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Pregnancy induces numerical and functional changes of CD4+CD25^{high} regulatory T cells in patients with rheumatoid arthritis

F Förger, N Marcoli, S Gadola, B Möller, P M Villiger, M Østensen

[1] Department of Rheumatology and Clinical Immunology and Allergology, Inselspital, University of Bern, Bern, Switzerland

Correspondence to: F Förger, Department of Nephrology, Klinikum rechts der Isar, Technische Universität München, Ismaninger St. 22, 81675 Munich, Germany; Frauke.Foerger@lrz.tu-muenchen.de

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ABSTRACT

Objective: In a prospective study we investigated whether numerical and functional changes of CD4+CD25^{high} regulatory T cells (Treg) were associated with changes of disease activity observed during pregnancy and post partum in patients with rheumatoid arthritis (RA).

Methods: The frequency of CD4+CD25^{high} T cells was determined by flow cytometry in 12 patients with RA and 14 healthy women during and after pregnancy. Fluorescence-activated cell sorting (FACS) was used to sort CD4+CD25^{high} T cells and CD4+CD25− T cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies alone or in co-culture to investigate proliferation and cytokine secretion.

Results: Frequencies of CD4+CD25^{high} Treg were significantly higher in the third trimester compared to 8 weeks post partum in patients and controls. Numbers of CD4+CD25^{high} Treg inversely correlated with disease activity in the third trimester and post partum. In co-culture experiments significantly higher amounts of IL10 and lowered levels of tumour necrosis factor (TNF)α and interferon (IFN)γ were found in supernatants of the third trimester compared to postpartum samples. These findings were independent from health or disease in pregnancy, however postpartum TNFα and IFNγ levels were higher in patients with disease flares.

Conclusion: The amelioration of disease activity in the third trimester corresponded to the increased number of Treg that induced a pronounced anti-inflammatory cytokine milieu. The pregnancy related quantitative and qualitative changes of Treg suggest a beneficial effect of Treg on disease activity.

Several autoimmune rheumatic diseases show a modulation of disease activity during and after pregnancy. Rheumatoid arthritis (RA) improves in the majority of patients early in pregnancy.[1] An aggravation of disease symptoms after delivery is commonly seen and occurs in general within the first 6 months post partum.[2]

Pregnancy induces a state of maternal tolerance against the semiallogeneic fetus. Among the mechanisms that protect the fetus are regulatory CD4+CD25^{high} T cells (Treg), which suppress maternal immune responses directed against fetal alloantigens.[3] It has been shown that among CD4+ T cells expressing CD25, only those expressing high levels of CD25 display regulatory function.[4] Cell contact- and cytokine-based mechanisms have been proposed in Treg mediated suppression.[5] The expression of the transcription factor forkhead box P3 (FOXP3) is regarded a characteristic of Treg that controls their development and function.[6 7] Studies of decidua from early and late human pregnancy have shown an accumulation of CD4+CD25^{high} T cells at the maternal–fetal interface either by active recruitment or by local expansion.[8 9] In human peripheral blood CD4+CD25^{high} T cells constitute 5–10% of the CD4+ T cells.[10] Several investigators found an increase of circulating Treg in pregnancy, peaking during the second trimester and declining post partum.[9 11]

Deficiency in number or functions of Treg may lead to autoimmune diseases.[12] Depletion and reconstitution experiments in mice have shown that Treg can prevent or ameliorate autoimmune disease such as diabetes or collagen induced arthritis.[13 14] Reduced circulating levels of Treg have been found in several rheumatic diseases such as juvenile idiopathic arthritis (JIA) and systemic lupus erythaematosus (SLE).[15 16] In patients with RA, decreased levels have been described in the peripheral blood in active disease, whereas an increase of Treg has been found after successful therapy.[17 18] Elevated numbers of Treg are present in the synovial fluid of patients with RA with an increased suppressive capacity.[19 20] However, synovial CD4+CD25− effector T cells show decreased susceptibility to the regulatory effect of Treg and fail to respond with a Th2 type cytokine secretion in co-culture experiments.[21]

Neither the factors contributing to the spontaneous improvement of disease activity in pregnant patients with RA nor to the rebound seen after delivery have yet been fully elucidated. One concept is that the immunomodulation induced by pregnancy acts at a systemic level and modifies the disease activity of rheumatic disease.[22] In concordance with this concept, a study of pregnant patients with multiple sclerosis found a significant increase of CD4+CD25^{high} T cells during pregnancy in parallel with improvement of neurological symptoms.[23] In the present study we investigated whether numerical and functional changes of Treg were associated with the changes of disease activity observed during pregnancy and post partum in patients with RA. We found that the natural modulation of Treg during pregnancy inversely correlated with the disease activity of RA during and after pregnancy.

