Frequency of Regulatory T Cells is Not Affected by Transient B Cell Depletion Using Anti-CD20 Antibodies in Rheumatoid Arthritis

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Abstract: *Objectives*: Transient B cell depletion with the monoclonal anti-CD20 antibody rituximab has shown favourable clinical responses in patients with rheumatoid arthritis (RA). Recently a characteristic regeneration pattern of B cell subpopulations has been reported. However, little is known about the impact of B-cell depletion on peripheral T cells in particular regulatory T cells.

Materials and Methodology: 17 patients with RA having failed anti-TNF were treated with rituximab. Four colour staining was performed using CD19, CD3, CD4, CD8, CD16, CD56, CD25, HLA-DR, HLA-G and intracellular Foxp3 at five time points spanning up to 12 months after rituximab. In addition, quantification of the soluble form of the HLA class I molecule HLA-G by ELISA has been performed.

Results: Peripheral B cell depletion lasted 6 to 9 months. The absolute number of CD3+, CD4+ and CD8+ lymphocytes showed no significant changes up to 1 year after B-cell depletion compared to before therapy. Only the relative frequency for CD3 and CD4 showed a significant increase ($p < 0.05$). In particular, CD4+CD25++ and Foxp3 positive regulatory T cells remained constant. The percentage of HLA-G positive cells in the CD4+ or CD8+ population did not change significantly either. The amount of sHLA-G remained without significant changes.

Conclusion: Absolute T cell counts showed no significant changes after rituximab compared to the time point before therapy. In particular, the frequency of regulatory T cells with a CD4+CD25++ phenotype as well as positive Foxp3 expression were numerically stable. Additionally, HLA-G positive regulatory T cells and soluble levels of HLA-G showed no significant changes.

INTRODUCTION

Rheumatoid arthritis (RA) represents a chronic inflammatory disease leading to progressive cartilage and joint destruction. RA is treated with DMARDs (disease modifying anti-rheumatic drugs) alone or in combination with glucocorticoids and/or so called biologicals, e.g. TNFalpha-antagonists. The introduction of TNFalpha blockers has revolutionized treatment of RA. Nevertheless, up to one third of by this means treated patients does not respond adequately [1]. Therefore there is still need for other treatment strategies like rituximab, a B-cell depleting anit-CD20 monoclonal antibody [2-4]. Within the last years growing evidence has emerged underlining the pathogenetical role of B lymphocytes in RA [5-9]. In several clinical trials B cell depletion with rituximab has been shown to be effective in treatment of RA and well tolerated by patients. Peripheral B cell depletion lasts usually 6-9 months. Recently a characteristic regeneration pattern of B cell subpopulations with a long lasting modulation of B cell subset composition has been reported [10, 11].

Regulatory T (Treg) cells represent a distinct subset of lymphocytes. They are attributed to have a key function in limiting immune responses against infectious agents and in avoiding pathologic autoimmunity. Defects in Treg function are discussed to play an important role in the pathophysiology of autoimmune diseases such as rheumatoid arthritis (RA) [12, 13].

There are still different concepts in defining Treg [14-17]. The best described Treg population is thought to be CD4+CD25++. In addition to this, Tregs have been defined by the expression of CD4 and the transcription factor Foxp3. Foxp3 seems to be characteristically expressed by Tregs and plays an important role in development of Tregs. Recently, a new subset of CD4 and CD8 positive T cells has been reported, characterized by the constitutive expression of the immunotolerogenic molecule HLA-G [18]. Besides the membrane-bound isoforms, HLA-G can be secreted and is found at detectable levels in the peripheral blood. Both, membrane bound and soluble HLA-G levels have been linked to the pathogenesis of autoimmune diseases and previous data suggest a positive correlation between soluble HLA-G (sHLA-G) and disease activity in rheumatoid arthritis patients [19].

The role of Treg in RA is still not exactly defined [20-22]. Tregs from synovial fluid showed increased expression

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of activation markers like CTLA-4 (both surface and intracellular), GITR and OX40, as well as Foxp3 transcripts [23].

B cells have multiple effects on the T cell compartment. They directly interact with T cells during antigen presentation, produce cytokines and have specific functions for the organization of tertiary lymphoid structures like germinal center formation [24]. However, very little is known about the impact of B-cell depletion on peripheral T cell subpopulations. Particularly regulatory T cells are important candidates which may be indirectly influenced by rituximab treatment. In this study, four colour staining was performed using CD19, CD27, CD3, CD4, CD8, CD16, CD56, CD25, HLA-DR, HLA-G and intracellular Foxp3 to study the effects of B cell depletion mediated by rituximab on different subsets of T cells with particular interest in regulatory T cells. In addition, quantification of sHLA-G in sera of five patients has been performed.

