovial Treg cells [35]. To our surprise, however, our experiments revealed a diminished suppression of T cell proliferation *in vitro*.

One explanation for this observation might be that CTLA-4Ig treatment directly targets Treg cells and blocks the interaction of CTLA-4 on Treg cells with B7 molecules on responder T cells. In line with this, the expression of CTLA-4 on Treg cells and binding to B7 molecules on other cells has been suggested to be required for normal Treg cell homeostasis and/or function [40, 41]. Alternatively the delivery of a suppressive signal by Treg cells has been shown to require the engagement of CD80 and CD86 on target T cells. A deficiency of CD86 and even more pronounced of CD80 was shown to result in

a diminished suppression of responder T cells, and B7-deficient cells were completely resistant to Treg cell suppression [14].

Our experiments revealed that the pre-incubation of only the responder T cell population but not of the Treg cell population with CTLA-4Ig caused a decrease of suppression, suggesting that CTLA-4Ig affects responder T cells but not Treg cells.

Furthermore blocking of anti-CD80/CD86 diminished the suppression of responder T cell proliferation to a comparable extent to that with CTLA-4Ig.

Although we observed a significant effect of CTLA4-Ig on $CD4^+$ T cells, including regulatory T cells, we did not see an improvement in clinical signs of arthritis. This observation is not surprising, considering that a clinical response should not be evaluated before 3 months of therapy.

In summary we were able to demonstrate for the first time that CTLA-4Ig in RA patients not only targets B7 molecules on APCs but also exerts effects via costimulatory molecules on T cells. Thereby CTLA-4Ig leads to reduced CD95-mediated apoptosis of T cells, including Treg cells, by downregulation of CD95. In addition binding of CTLA-4Ig on B7 molecules on T cells leads to a reduced susceptibility of T cells for Treg cell suppression. A direct binding of CTLA-4Ig on T cells with downstream effects has to be taken into account when treating patients with CTLA-4Ig.

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Supplementary data

Supplementary data are available at Rheumatology Online.

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