METHODS

Study population

The study had a longitudinal design, required informed consent and was approved by the ethical committee of the University of Berne. Included in

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the study were 12 pregnant patients with RA as well as 14 age-matched healthy pregnant women. To be eligible, patients with RA had to fulfil the American College of Rheumatology (ACR) criteria.[24] Medication allowed during pregnancy were non-steroidal anti-inflammatory drugs (NSAIDS) until week 32 of gestation, prednisone (<10 mg), antimalarials and sulfasalazine. Table 1 shows clinical characteristics and drug therapy of the included study population. Two patients with RA were excluded from the data analysis due to a prednisone dosage above 15 mg at postpartum flare.

In the group of patients analysed for numerical changes of Treg, nine were on low-dose prednisone, three on sulfasalazine and two on hydroxychloroquine. In patients analysed for functional changes of Treg, seven were on low-dose prednisone, one on sulfasalazine and one on hydroxychloroquine. The therapy remained unchanged throughout the period of investigation.

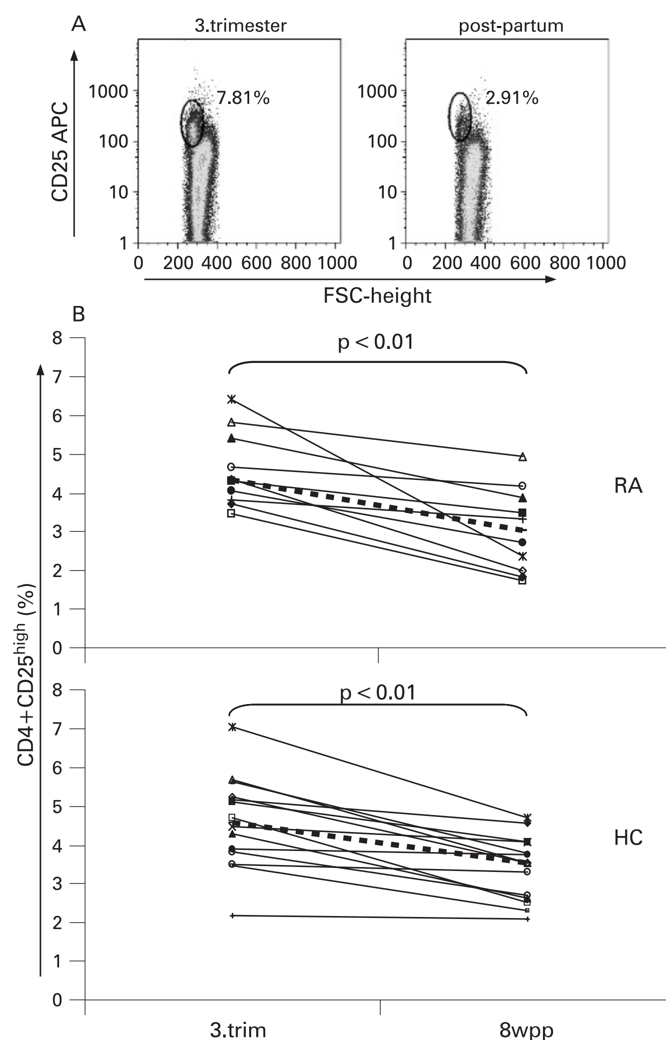

Figure 1 Increased numbers of CD4+CD25^{high} T cells in the third trimester. (A) Representative fluorescence-activated cell sorting (FACS) plot showing the percentage of CD4+CD25^{high} T cells during and after pregnancy (cells were gated on CD4+ T cells). (B) Freshly isolated peripheral blood mononuclear cells (PBMCs) from 10 patients with rheumatoid arthritis (RA) as well as from 14 healthy controls (HC) were analysed in the third trimester (3.trim) and 8 weeks post partum (8wpp) for the frequency of CD4+CD25^{high} T cells (expressed as percentage of CD4+ T cells). Solid lines represent each individual. Dotted lines show the medians. APC, allophycocyanin; FSC, forward scatter channel.

All patients and all healthy pregnant women had blood sampling at the third trimester and 8 weeks post partum. None of the pregnant women were close to labour at the time of sampling. Clinical evaluation of pregnant patients was performed using the RA Disease Activity Index (RADAI).[25] Serum C-reactive protein (CRP) levels were determined by a sensitive latex agglutination method (Sentinel Diagnostics, Milan, Italy; analytical range: 0.2–320 mg/litre) in patients with RA (20 sera) and healthy controls (24 sera) at the third trimester and 8 weeks post partum.

