The immunological phenotype of rituximab-sensitive chronic graft-*versus*-host disease: a phase II study

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ABSTRACT

Chronic graft-*versus*-host disease is the major long-term complication after allogeneic stem cell transplantation with a suboptimal response rate to current treatments. Therefore, clinical efficacy and changes in lymphocyte subsets before and after rituximab treatment were evaluated in a prospective phase II study in patients with steroid-refractory chronic graft-*versus*-host disease. Overall response rate was 61%. Only responding patients were found to have increased B-cell numbers prior to treatment. B cells had a naïve-antigen-presenting phenotype and were mainly CD5 negative or had a low CD5 expression. Normal B-cell homeostasis was reestablished in responding patients one year after ritxumab treatment and associated with a significant decline in skin-infiltrating $CD8^+$ T cells, suggesting that host B cells play a role in maintaining pathological $CD8^+$ T-cell responses. Imbalances in B-cell homeostasis could be used to identify patients *a priori* with a higher chance of response to rituximab treatment (Eudra-CT 2008-004125-42).

Key words: graft-*versus*-host-disease, B cell, rituximab, transplantation.

*Citation: van Dorp S, Resemann H, te Boome L, Pietersma F, van Baarle D, Gmelig-Meyling F, de Weger R, Petersen E, Minnema M, Lokhorst H, Ebeling S, Beijn SJP, Knol EF, van Dijk M, Meijer E, and Kuball J. The immunological phenotype of rituximab-sensitive chronic graft-*versus*-host disease: a phase II study. Haematologica 2011;96(9):1380-1384. doi:10.3324/haematol.2011.041814*

Introduction

Graft-*versus*-host-disease (GVHD) is the most common and life-threatening complication after allogeneic stem cell transplantation (allo-SCT).[1,2] B-cell depletion with rituximab (RTX) has been successful in steroid-refractory chronic GVHD, showing response rates of 43-80%.[3-8] However, the nature of B-cell contribution, as well as to what extent B-cell depletion can restore physiological conditions, has so far not been clarified. Hypotheses obtained from mouse models of chronic GVHD and retrospective analysis of patient materials have been conflicting. For example, a correlation was found between high levels of B-cell activating factor (BAFF) in a retrospective analysis of 45 patients with active chronic GVHD.[9] However, no significant correlation could be found between the levels of BAFF and RTX-responsiveness in the most recently published prospective phase II study of 37 patients.[10] In active chronic GVHD prior to response to immunosuppressants, expansion of activated $CD27^+$ B cells has been observed.[9] Another retrospective study of 35 patients suffering from active chronic GVHD, in which treatment included prednisone and calcineurin inhibitors, showed significantly lower numbers of memory B cells ($CD27^+$).[11] To our knowledge, no prospective comparisons have been made between immune subsets before and after B-cell depletion therapy of steroid-refractory chronic GVHD, so details on reestablishment of normal B-cell pools after RTX treatment, as well as changes in immune-pathology of the skin, still have to be clarified. Consequently, the aim of this study was to demonstrate effectiveness of B-cell depletion therapy in steroid-refractory chronic GVHD and to identify potentially involved cell subsets by a comprehensive immunological analysis in a prospective clinical trial.

Design and Methods

Patients and patient material

In the course of a prospective study (Eudra-CT 2008-004125-42), a cohort of 20 chronic GVHD patients who received allo-SCT due to various hematologic malignancies was treated with 4 weekly doses of 375 mg/m^2 RTX (F. Hoffmann-La Roche Ltd., Basel, Switzerland). All patients had chronic GVHD with at least skin symptoms. Inclusion criteria were age over 18 years, life-expectancy of more than six months, and a World Health Organization (WHO)

The online version of this article has a Supplementary Appendix.