MATERIALS AND METHODOLOGY

Patient samples, patient characteristics, and study design. Peripheral blood samples were obtained from 17 patients with RA, at the indicated time points whose B cell regeneration pattern has been published recently [11]. For immunfluorescence staining of Foxp3 peripheral blood samples were obtained from seven patients out of 17. Furthermore, in these seven patients HLA-G staining was performed and sHLA-G was determined in sera of five out of these seven patients. All patients met the American College of Rheumatology revised criteria for the classification of RA. In all patients, RA was refractory to standard treatment with disease-modifying antirheumatic drugs, including methotrexate (MTX) and/or tumor necrosis factor alpha antagonists (etanercept, adalimumab or infliximab). Informed consent was obtained from all patients before entering the study, in accordance with the protocol approved by the ethics committee of the University of Wuerzburg. More than 70% (12 of 17) of patients continued to receive concomitant MTX. Rituximab was administered as follows: patients 1-6 received 4 weekly infusions of rituximab at a dose of 375 mg/m2 (range 2.4-3.6 gm); patients 7-17 received 2 infusions of 1,000 mg rituximab, 2 weeks apart. Table **1** summarizes the characteristics of the patients. Peripheral blood was collected from the patients at the time points before B cell depletion and 1-3, 4-6, 7-9 and 10-12 months after rituximab, respectively.

Cell preparation: Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Paque Plus separation (Pharmacia Biotech, Freiburg, Germany).

Monoclonal antibodies: For immunofluorescence staining, the following cell surface markers were used: CD19 (phycoerythrin [PE]), CD27 (phycoerythrin [PE]), CD3 (peridinin chlorophyll protein [PerCP]), CD4 (SK3, [PerCP]), CD8 (SK1, [APC]) from Becton Dickinson (Heidelberg, Germany); CD4 (fluorescein isothiocyanate [FITC]), CD8 (fluorescein isothiocyanate, [FITC]), HLA-DR (phycoerythrin [PE]) from Beckmann & Coulter; CD25 (allophycocyanin [APC]), CD56 ([PE]), CD16 ([PE]) from Becton Dickinson; anti-human Foxp3 ([PE]) from eBioscience (San Diego, USA); anti-human HLA-G (MEM-G/9, [PE], Exbio, Praha, Czech Republic), rat IgG2a (phycoerythrin [PE]) from ACRIS, rat IgG1 ([PE]) and mouse IgG1 ([PE], [PerCP] and [APC]) from Becton Dickinson.

Flow cytometric analysis: Immunofluorescence staining for flow cytometric analysis was performed by incubating PBMCs in phosphate buffered saline (PBS) with 10 μl of monoclonal antibodies for 20 minutes on ice. In each tube, 1×10^6 cells were suspended. The cells were then washed in PBS. Immunfluorescence staining for Foxp3 was performed using a commercially available kit from eBioscience (San Diego, USA). Four-color staining was performed and followed by analysis using the FACSCalibur system (Becton Dickinson, San Jose, CA). T and B cells were identified by forward versus side scatter (FSC/SSC) gating on viable lymphocytes in combination with gating on CD19+ and CD3+ cells, respectively. A total of minimum 5,000 events were collected for each analysis. The frequency of cell populations was calculated using CellQuest software (Becton Dickinson). The total numbers of cells were calculated per millilitre of blood, based on the frequencies of these cells among lymphocytes and the white blood cell count.

Table 1. Characteristics of the Patients

Characteristic	Baseline	6 months	12 months
Age, mean (range) years	48 (28-77)		
Female sex, %	88		
Disease duration, mean (range) years	11.4 (1-28)		
Receiving MTX, %	70.6		
RF positivity, %	88		
DAS28 score, mean (range)	6.1 (3.6-7.8)	4.1 (3.0-6.9)	4.9 (2.4-7.7)
RF, mean (range) IU/ml	292.7 (17-1,180)	110.5 (12-341)	155.8 (12-617)
ESR, mean (range) mm/hour	28 (12-38)	15 (7-33)	21 (8-46)
CRP, mean (range) mg/dl	3.93 (0.6-10.4)	0.79 (0.03-2.24)	1.6 (0.09-5.9)
	Before therapy	6-8 months	12-16 months
CD19+ B cells/μl, mean (range)	77 (43-335)	16 (0-28)	92 (11-132)

MTX = methotrexate; RF = rheumatoid factor; DAS28 = Disease Activity Score in 28 joints; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; CD19+ B cells/μl in peripheral blood; n=17.

For the analysis of HLA-G positive T cells gates were set on lymphocytes according to their characteristic properties in the FSC/SSC. Additional gates were set on FSC/CD4 or FSC/CD8 dot plots to identify the desired subsets. HLA-G expression was assessed in comparison to the respective isotype control staining. Due to the low frequency of HLA-G+ T cells at least 75.000 events in the lymphocyte gate were collected for each analysis.