Cell isolation and sorting

Peripheral blood mononuclear cells (PBMC) were prepared by density gradient centrifugation over Ficoll-Hypaque gradient. Cells were stained with PE-anti-CD25 (clone 2A3) (BD Bioscience, San Jose, California, USA) and anti-PE magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD25+ cells were enriched using the Midi-MACS system (Miltenyi Biotec). CD25-enriched and CD25-depleted cell populations were stained with FITC-anti-CD4 (clone RPA-T4) (BD Bioscience) and sorted into CD4+CD25− and CD4+CD25^{high} T cells on a FACS Vantage SE (BD Bioscience). Cell analyses after sorting showed a purity of more than 98%.

Flow cytometric analysis

For the frequency analysis of CD4+CD25^{high} T cells, the following directly labelled monoclonal antibodies were used: FITC-conjugated anti-CD45RO (clone UCHL1), PE-conjugated anti-CD3 (clone HIT3a), PerCP-conjugated anti-CD4 (clone SK3) and allophycocyanin (APC)-conjugated anti-CD25 (clone 2A3). All antibodies were obtained from BD Bioscience Pharmingen. Immunofluorescence staining was performed after washing the cells twice with phosphate-buffered saline (PBS) containing 1% human serum. Cells were incubated for 20 min at 4°C with each monoclonal antibody (mAb) (10^5 cells/staining). To better identify CD4+CD25^{high} T cells, which tend to be slightly smaller in size compared to the CD4+CD25^{intermediate} T cells, the forward scatter channel (FSC) was used for a more precise quantification of this particular population. The number of CD4+CD25^{high} T cells was always expressed as percentage of CD4+ T cells.

Proliferation assay

To assess proliferation, 2.5×10^4 purified fluorescence-activated cell sorting (FACS)-sorted CD4+CD25− and CD4+CD25^{high} T cells were incubated alone or co-cultured at a 1:1 ratio in complete medium with 1 μg/ml plate bound anti-CD3 (OKT-3, eBioscience, San Diego, California, USA) and 5 μg/ml soluble anti-CD28 (BD Bioscience) in 96-well U-bottom plates. Where indicated cells were additionally stimulated with or without oestradiol at 3×10^4ng/ml. Cell culture medium contained RPMI 1640 with 1% sodium pyruvate, 1% non-essential amino acids, 1% glutamax, 1% kanamycin, 0.1% β-mercaptoethanol (Invitrogen, Basel, Switzerland) and 5% human pooled AB serum (Swiss Red Cross, Bern, Switzerland). After 6 days of culture, cells were labelled with 1.0 μCi [^{3}H] thymidine (Amersham Bioscience, Little Chalfont, UK) per well for the last 18 h. Proliferation was measured by liquid scintillation counting. The suppressive capacity of CD4+CD25^{high} T cells was calculated as 1−(cpm incorporated in the co-cultures/cpm of CD4+CD25− effector T cells cultured alone)×100.

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Table 1 Clinical characteristics and drug therapy of the included study population

	Rheumatoid arthritis (n = 12)	Healthy controls (n = 14)
Age (years), median (range)	34 (28–41)	34 (28–39)
Duration of disease (years), median (range)	5 (1–15)	–
Rheumatoid factor positive, n (%)	10 (83.3)	–
RADAI, median (range):		
Third trimester/8 wpp	0.9 (0–4.9)/3.8 (0–7.4)	–
Breast feeding, n (%)	10 (83.3)	12 (85.7)
Medication:†		
NSAID, n	4/3	0/0
Prednisone low dose*, n	9/9	0/0
Sulfasalazine, n	3/3	0/0
Hydroxychloroquine, n	2/2	0/0

*<10 mg prednisone. †Values are third trimester/8 weeks post partum.
NSAID, non-steroidal anti-inflammatory drug; RADAI, Rheumatoid Arthritis Disease Activity Index.

Multiplex cytokine assay

For the analysis of cytokine production, CD4+CD25− T cells were stimulated for 48 h either alone or at a 1:1 ratio with CD4+CD25^{high} T cells as mentioned previously. The Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, USA) was used to simultaneously detect the cytokines interleukin (IL)4, IL5, IL13, IL10, interferon (IFN)γ and tumour necrosis factor (TNF)α in each cell culture supernatant. The tests were run according to the manufacturer's instructions and data analysis was calculated using Bio-Plex Manager software (Bio-Rad). The TNFα suppression was calculated as 1−(TNFα secretion in the co-cultures/TNFα secretion in CD4+CD25− effector T cells cultured alone)×100.