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Acknowledgments: we thank Roche Nederland B.V. for kindly providing the study medication. We thank Wilco de Jager (UMC Utrecht, The Netherlands) for performing the multiplex immunoassay. We thank Andries Bloem (UMC Utrecht, The Netherlands) for critical reading of the manuscript.
Funding: supported by grants KWF 2006-3685 to SE and EM and LSBR 0902 to JK.
Manuscript received on February 3, 2011. Revised version arrived on April 11, 2011. Manuscript accepted May 2, 2011.
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performance status of 2 or under. All patients were steroid-refractory or steroid-dependent. Refractory chronic GVHD was defined as progression of disease after at least two weeks of prednisone treatment (approximately 1 mg/kg) or no response after four weeks of prednisone treatment. Steroid-dependent chronic GVHD was defined as an inability to completely taper immunosuppressive treatment. Patients received RTX treatment at the University Medical Center Utrecht between March 2007 and February 2010 according to clinical protocols approved by the local ethics board. In cases of progression or recurrence of chronic GVHD, for which an alternative systemic therapy was needed, patients were excluded from the study and follow up was ended. Response criteria were set as the following: complete response (CR) and partial response (PR) defined according to the recently published National Institutes of Health (NIH) criteria.[12] For laboratory analysis, blood samples were taken from each patient before and after treatment, and consecutively every two months until one year after treatment. Peripheral blood mononuclear cells (PBMCs) were immediately isolated and lymphocyte numbers measured by TruCount (according to the manufacturer's protocol, BD Biosciences). PBMCs were then frozen and stored in liquid nitrogen until further analysis. Plasma and serum were stored at -80°C until further analysis. Furthermore PBMC, plasma and serum samples from allo-SCT recipients without GVHD (No-GVHD) and healthy donors were obtained. An informed consent was obtained for other control samples to be used for analysis. Skin biopsies were obtained before RTX therapy and five months after treatment from adjacent sites if possible, otherwise from the most active site, and stored in 4% formalin and embedded in paraffin. Eye involvement was measured before RTX, three and seven months after treatment, using a Schirmer's test.

FACS staining, cytokine analysis,[13] B-cell clonality, chimerism,[14] and auto-antibodies,[15] histological stainings[3] and the control groups used are described in the *Online Supplementary Design and Methods* section and *Online Supplementary Tables S1 and S2*.

Statistical analysis

For prospective data analysis of the cohort of 20 patients SPSS (IBM, Chicago, USA) was used. Receiver operating characteristic (ROC) curve was used to determine a cut-off point for B-cell numbers that was predictive for responsiveness to treatment. Hazard ratios were calculated using a Cox's regression analysis. Incidence of response was calculated using the Kaplan-Meier method. A Kaplan-Meier curve was used to illustrate responsiveness and a log rank test was used to compare incidence between patients with high and low B-cell numbers. Data from FACS staining, plasma and serum analysis were analyzed using GraphPad Prism 5 for Windows (GraphPad Software, La Jolla, USA). Differences in lymphocyte subsets were compared using two-way ANOVA for normally distributed data. Gaussian-distributed groups were compared using Student's t-test. Groups of data which were not normally distributed were compared using Mann-Whitney U tests. In either case, a probability level of 5% ($P<0.05$) was found to be significant.

Results and Discussion

In order to prospectively test clinical efficacy of B-cell depletion therapy in steroid-refractory chronic GVHD, a cohort of 20 patients presenting with at least skin involvement was treated with rituximab and followed until one year after treatment or until relapse of chronic GVHD. Two patients had to be excluded from further study; one due to an allergic reaction to rituximab and one due to relapse of leukemia. Eighteen patients could, therefore, be included for further analyses. Patients' characteristics are shown in the *Online Supplementary Table S3*. Overall response rate was 61% (n=11). Only partial responses were seen during the time of follow up. Median time to response was three months (range 1-4 months) and 55% of responders had an ongoing response (n=6). Median response duration, measured until last time of follow up, was 12 months (range 1-12 months) (Table 1). Dosage of prednisone could be reduced in 50% of patients (n=9) and completely stopped in 4 patients (22%). Median time to dose reduction of prednisone was three months (range 1-7 months) (Table 1).

To investigate whether the production of auto-antibod-

Table 1. Total response rates, response rates per organ and dose reduction of immunosuppressants after treatment with RTX.