Soluble HLA-G: To quantify soluble HLA-G (sHLA-G) in serum we used a commercially available ELISA Kit (Exbio, Praha, Czech Republic) with a lower limit of detection of 0.5 to 1 ng/millilitre according to the manufacturer's protocol. Serum samples were diluted 1 : 3 in PBS and ELISA was performed according to the manufacturer's instructions. All samples were run in duplicates. Cell culture supernatant of HLA-G5 transfected HeLA cells (kindly provided by E. Weiss, Munich) was used as an internal standard.

Statistical analysis: Statistical analysis was performed using an unpaired 2-tailed t-test with bonferroni correction. Values are expressed as the mean ± SEM and were calculated using Excel software and SPSS - software (Statistical Product and Service Solutions; SPSS Inc., Chicago, USA). P values less than 0.05 were considered significant.

RESULTS

B-Cell Compartment

After treatment with rituximab all patients showed a depletion of CD19+ B lymphocytes (to < 0.1%) in peripheral blood. Peripheral B cell depletion lasted 6 to 9 months after Rituximab (mean ± SEM 7.6 ± 0,6 months) [11]. The absolute numbers of CD19+ B lymphocytes returned to baseline levels 12 months after therapy (Table **1**). Only 1 patient did not recover normal B cell numbers, and in that patient the levels remained reduced at 11.2 cells/µl. The numerically low B cell levels were not accompanied by a special clinical course or a different B cell regeneration pattern.

B cell depletion was accompanied by a significant clinical improvement regarding disease activity like decrease in tender and swollen joint count, CRP or ESR (data not shown).

T-Cell Compartment

The absolute number of CD3 positive lymphocytes showed no significant changes during the time of B cell depletion and during the regeneration phase up to 1 year after B cell depletion compared to before therapy (Table **2**). The absolute numbers for CD4+ and CD8+ cells showed no significant changes either. Nevertheless we observed some changes in the composition of the peripheral lymphocyte pool. The relative frequency of CD3+ cells was significantly increased within 1-12 months after Rituximab (p < 0.05). This was related to a statistically significant increase of CD4+ T cells. CD8+ T cells did neither show significant changes in the absolute numbers nor in the relative frequency. For additional subtypes of T cells neither the relative frequency nor the absolute cell number changed significantly compared to the timepoint before therapy. This was found for CD3+HLA-DR+, CD3+CD16+ and/or 56+, CD3-CD16+ and/or 56+, CD8+CD25+, CD8+HLA-DR+, CD4+HLA-DR+ and CD3+γδ+ (data not shown).

CD4+CD25+ Compartment and Foxp3

Since regulatory T cells are thought to be more important for the pathophysiology of RA, we studied the CD4+CD25+ compartment more in detail. We did not observe any significant increase or decrease in the absolute numbers or the relative frequency of CD4+CD25+ cells (Table **2**). In particular, also the percentage and absolute cell count for regulatory T cells defined as CD4+CD25++ T-cells remained constant over the period under review and did not show any statistically significant changes compared to the timepoint before administration of rituximab.

Next we studied Fox P3 positive CD4+ T cells. Both, the absolute number and the relative frequency of Fox P3 positive T-cells did not change statistically significant over the period under review (Fig. **1**).

HLA-G

HLA-G defines a novel subset of regulatory T cells. Therefore we studied the numbers of CD4+ and CD8+ HLA-G+ cells under rituximab treatment. Before initiating rituxi-

Table 2.   Time Course of T-Cell Subsets After B-Cell Depletion with Rituximab Compared to Baseline

%	Before Therapy	1-3	4-6	7-9	10-12
CD3+	66 ± 10 %	74 ± 11 %*	73 ± 12 %*	73 ± 8 %*	73 ± 8 %*
CD4+	47 ± 11 %	55 ± 10 %*	51 ± 9 %*	51 ± 10 %*	52 ± 9 %*
CD8+	26 ± 7 %	25 ± 6 %	28 ± 9 %	27 ± 7 %	26 ± 9 %
CD4+CD25+	23 ± 8 %	24 ± 13 %	22 ± 8 %	20 ± 7 %	20 ± 10 %
CD3+HLA DR+	6 ± 5 %	6 ± 7 %	6 ± 5 %	5 ± 3 %	5 ± 4 %
cells/microlitre					
CD3+	867 ± 537	951 ± 419	923 ± 404	996 ± 584	951 ± 624
CD4+	630 ± 417	720 ± 299	642 ± 270	718 ± 474	689 ± 475
CD8+	318 ± 176	304 ± 126	348 ± 161	350 ± 154	324 ± 215
CD4+CD25+	314 ±196	298 ± 209	265 ± 121	248 ± 108	266 ± 232
CD3+HLA DR+	69 ± 63	49 ± 25	75 ± 82	59 ± 30	61 ± 68