Real-time quantitative PCR

Total RNA was extracted from freshly isolated CD4+CD25^{high} cells and CD4+CD25− T cells using the RNeasy Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. cDNA was prepared with random hexamers using Superscript II reverse transcriptase (Invitrogen, Paisley, UK). FOXP3 mRNA quantitative analysis was performed using TaqMan gene expression assays (Applied Biosystems, Foster City, California, USA). The following FOXP3 primer pairs were used: forward 5′-GAAACAGCACATTCCCAGAGTTC-3′ and reverse 5′-ATGGCCCAGCGGATGAG-3′. The amount of FOXP3 mRNA was normalised to the expression of 18S rRNA and calculated relative to the expression of FOXP3 in EBV-transfected B cells.

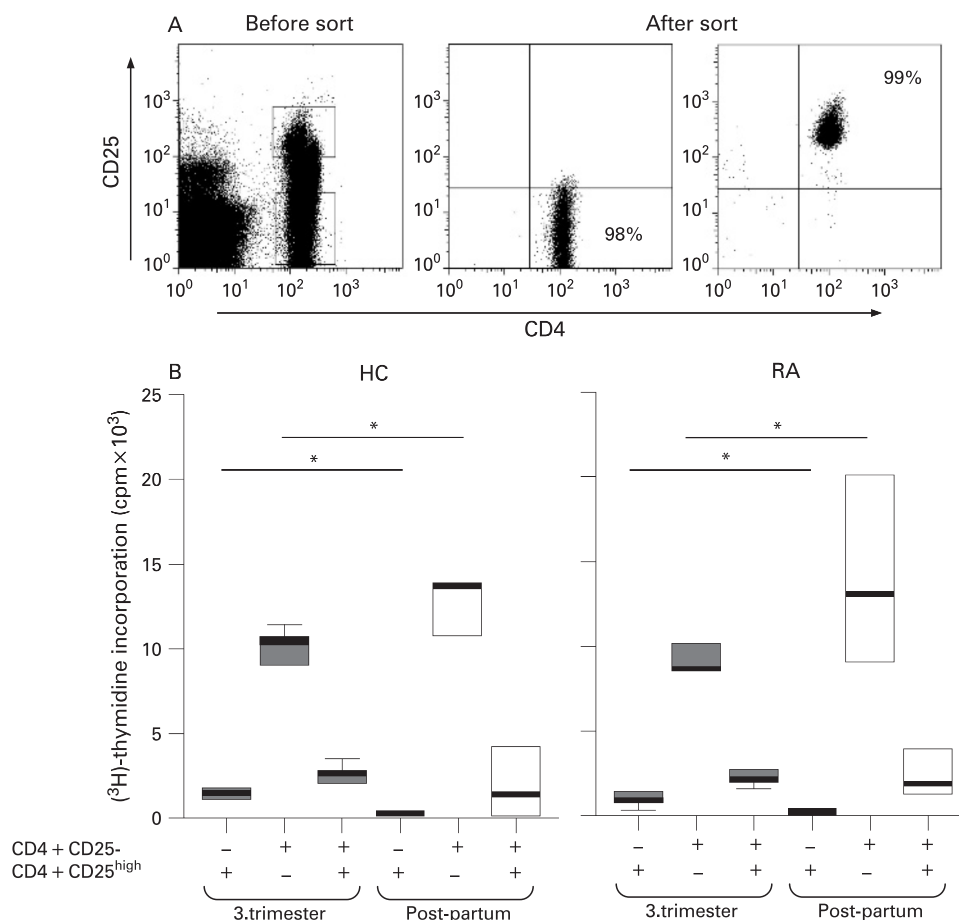

Figure 2 Though less anergic, CD4+CD25^{high} T cells of the third trimester are able to suppress proliferation of effector T cells. (A) Fluorescence-activated cell sorting (FACS) plots showing the expression of CD4 and CD25 as well as the purity of isolated CD4+CD25− and CD4+CD25^{high} T cells. (B) For the co-culture experiments, CD4+CD25^{high} T cells (2.5×10^4 cells) and CD4+CD25− (2.5×10^4 cells) were cultured either alone or at a 1:1 ratio and stimulated with 1 μg/ml plate-bound anti-CD3 and 5 μg/ml soluble anti-CD28. After 6 days, proliferation was determined by ^{3}[H]thymidine incorporation. The experiments were performed at the third trimester and 8 weeks post partum in five healthy controls (HC) and seven patients with rheumatoid arthritis (RA). Box plots represent the median (bold line) with 25th and 75th percentiles and whiskers 10th and 90th percentiles. *$p<0.05$.

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Statistical analysis

The frequency of CD4+CD25^{high} T cells of the study groups were compared by the unpaired Mann–Whitney U test. To analyse longitudinal changes, we performed the Wilcoxon test for paired samples. The linear regression analysis was calculated to describe the relationship between CD4+CD25^{high} T cells and sensitive CRP as well as the relationship between the suppression of TNFα in the co-culture and the RADAI scores. In case of skewed distributions of variables logarithmic transformation was applied to get an approximately normal distribution. All analyses were performed on a two-sided 5% level of significance.