	Response [n (%)]					Median TTR Months (range)	Response duration Months, (range)
	OR	CR	PR	SD	Progression		
Total (n=18)	11 (61)	0	11 (61)	3 (17)	4 (22)	3 (1-4)	12 (1-12)
Organ involvement	OR	CR	PR	SD	Progression		
Skin (n=18)							
Erythema (n=17)	13 (76)	4 (24)	9 (53)	2 (12)	2 (12)	3 (1-8)	6 (3-12)
Ulcera (n=3)	0	0	0	1 (33)	2 (67)	x	x
Movable sclerosis (n=5)	4 (80)	2 (40)	2 (40)	0	1 (20)	1 (1-3)	10 (3-12)
Deep sclerosis (n=12)	9 (75)	4 (33)	5 (42)	2 (17)	1 (8)	1.5 (1-3)	8 (3-12)
Eyes (n=15)	6 (40)	4 (27)	2 (13)	7 (47)	2 (13)	2 (1-6)	6.5 (4-12)
Oral mucosa (n=8)	3 (38)	1(12)	2 (25)	5 (75)	0	1 (1-6)	n.d.
GI tract (n=7)	3 (43)	2 (29)	1 (14)	4 (57)	0	1 (x)	n.d
Dose reduction							n.d
Prednisone (n=18)	9 (50)	4 (22)	5 (28)	9 (50)	x	3 (1-7)	
Cyclosporine A (n=5)	2 (40)	2 (40)	0	3 (60)	x	3 (2-4)	
MMF (n=4)	2 (50)	1 (25)	1 (25)	2 (50)	x	3.5 (3-4)	

CR indicates complete response/reduction; GI: gastrointestinal; MMF, mycophenolate mofetil; OR: overall response/reduction; PR: partial response/reduction; SD: stable disease/dose; TTR: time to response/reduction.

ies was associated with symptoms of chronic GVHD as reported,[16,17] serum before and after rituximab treatment of patients and No-GVHD controls was tested for a panel of antibodies correlated with Systemic Sclerosis (SSc) in terms of quality (type) and quantity. Several auto-antibodies were found in serum of both responders and non-responders, as well as in serum of No-GVHD controls. However, no significant associations between presence of antibodies and chronic GVHD could be found (*data not shown*) as also reported by others.[18] Conflicting data have also been reported on the correlation between BAFF-levels or BAFF-to-B-cell ratios in RTX-responding patients, as the recently published prospective study of 37 patients[10] did not show a significant correlation in contrast to a ret-

Table 2. Predictive value of various factors for responsiveness of RTX treatment.

Factor	HR	95% CI	*P* value
Age	0.985	0.914-1.061	0.689
Sex	0.796	0.210-2.980	0.728
ATG	0.925	0.198-4.307	0.920
Acute GVHD	1.585	0.418-6.009	0.498
B cells >0.40×10⁹/L	10.683	1.237-92.235	0.031
Low IL-21	3.615	0.435-30.057	0.234
Ulcerative skin	0.033	0.000-16.323	0.281

ATG indicates anti-thymocyte globuline as part of conditioning regimen; CI: confidence interval; GVHD: graft-versus-host disease; HR: hazard ratio. HR and P values: univariate Cox's regression model.