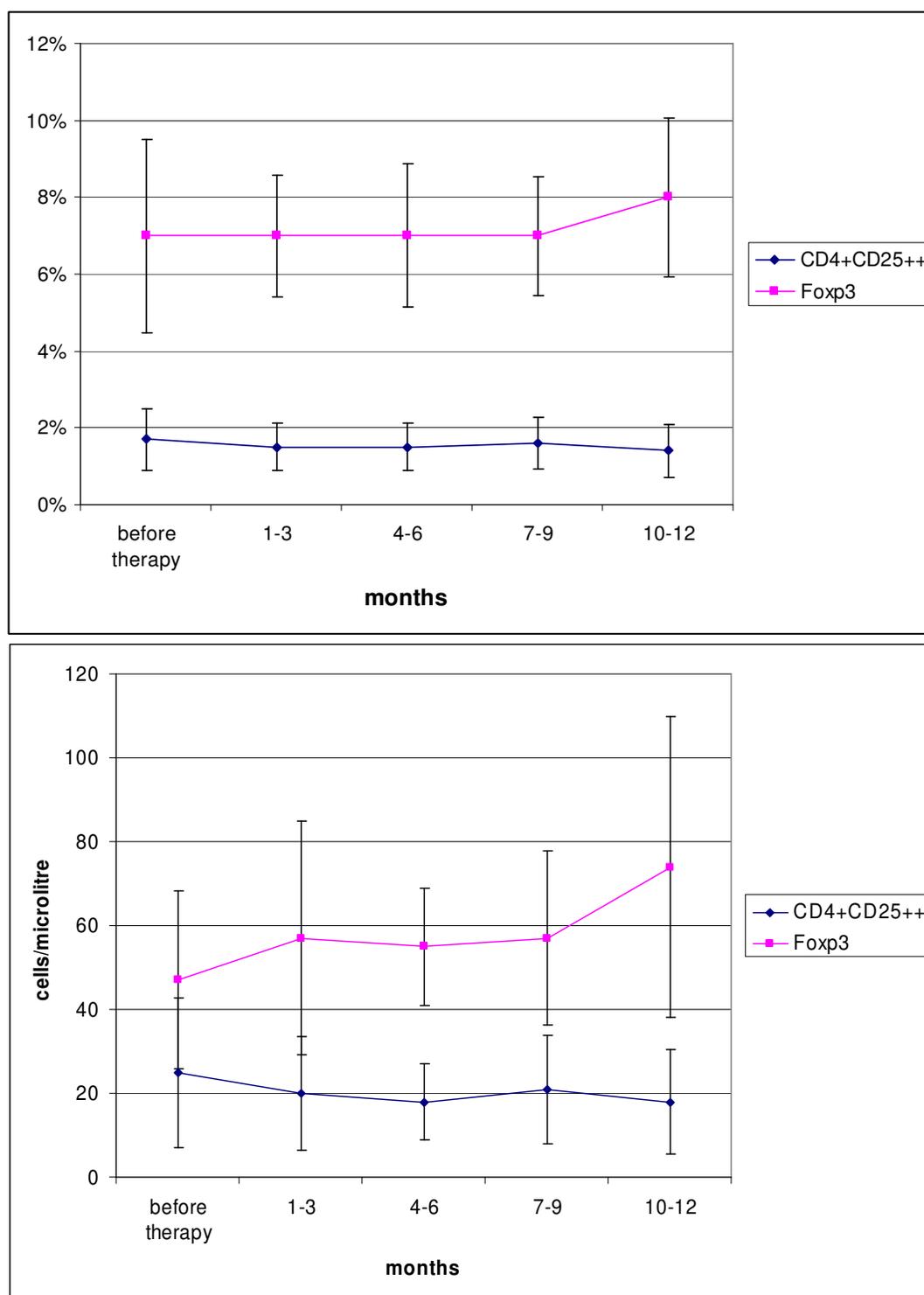Relative frequency within lymphocytes and absolute numbers; mean ± SEM, *p<0.05.

Fig. (1). Time course of CD4+CD25++ and Foxp3 positive T cells (relative frequency within lymphocytes, Foxp3 within CD4+ lympho-cytes) and absolute numbers after B-cell depletion with rituximab compared to baseline. (mean ± SEM).

mab an average of 0.95% of CD4+ T cells in the peripheral blood of RA patients expressed HLA-G (range 0.6% to 1.7%, n=7). Analysis of HLA-G expression on CD8 revealed an average of 1.56 % HLA-G positive CD8+ T cells in RA patients (range 0.9% to 3.6%, n= 7). Although the frequency of CD4+HLA-G+ and CD8+HLA-G+ varied in between the different time points in single patients we could not find a significant change for these subpopulations (Fig. **2**). The

absolute cell counts did not show significant changes neither for CD4+HLA-G+ nor CD8+HLA-G+ (data not shown).