RESULTS

Increased percentage of CD4+CD25^{high} T cells during pregnancy

As shown in fig 1, in 10 patients with RA as well as in 14 healthy controls (HC), the percentage of Treg was significantly higher in the third trimester (mean (SD): RA 4.6 (1)%; HC 4.6 (1)%) than 8 weeks post partum (mean (SD): RA 3.2 (1)%; HC 3.4 (1)%). Similar results were obtained when CD4+CD25^{high} T cells were based on absolute cell counts. Comparing the percentages of CD4+CD25^{high} T cells between patients and healthy subjects, no significant differences could be seen at any timepoint. These data show that pregnancy leads to a marked increase of Treg in healthy and diseased women. By contrast, the percentage of CD3+ T cells in both groups tended to be lower at the third trimester than 8 weeks post partum (mean (SD), third trimester: HC 23 (5)%, RA 25 (7)%; post partum: HC 29 (7)%, RA 30 (9)%).

Suppressive function of CD4+CD25^{high} T cells during and after pregnancy

Next, we investigated the capacity of Treg to suppress the proliferation of CD4+CD25− effector T cells during and after pregnancy in a co-culture setting. For this reason CD4+CD25^{high} T cells and CD4+CD25− T cells from seven RA and five healthy controls were sorted by FACS (fig 2A). CD4+CD25^{high} T cells and CD4+CD25− T cells were cultured either alone or in a 1:1 ratio. As shown in fig 2B, CD4+CD25^{high} T cells were able to suppress the proliferation of effector T cells effectively in patients and controls. The suppressive capacity did not differ between the third trimester (mean (SD) HC 73 (4)%, RA 74 (2)%) and the postpartum period (mean (SD), HC 87 (16)%, RA 86 (9)%). Remarkably, in both groups CD4+CD25^{high} T cells cultured alone displayed a significantly less anergic phenotype when isolated in the third trimester than 8 weeks post partum. By contrast, CD4+CD25− T cells of the third trimester showed a lower proliferation rate as compared to those of the postpartum timepoint.

These different proliferative behaviours of Treg and T effector cells could not be seen when CD4+CD25^{high} and CD4+CD25− T cells isolated from non-pregnant healthy women were additionally stimulated with oestradiol at the concentration of late pregnancy (data not shown).

CD4+CD25^{high} T cells were further analysed for the expression of the transcription factor FOXP3. As shown in fig 3, CD4+CD25^{high} T cells of both groups revealed higher amounts of FOXP3 mRNA at the third trimester than post partum. Due to the low number of samples analysed this tendency did not reach the level of significance.

Collectively, these findings indicate that FOXP3 expressing CD4+CD25^{high} T cells isolated in the third trimester reveal a less anergic phenotype and provide active suppression of proliferating CD4+CD25− effector T cells in patients and healthy controls

Cytokine profile of CD4+CD25^{high} cells and CD4+CD25^{high}/CD4+CD25− co-cultured cells

To address the question, whether gestational CD4+CD25^{high} T cells co-cultured with CD4+CD25− T cells induce a more tolerogenic milieu, cytokine secretion was analysed during and after pregnancy. As shown in fig 4, IL10 was preferentially detected in supernatants of CD4+CD25^{high} cells or CD4+CD25^{high}/CD4+CD25− co-cultured cells that were isolated at the third trimester. By contrast, only slight amounts of IL10 could be measured in the co-culture supernatants at 8 weeks post partum. The highest levels of IL10 were detected in the group of healthy pregnant women. Other Th2 cytokines such as IL4, IL5 and IL13 were hardly detectable in any of the supernatants of cultured or co-cultured cell populations (data not shown).

IFNγ and TNFα pro-inflammatory cytokines were preferentially produced by CD4+CD25− T effector cells. In both groups of women, the suppression of TNFα and IFNγ was significantly higher in the third trimester (mean (SD); TNFα: HC 83 (4)%, RA 78 (12)%; IFNγ: HC 81 (10)%; RA 77 (9)%) compared to the postpartum period (mean (SD); TNFα: HC 64 (8)%; RA 52 (12)%; IFNγ: HC 64 (5)%, RA 46 (13)%). However, patients with RA displayed a significantly less intense postpartum suppression of TNFα and IFNγ compared to healthy controls. In summary, these experiments clearly demonstrate that during pregnancy, CD4+CD25^{high} T cells induce a more pronounced anti-inflammatory cytokine profile compared to the postpartum timepoint.