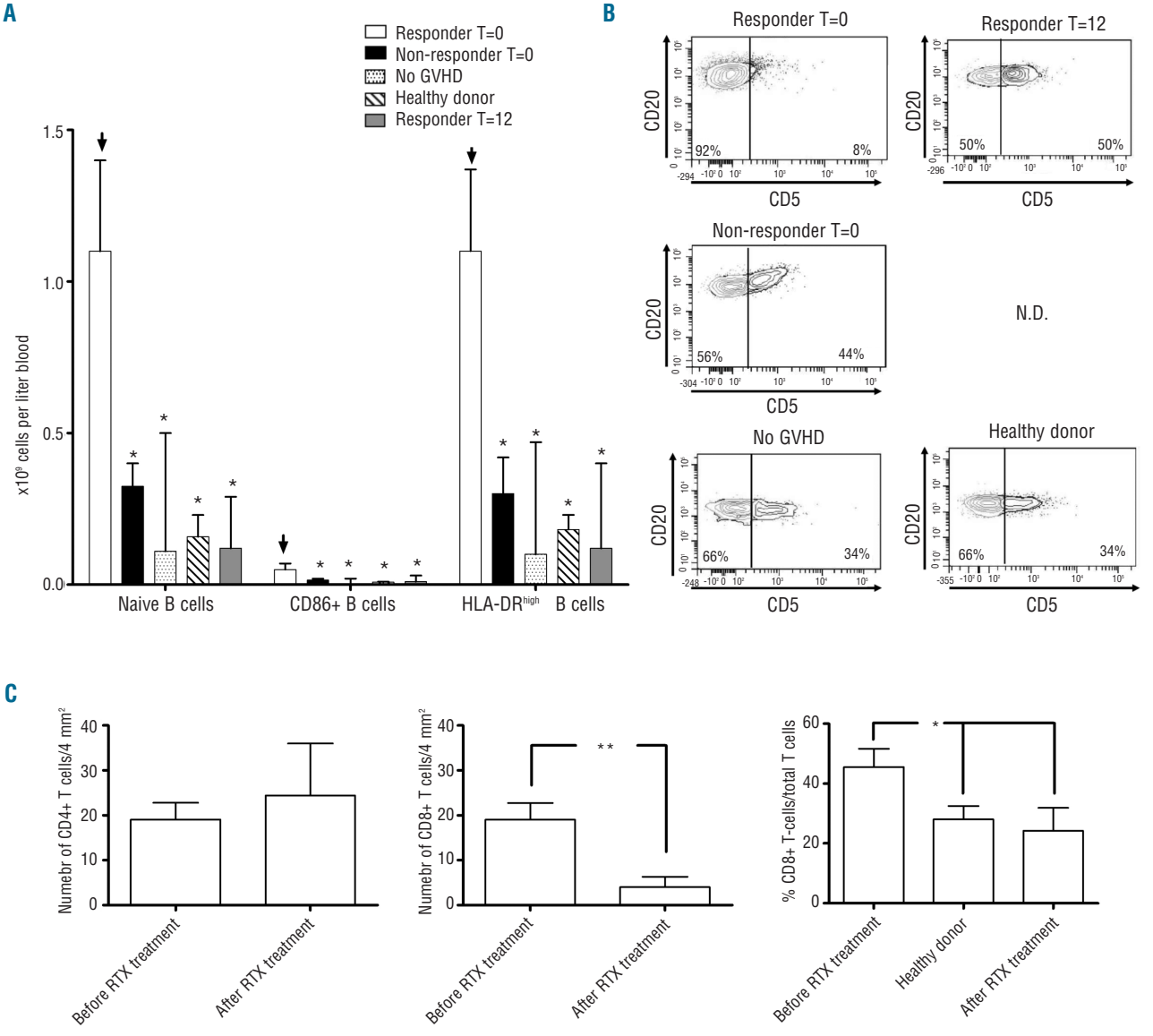

Figure 1. Possible predictive markers for responsiveness to RTX treatment and immunological changes after RTX treatment. (A) Absolute numbers of naïve (CD27⁻), and activated (HLA-DR^{high}) B cells in responding, non-responding patients, No-GVHD and healthy donor controls. Arrows indicate the control group on which statistical analysis was performed by a Mann-Whitney-U test (**P*<0.05). (B) Representative dot plots of cell surface expression of CD5 and CD20 in responding patients at T=0 and T=12, non-responding patients at T=0, No-GVHD and healthy-donor controls. Number of skin-infiltrating (C) CD4⁺ and CD8⁺ T cells before and five months after RTX treatment in responding patients as well as percentage of skin-infiltrating CD8⁺ T cells before and after RTX treatment in responders and healthy controls. Statistical analysis was performed using an unpaired t-test and a two-way ANOVA (**P*<0.05, ***P*<0.01).

rospective study of 20 patients.[19] Also, our prospective study of 18 patients did not show any correlation between BAFF-levels and RTX-response. However, differences in these studies and our data could also be partially a consequence of the fact that patients received different doses of corticosteroids in different studies, and high doses of corticosteroids as used in our study have been reported to partially inhibit BAFF.[20] In our study, only IL-21 was significantly decreased in responding as compared to non-responding patients (*data not shown*).