sHLA-G

Serum levels of sHLA-G were previously found to be decreased in RA patients and have been linked to disease activity [12]. However, we did not observe a consistent sig-nificant change in soluble HLA-G as measured by ELISA in
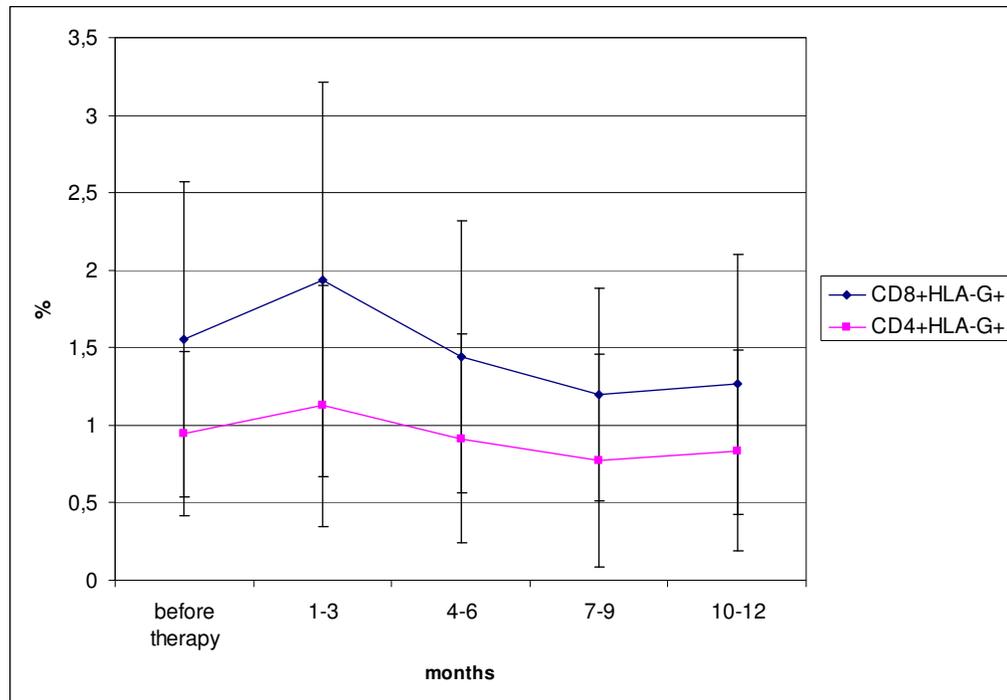
Fig. (2). Mean frequencies of CD4+HLA-G+ and CD8+HLA-G+ as percentages of CD4+ or CD8+ respectively at different time points during the first year follow up after initiation of rituximab treatment. (mean ± SEM, n=7).

the sera of five patients under rituximab treatment during the first year of follow-up (Fig. **3**).

DISCUSSION

Both, B and T cells have been shown to be of crucial importance in the pathogenesis of autoimmune disorders e.g. RA [2, 5, 25]. B cell depletion by rituximab has been approved for treatment of severe RA refractory to conventional DMARDs including TNFalpha inhibitors in combination with methotrexate underlining the key function of B cells in the pathogenesis in RA [3, 4, 7, 9, 26-28]. Undoubtedly there is a linkage between the B and T cell compartment under
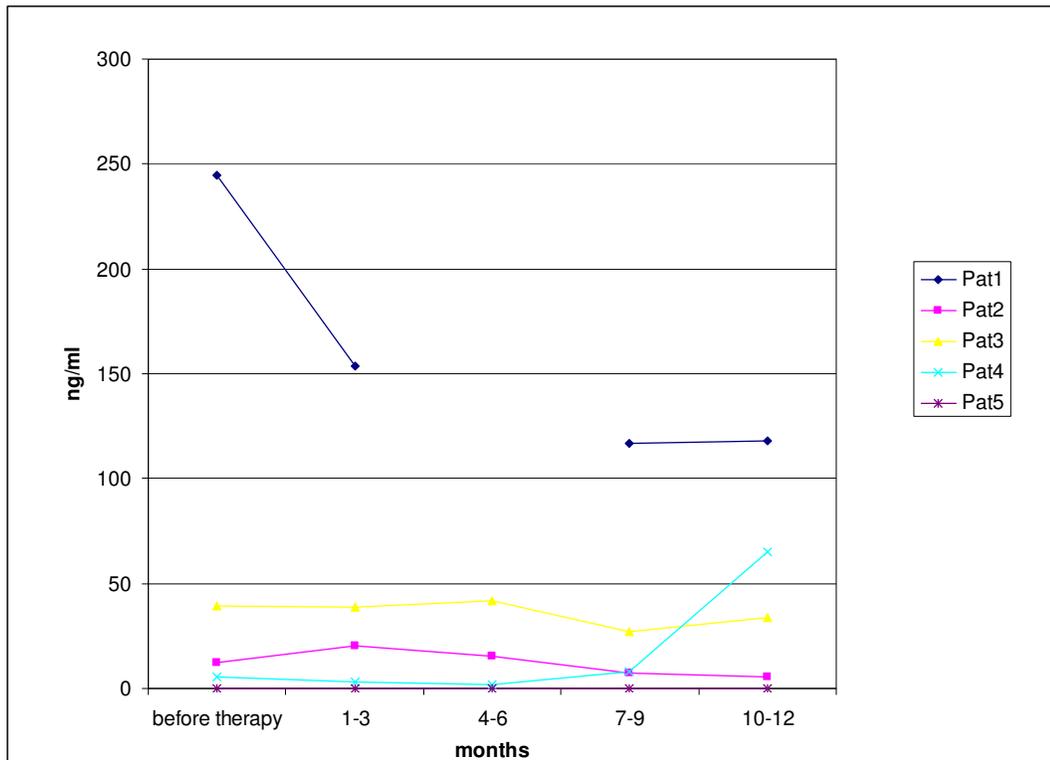