CD4+CD25^{high} T cells and disease activity

Disease activity as measured by RADAI and sensitive CRP was lowest in the third trimester, but followed by postpartum flare

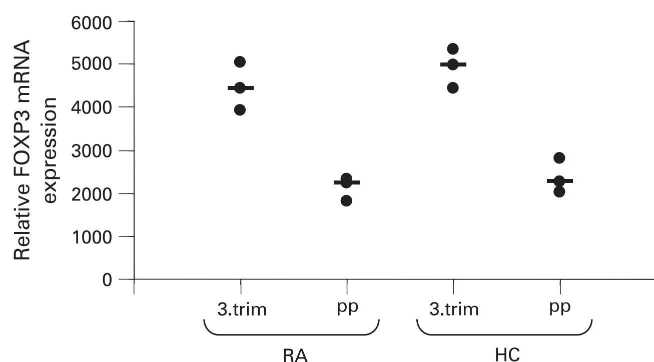

Figure 3 Transcription factor forkhead box P3 (FOXP3) expression of CD4+CD25^{high} T cells during and after pregnancy. Relative FOXP3 mRNA expression of sorted CD4+CD25^{high} cells analysed at the third trimester (3.trim) and post partum (pp) in three patients with rheumatoid arthritis (RA) and three healthy controls (HC). Median values are marked by horizontal lines.

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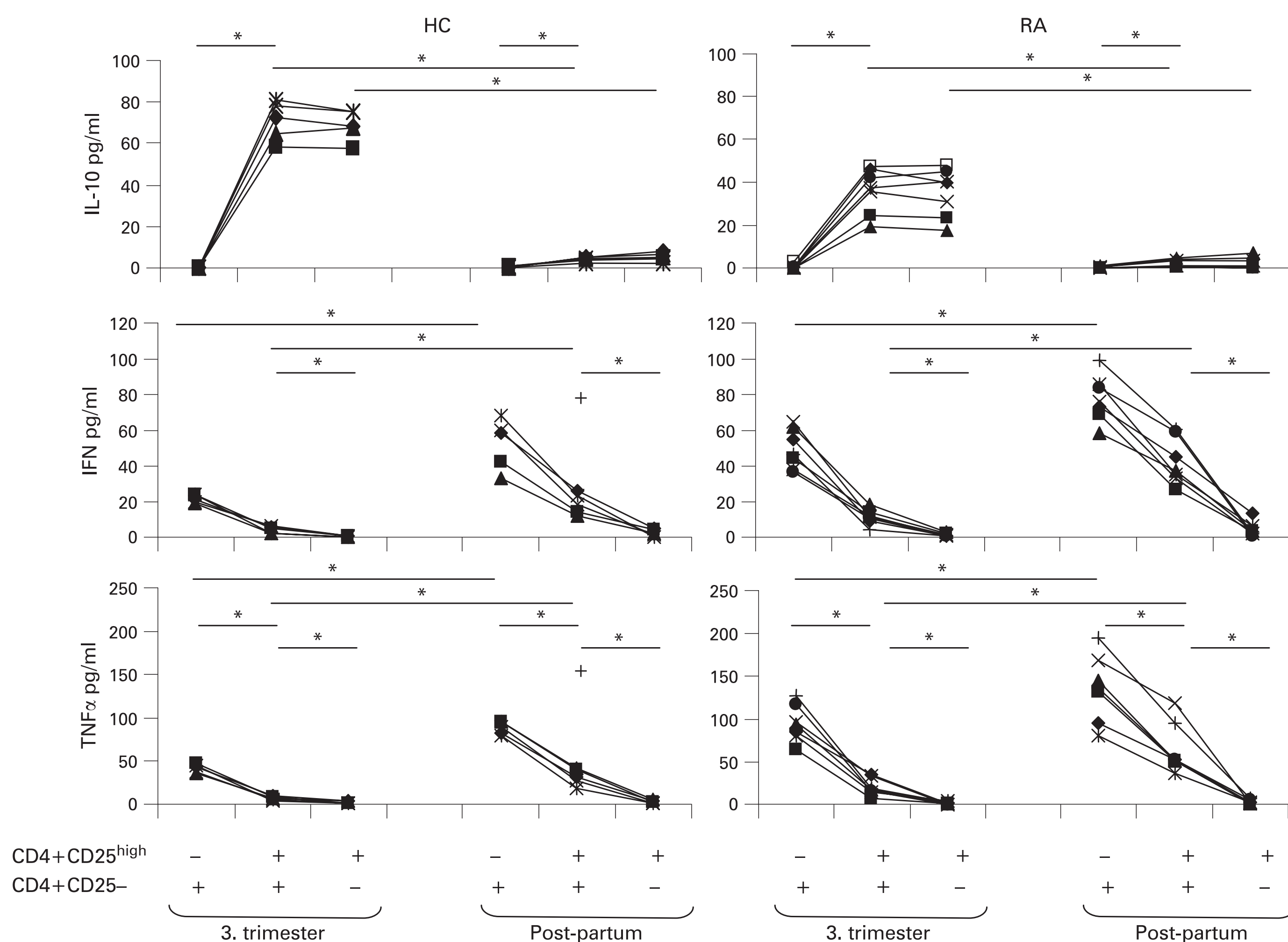