Peripheral blood mononuclear cells from different groups were analyzed by flow cytometry for lymphocyte subsets, and there was no significant difference in total lymphocyte numbers between patient groups, No-GVHD and healthy donor controls. No significant differences were observed between responders and non-responders when comparing $CD8^+$ and $CD4^+$ T cells in the peripheral blood. Also, regulatory T cells (Tregs, $CD3^+CD4^+CD25^-CD127^+FoxP3^+$), naïve, effector memory and central memory, distinguished on the basis of CD62L and CD45RO expression, did not show any significant difference between all groups at any time point, as well as T cells expressing early (CD69), intermediate (CD137) and late (HLA-DR) activation markers (*data not shown*). B-cell numbers in responding patients before treatment (T=0) were increased with significantly higher absolute numbers of naïve B cells ($CD19^+CD20^+CD27^-$) and $CD86^+$ and HLA-DR^{high} B cells when compared to both non-responders, No-GVHD, and healthy donor controls (against all controls all <0.05, Figure 1A). A cut-off point for B cells of 0.40 $\times 10^9$/L was calculated using a ROC curve. The relative risk of responsiveness in patients with absolute B-cell numbers more than 0.40×10^9/L was estimated using a univariate Cox's regression analysis. Patients with absolute B-cell numbers of more than 0.40×10^9/L had a 10.7 times higher chance of being responsive to rituximab treatment (HR 10.7 [95% CI 1.2-92.3]; P=0.031). B-cell number was the only factor found to be of significant predictive value (Table 2). Rituximab sufficiently depleted all B cells in the peripheral blood of all patients as indicated by the lack of CD19-positive cells at one month after the first rituximab dose (T=1; *data not shown*). B-cell reconstitution in peripheral blood could only be observed 10-12 months after start of rituximab. The MFI of $CD5^+$ B cells was significantly decreased in responding patients before treatment (T=0) and normalized until T=12 (P<0.05), and was, therefore, then again comparable to all controls. These differential expression patterns of CD5 in patients and all controls are shown in representative dot plots in Figure1B. At T=12, also CD5- B-cell numbers, naive B-cell numbers, and HLA-DRhigh B-cell numbers in responding patients were again comparable to all controls, thus with a significant difference when compared to T=0 (P<0.05). Knowledge about CD5 and its signaling functions in lymphocytes is still very limited and the biological role of CD5- and CD5+ B cells differ in mice and men.[21,22] In contrast to our study, an increase in CD5+ B cells has been observed in patients with other autoimmune disease such as lupus erythematosus.[22] However, regardless of its function, loss of CD5 expression on B cells might assist in identifying patients who will benefit from B-cell depletion.

To investigate whether responding and non-responding patients also show differences in infiltrating immune cells of the skin, skin sections of responders, non-responders, and healthy donors were additionally stained for T- and B-cell markers. As reported,[23] only minimal immune cell infiltrates could be observed in patients with chronic GVHD: total numbers of T cells were within the range of T-cell infiltrates usually observed in healthy individuals (*data not shown*). However, the percentage of skin-infiltrating $CD8^+$ T cells was significantly higher when compared to healthy controls (P<0.05; Figure 1C). No significant difference was observed in skin infiltrating T cells before rituximab treatment between responding and non-responding patients (*data not shown*). There was no difference in numbers of skin infiltrating B cells between responding and non-responding patients, and B cells disappeared after rituximab treatment (*data not shown*). After rituximab treatment, only ongoing responding patients were analyzed at five months after study entry as non-responding patients had already undergone other therapies and were thus no longer eligible. A significant decrease in $CD8^+$ T cells was observed after five months in responding patients (P<0.01), whereas there was no significant change in $CD4^+$ T-cell numbers (Figure 1C and D). This resulted again in normalization of the percentage of skin-infiltrating $CD8^+$ T cells when compared to the healthy control group (Figure 1C). This suggests that B-cell depletion by rituximab not only reduces the number of potentially antigen-presenting B cells, but also reduces the skin infiltrating $CD8^+$ T-cell compartment. This, therefore, supports recent hypotheses of a T-cell to B-cell crosstalk in the setting of chronic graft-*versus*-host disease in man.[18]

In summary, to our knowledge this is the first prospective comprehensive study which describes the immunological phenotype of RTX-sensitive as compared to RTX-unresponsive chronic graft-*versus*-host disease in the peripheral blood and the skin. Elevation of B-cell numbers with a dominant naïve, antigen-presenting phenotype, as well as skewing towards CD5- B cells and B cells with a low CD5 expression was selectively found in responding patients and was the only predictive factor of responsiveness to rituximab. Physiological B-cell homeostasis was re-established in responding patients one year after treatment and associated with a reduced skin infiltration of $CD8^+$ T cells in responding patients. This suggests that an imbalanced B-cell repertoire can contribute to chronic graft-*versus*-host disease by sustaining skin-infiltrating $CD8^+$ T cells. These findings could also be useful in identifying in advance those patients who will benefit from rituximab treatment and provide a basis for larger confirmatory prospective clinical trials.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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