Fig. (3). Serum levels of sHLA-G during the first year of follow-up after initiation of rituximab therapy as measured by ELISA. (n=5).

both physiological and pathological conditions. B cells e.g. support the activation of T cells by expressing costimulatory molecules such as CD80/86 and CD40 or by the secretion of cytokines. Furthermore they function as antigen presenting cells and have essential effects on other cells required for the development of tertiary lymphoid tissues [24]. Rituximab treatment causes a profound peripheral B cell depletion which can be observed usually for about 6 months. In addition it causes dramatic changes in the homeostasis of B cell subsets which can be observed for more than a year after rituximab treatment [10, 11, 27, 29-31].

There is also evidence for a disturbed T cell homoeostasis within autoimmune diseases, in particular, a disturbance of T cells with immunosuppressive and regulatory function [12]. Several subsets of T cells with suppressive and regulatory functions have been described within the last years. To date, only 2 populations of "natural" regulatory T cell populations can be identified in peripheral blood. 1) the population of CD4+CD25++ coexpressing Foxp3 and 2) the recently identified HLA-G-expressing CD4 or CD8 Tregs.

Valencia *et al.* demonstrated differences regarding the expression of Foxp3 in patients with SLE [32]. In rheumatoid arthritis, Mottonen *et al.* studied the characteristics of CD4+CD25+ T cells in peripheral blood (PB) and synovial fluid (SF) compared to normal controls [23]. They found an increased frequency of CD4+ cells T cells expressing CD25 in SF compared to PB from patients with RA. However, no significant difference was observed in the numbers of CD4+CD25+ T cells in PB from patients and controls.

There is evidence that CD4+CD25++Foxp3+ T regs are modulated by anti-TNF therapies [33]. CD4+CD25++ Tregs isolated from patients with active rheumatoid arthritis (RA) expressed reduced levels of Foxp3 mRNA and protein which was ameliorated by inhibition of TNFalpha with infliximab [34]. In another study, treatment with infliximab also restored the capacity of regulatory T cells to inhibit cytokine production and to induce a suppressive phenotype on CD4+CD25- T cells [35]. Furthermore, anti-TNFalpha treatment led to a significant rise in the number of peripheral blood regulatory T cells in RA patients responding to this treatment. These data so far suggest that particularly regulatory T cells are of importance also for RA, which can be influenced to a certain extent by antiinflammatory treatment.

Only little is known about the influence of rituximab mediated B-cell depletion on the T-cell compartment and in particular on regulatory T-cells.

Leandro *et al.* could not find significant changes in the frequency and absolute number of T cells (CD3+) and NK cells (CD3-CD56+) at 1 month and 3 months after rituximab in RA. In addition, no significant differences were detected in the different subpopulations of T cells like CD4+, CD8+ and CD4+ T cells expressing CD25 [10]. Since the clinical response to rituximab is somewhat delayed and not yet fully developed at 3 months after rituximab and B cell homeostasis is disturbed for more than a year, changes in the T cell compartment may also evolve later than three months after Rituximab. Therefore we extended these data by analysing T cell subpopulation over a period of one year. Both, the relative frequency and also the absolute number of CD4+CD25++ T cells in our population remained constant

without any significant changes. Despite the fact that we saw a significant increase in the relative frequency of CD3+ cells and in the CD4+ subset within 1-12 months after Rituximab ($p < 0.05$), the absolute numbers of CD3+ and CD4+ T cells as well as CD3+HLA-DR+, CD3+CD16/56+, CD8+CD25+, CD8+HLA-DR+, CD4+HLA-DR+ and CD3+$\gamma\delta$+ and of NK cells (CD3-CD56+) were not significantly altered. This can be explained by the lack or reduced levels of B-cells during period under review, respectively. Due to extensive pretreatment before Rituximab investigated patients in our study tended to have low lymphocyte counts. As a consequence absolute numbers of T cells were generally low.