Figure 4 In the third trimester, CD4+CD25^{high} T cells induce a more pronounced anti-inflammatory cytokine profile compared to the postpartum period. Peripheral blood mononuclear cells (PBMCs) were collected from five healthy controls (HC) and seven patients with rheumatoid arthritis (RA) at the third trimester and 8 weeks post partum. CD4+CD25^{high} T cells and CD4+CD25− T responder cells were isolated by fluorescence-activated cell sorting (FACS) and cultured alone (2.5×10^4 cells) or at a 1:1 ratio (2.5×10^4 cells of each cell type) and stimulated with 1 μg/ml plate-bound anti-CD3 and 5 μg/ml soluble anti-CD28 for 48 h. Cytokines from the supernatants were measured by the Bio-Plex suspension array system. *$p<0.05$ (comparison within each group); +$p<0.05$ (comparison between patients with HC and RA). IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

in eight out of 10 patients with RA. In the investigated time period, one patient with RA remained clinically inactive, a second had persistent active disease. The relationship between disease activity during and after pregnancy and number as well as function of Treg was analysed by linear regression analysis. A significant association of the sensitive CRP with the logarithmically transformed percentages of Treg could be shown in patients with RA only, but not in healthy controls (fig 5A). Therefore, an overall effect of pregnancy seemed unlikely. In addition, improved gestational RADAI scores were significantly associated with the increased suppression of TNFα (fig 5B). Thus, a relationship between improved disease activity and pregnancy related modified number and function of CD4+CD25^{high} T cells could be shown.

DISCUSSION

This is the first study investigating the role of regulatory CD4+CD25^{high} T cells in pregnant patients with RA. Pregnancy served as a model of natural immune modulation in patients, who were receiving no aggressive immunosuppressive therapy. Our results demonstrate that pregnancy induces a change in the number and function of Treg, with a reversal of these changes after delivery. The CD4+CD25^{high} T cell population that we studied exhibited the typical properties of human Treg including high expression of CD25 and of FOXP3, as well as the suppression of the proliferation and the pro-inflammatory cytokine secretion of CD4+CD25− effector T cells.

Our findings demonstrate that circulating Treg were significantly increased during pregnancy compared to the postpartum period in patients and controls. In a previous study, a gestational rise of CD4+CD25+ T cells could be found in a cohort of healthy pregnant women, however, no further analysis was performed on the function of the CD4+CD25^{high} T cell subset.[11] In our patient cohort, the percentage of CD4+CD25^{high} T cells was highest in the third trimester at a time when disease activity was lowest in most patients with RA. Furthermore, the drop of Treg after delivery coincided with an aggravation of the disease. The inverse correlation of circulating CD4+CD25^{high} T cells and CRP levels found in our patients as well as in a previous study investigating non-pregnant patients with RA supports the association between disease activity and the number of Treg.[26] Another study disclosed that high numbers of peripheral blood CD4+CD25^{high} T cells correlated with a benign disease course in patients with

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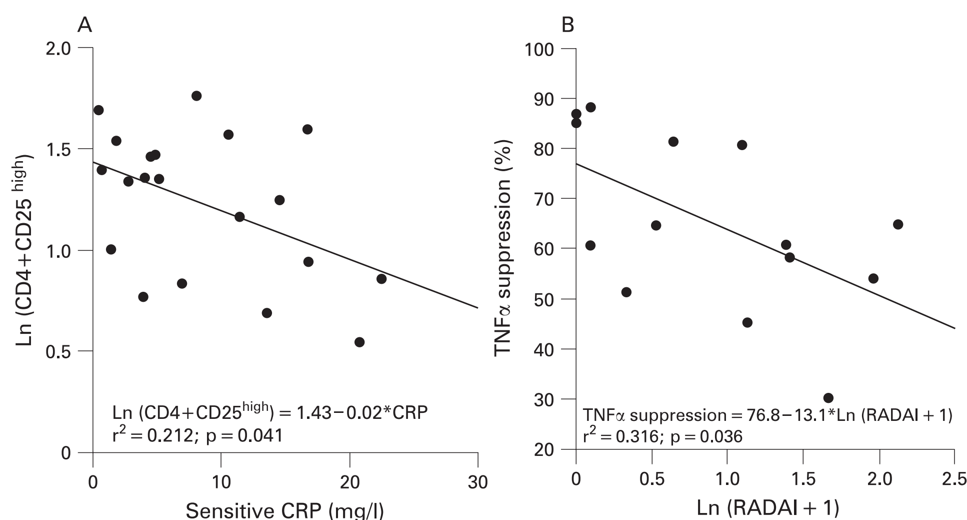

Figure 5 Pregnancy related improved disease activity is inversely associated with increased number and enhanced anti-inflammatory milieu of CD4+CD25^{high} T cells. (A) Scatter plot showing a significant correlation between the C-reactive protein (CRP) and the logarithmically transformed percentages of CD4+CD25^{high} T cells in 10 patients with rheumatoid arthritis (RA) analysed at the third trimester and 8 weeks post partum. (B) Scatter plot showing a significant correlation between the relative tumour necrosis factor (TNF)α suppression by CD4+CD25^{high} T cells and the logarithmically transformed Rheumatoid Arthritis Disease Activity Index (RADAI) scores of seven patients with RA analysed at the third trimester and 8 weeks post partum.

oligoarticular JIA.[15] Conversely, decreased levels of Treg were found in patients with early active RA prior to treatment with steroid or disease modifying drugs.[27] Thus, increased levels of CD4+CD25^{high} T cells may be beneficial in chronic inflammatory diseases.

In our study, CD4+CD25^{high} T cells from the same donor stimulated with anti-CD3/anti-CD28 were more ready to proliferate when isolated in the third trimester compared to cells isolated post partum. Different factors have been identified in the natural expansion of Treg during pregnancy. One is the elevated oestradiol level, that was previously shown to promote the proliferation of male CD4+CD25+ T cells in response to CD3/CD28 engagement.[28] However, we could not find a proliferative effect of oestradiol when added to anti-CD3/anti-CD28 stimulated CD4+CD25^{high} T cells of non-pregnant women. These discrepant findings could be due to the different purity of Treg. Since the CD4+CD25+ T cells contain the CD4+CD25^{high} Treg cells and the CD4+CD25^{intermediate} cells, the proliferative effect of oestradiol could result from a stimulation of IL2 producing CD4+CD25^{intermediate} T cells. Another factor that could account for the expansion of Treg during pregnancy are circulating fetal alloantigens.[29] In this context, the observation of a positive correlation of high levels of circulating fetal DNA with disease improvement in pregnant patients with rheumatoid arthritis is of interest.[30]

Recent studies investigating Treg in active non-pregnant patients with RA suggested an impaired function of CD4+CD25^{high} T cells as demonstrated by their inability to suppress the proliferation and the production of pro-inflammatory cytokines of autologous CD4+CD25− T cells.[26 31] Accordingly, in our study, Treg isolated from patients with RA with more active disease showed an impaired capacity to suppress pro-inflammatory cytokines, whereas the ability to suppress proliferation was unaltered.

Interestingly, in the third trimester, higher levels of IL10 were measured in the monoculture supernatants of CD4+CD25^{high} T cells or in the co-culture supernatants of CD4+CD25^{high} and CD4+CD25− T cells, yet almost undetectable levels were found post partum. This effect was seen irrespective of health status. IL10 producing Treg are known to suppress autoimmune disease and mediate tolerance towards alloantigens.[32 33] Moreover, Treg isolated at the third trimester showed an increased capacity to suppress Th1 cytokines. This finding correlated with improved disease activity in our cohort of pregnant patients with RA, who did not receive any aggressive immunosuppressive therapy. This pregnancy related effect was similar to what was seen in patients with RA after anti-TNFα treatment.[26 31] We therefore suggest that by enhancing an anti-inflammatory cytokine pattern during pregnancy, CD4+CD25^{high} T cells may contribute to the low disease activity seen in patients with RA at the third trimester.

In conclusion, pregnancy is a state of natural immunomodulation that induces tolerance towards the semiallogeneic fetus. In this context, pregnancy gives rise to a population of inducible FOXP3 expressing CD4+CD25^{high} Treg that secrete IL10 and provide an enhanced suppression of pro-inflammatory cytokines produced by effector T cells. As an epiphenomenon, this pronounced tolerogenic microenvironment of pregnancy induce Treg that may partly revert a Th1-biased immune response and thus contribute to the amelioration of disease signs and symptoms in chronic inflammatory diseases.

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Competing interests: None.

Ethics approval: Informed consent was sought and the study was approved by the ethics committee of the University of Bern.

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