Of particular interest was the analysis of the Treg compartment. In the study by Leandro the frequency of CD4+CD25++ T cells decreased 1 month after treatment significantly ($p < 0.05$), but the total numbers did not, and no significant differences were found at 3 months. In our population we could not find a decrease in the frequency of CD4+CD25++ within 3 months after Rituximab. However, we did not specifically look within the first 4 weeks after rituximab since we thought this period to be confounded by the i.v prednisolone used during antibody infusions. The relative frequency and also the absolute number of CD4+CD25++ T cells remained constant without any significant changes up to one year after initiating B cell depletion. Also the numbers of Foxp3+CD4+ T cells remained constant under rituximab treatment. In lupus nephritis Sfikakis *et al.* could show increased Foxp3 mRNA after B cell depletion by Rituximab in peripheral blood of 7 patients [36]. During follow-up, increased Foxp3 mRNA persisted in those patients in clinical remission, while in those patients with active disease subsequent decreases were noted. It is not clear whether the different techniques for defining Foxp3+ Treg or the different pathophysiology between RA and lupus accounts for these differences.

An interesting new subset of regulatory T cells which does not share the CD25++ Foxp3 phenotype are the HLA-G expressing Treg. HLA-G expressing T cells represent a novel subset of natural occurring regulatory T cells, that efficiently inhibit autologous T cell proliferation *via* a yet to be defined cell-contact independent mechanism. HLA-G positive T cells can be found at low percentages in the peripheral blood of healthy volunteers and the presence of HLA-G+ T cells at sites of inflammation due to infections or autoreactivity like neuroinflammatory disorders (e.g. multiple sclerosis, vasculitis, encephalitis or myositis) suggests an important function of these cells in modulating inflammatory responses *in vivo* [37, 38]. Similar to membrane bound HLA-G on T cells Verbruggen *et al.* were able to demonstrate that soluble HLA-G was lower in RA but positively correlated with parameters of RA disease activity [19]. However, we did not observe a consistent significant change in soluble HLA-G as measured by ELISA in the sera of five patients under rituximab treatment.

From our data, there seems to be no evidence for any numerical influence of CD20 mediated B cell depletion on the amounts and frequency of regulatory T cells in peripheral blood in RA. For future studies it will be relevant to extend the analysis of Tregs to their function, since the available data give no indication whether B cell depletion is able to restore defective regulatory T cell activity. Results regarding

T cell subsets after B cell depletion *via* Rituximab may also vary depending on the underlying disease. To address this, there is an obvious need for further studies on the impact of therapeutic B cell depletion on T cell subsets.

CONCLUSION

Our data for patients with RA indicate that absolute T cell counts do not change significantly after rituximab compared to the time before therapy for all studied populations in peripheral blood. In particular, the frequency and the absolute number of regulatory T cells defined as T cells characterized by CD4+/CD25++, Fox P3 or HLA-G expression were numerically stable during transient B-cell depletion using anti-CD20 antibodies up to 12 months in the peripheral blood.

ABBREVIATIONS

RA	=	Rheumatoid arthritis
SLE	=	Systemic lupus erythematosus
CD	=	Cluster of differentiation
TNFalpha	=	Tumor necrosis factor alpha
DMARDs	=	Disease modifying anti-rheumatic drugs
MTX	=	Methotrexate
PB	=	Peripheral blood
Treg	=	Regulatory T cells
APC	=	Antigen presenting cells
PBMCs	=	Peripheral blood mononuclear cells
DAS28	=	Disease Activity Score in 28 joints
ESR	=	Erythrocyte sedimentation rate;
CRP	=	C-reactive protein
SF	=	Synovial fluid
NK cells	=	Natural killer cells

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REFERENCES

[1] Van der Kooij SM, de Vries-Bouwstra JK, Goekoop-Ruiterman YP, *et al.* Limited efficacy of conventional DMARDs after initial methotrexate failure in patients with recent onset rheumatoid arthritis treated according to the disease activity score. Ann Rheum Dis 2007; 66(10): 1356-62.

[2] Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat Rev Immunol 2006; 6(5): 394-403.

[3] Leandro MJ, Edwards JC, Cambridge G. Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion. Ann Rheum Dis 2002; 61(10): 883-8.

[4] Edwards JC, Cambridge G. Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes. Rheumatology (Oxford) 2001; 40(2): 205-11.

[5] Kim HJ, Berek C. B cells in rheumatoid arthritis. Arthritis Res 2000; 2(2): 126-31.

[6] Cohen SB. Updates from B Cell Trials: Efficacy. J Rheumatol Suppl 2006;77: 12-7.

[7] Cohen SB, Emery P, Greenwald MW, *et al.* Rituximab for rheumatoid arthritis refractory to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. Arthritis Rheum 2006; 54(9): 2793-806.

[8] Edwards JC, Szczepanski L, Szechinski J, *et al.* Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. N Engl J Med 2004; 350(25): 2572-81.

[9] Emery P, Fleischmann R, Filipowicz-Sosnowska A, *et al.* The efficacy and safety of rituximab in patients with active rheumatoid arthritis despite methotrexate treatment: results of a phase IIB randomized, double-blind, placebo-controlled, dose-ranging trial. Arthritis Rheum 2006; 54(5): 1390-400.

[10] Leandro MJ, Cambridge G, Ehrenstein MR, Edwards JC. Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis. Arthritis Rheum 2006; 54(2): 613-20.

[11] Roll P, Palanichamy A, Kneitz C, Dorner T, Tony HP. Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis. Arthritis Rheum 2006; 54(8): 2377-86.

[12] Sarkar S, Fox DA. Regulatory T cell defects in rheumatoid arthritis. Arthritis Rheum 2007; 56(3): 710-3.

[13] Janossy G, Panayi G, Duke O, Bofill M, Poulter LW, Goldstein G. Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation. Lancet 1981; 2(8251): 839-42.

[14] Bluestone JA, Tang Q. How do CD4+CD25+ regulatory T cells control autoimmunity? Curr Opin Immunol 2005; 17(6): 638-42.

[15] Costantino CM, Baecher-Allan CM, Hafler DA. Human regulatory T cells and autoimmunity. Eur J Immunol 2008; 38(4): 921-4.

[16] Hori S. Rethinking the molecular definition of regulatory T cells. Eur J Immunol 2008; 38(4): 928-30.

[17] Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 2008; 9(3): 239-44.

[18] Feger U, Tolosa E, Huang YH, *et al.* HLA-G expression defines a novel regulatory T-cell subset present in human peripheral blood and sites of inflammation. Blood 2007; 110(2): 568-77.

[19] Verbruggen LA, Rebmann V, Demanet C, De Cock S, Grosse-Wilde H. Soluble HLA-G in rheumatoid arthritis. Hum Immunol 2006; 67(8): 561-7.

[20] Cope AP, Schulze-Koops H, Aringer M. The central role of T cells in rheumatoid arthritis. Clin Exp Rheumatol 2007; 25(5 Suppl 46): S4-11.

[21] Kelchtermans H, Geboes L, Mitera T, Huskens D, Leclercq G, Matthys P. Activated CD4+CD25+ regulatory T cells inhibit osteoclastogenesis and collagen-induced arthritis. Ann Rheum Dis 2008; [Epub ahead of print]

[22] Londei M. Role of regulatory T cells in experimental arthritis and implications for clinical use. Arthritis Res Ther 2005; 7(3): 118-20.

[23] Mottonen M, Heikkinen J, Mustonen L, Isomaki P, Luukkainen R, Lassila O. CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis. Clin Exp Immunol 2005; 140(2): 360-7.

[24] Takemura S, Klimiuk PA, Braun A, Goronzy JJ, Weyand CM. T cell activation in rheumatoid synovium is B cell dependent. J Immunol 2001; 167(8): 4710-8.

[25] Kotzin BL. The role of B cells in the pathogenesis of rheumatoid arthritis. J Rheumatol Suppl 2005; 73: 14-8; discussion 29-30.

[26] Dass S, Vital EM, Emery P. Rituximab: novel B-cell depletion therapy for the treatment of rheumatoid arthritis. Expert Opin Pharmacother 2006; 7(18): 2559-70.

[27] Kneitz C, Wilhelm M, Tony HP. Effective B cell depletion with rituximab in the treatment of autoimmune diseases. Immunobiology 2002; 206(5): 519-27.

[28] Kneitz C, Wilhelm M, Tony HP. Improvement of refractory rheumatoid arthritis after depletion of B cells. Scand J Rheumatol 2004; 33(2): 82-6.

[29] Anolik JH, Barnard J, Owen T, *et al.* Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. Arthritis Rheum 2007; 56(9): 3044-56.

[30] Lundy SK, Sarkar S, Tesmer LA, Fox DA. Cells of the synovium in rheumatoid arthritis. T lymphocytes. Arthritis Res Ther 2007; 9(1): 202.

[31] Thurlings RM, Vos K, Wijbrandts CA, Zwinderman A, Gerlag DM, Tak PP. Synovial tissue response to rituximab: mechanism of action and identification of biomarkers of response. Ann Rheum Dis 2008; 67(7): 917-25.

[32]   Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. J Immunol 2007; 178(4): 2579-88.

[33]   Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis *via* TGF-beta. J Exp Med 2007; 204(1): 33-9.

[34]   Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 2006; 108(1): 253-61.

[35]   Ehrenstein MR, Evans JG, Singh A, *et al.* Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med 2004; 200(3): 277-85.

[36]   Sfikakis PP, Souliotis VL, Fragiadaki KG, Moutsopoulos HM, Boletis JN, Theofilopoulos AN. Increased expression of the FoxP3 functional marker of regulatory T cells following B cell depletion with rituximab in patients with lupus nephritis. Clin Immunol 2007; 123(1): 66-73.

[37]   Kroner A, Grimm A, Johannssen K, Maurer M, Wiendl H. The genetic influence of the nonclassical MHC molecule HLA-G on multiple sclerosis. Hum Immunol 2007; 68(5): 422-5.

[38]   Wiendl H. HLA-G in the nervous system. Hum Immunol 2007; 68(4): 286-93.