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ORIGINAL ARTICLE

The lack of memory B cells including T cell independent IgM$^+$ IgD$^+$ memory B cells in chronic graft-versus host disease is associated with susceptibility to infection

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Abstract

The chronic graft-versus host disease (cGVHD) is associated with a perturbed B cell homeostasis and an increased infection rate. Aiming to determine the impact of lymphocyte subsets on cGVHD, blood samples from 98 patients at least 100 days following allogeneic haematopoietic stem cell transplantation (median 1066 days) were analyzed, serum levels of immunoglobulins measured and the incidence of severe infections retrospectively documented. Absolute CD19$^+$ B cell counts, including counts of immature (CD10$^+$ CD38^{++} CD20$^+$ IgM^{++}) and transitional (CD10$^-$ CD38^{++} CD20$^+$ IgM^{++}) as well as class switched memory (CD19$^+$ CD27$^+$ IgM$^-$ IgD$^-$) B cells in patients with active cGVHD ($n = 52$) were significantly decreased as compared to those with inactive ($n = 18$) or without cGVHD ($n = 28$). In addition, nonclass switched IgM$^+$ memory B cells (CD19$^+$ CD27$^+$ IgM$^+$ IgD$^+$) were absent in patients with cGVHD, but not in patients with inactive (0.4×10^6/l) or without (1.7×10^6/l) cGVHD (both $P < 0.001$). In line with these results we found significantly decreased lgG levels in patients with cGVHD, which was associated with a significantly higher rate of severe infections in cGVHD patients. Our data underline the close association of diminished B cell counts with cGVHD and the onset of severe infections. The lack of IgM$^+$ memory B cells in patients with cGVHD may indicate functional asplenia.

Introduction

The chronic graft-versus host disease (cGVHD) presents as a multifaceted condition with a broad variety of clinical manifestations. The role of B cells in cellular immune responses is now receiving renewed interest [1] owing to the emergence of clinical data showing that B cell depletion with rituximab may have beneficial effects in cGVHD [2–4].

Apart from antibody production, B cells may contribute to the immune response by antibody-independent mechanisms, such as presentation of antigen, the production of chemokines and cytokines as well as acting as immunmodulatory cells [5]. Accumulating evidence from animal [6,7] and human studies [8–10], increased levels of B cell activating factor (BAFF) in patients with cGVHD [9] and the presence of antibodies to minor histocompatibility antigens encoded on the Y-chromosome in male patients receiving female grafts [11], suggest a crucial role of B cells in the pathogenesis of cGVHD.

In this study, we compared lymphocyte subsets in patients with active cGVHD with those patients who had either never experienced cGVHD, or had resolved cGVHD. Healthy donors were included to control for the

effect of allogeneic hematopoietic stem cell transplantation (HSCT) itself. We put special focus on the analysis of different B cell subsets with the aim to further characterize the nature of the observed B cell deficiency. As increased rates of infectious complications have been reported in patients with B cell deficiencies [10,12,13], the incidence of severe infections was retrospectively documented. Moreover, serum levels of IgG, including IgG subclasses were determined.

Methods

Patients

Consecutive adult patients ($n = 98$) who were, at least 100 days, following allogeneic HSCT, in complete remission of their underlying hematologic malignancy at the time of analysis and attending the outpatient unit, as well as healthy donors ($n = 16$) who never experienced HSCT, were recruited for this study. The difference in time post-HSCT was not significant ($P = 0.720$) among the groups. In patients lacking a HLA-genotypically identical sibling ($n = 47$), transplantation from a HLA A/B/C/DRB1/DQB1 (10/10) matched ($n = 40$) or mismatched unrelated donor ($n = 11$) was performed.

A summary of the patient characteristics are shown in Table 1. All patient samples were collected after written informed consent was obtained. The study was conducted in accordance with the Declarations of Helsinki and Istanbul and had been approved by the local ethic committees.

At the time point of blood sampling, cGVHD was evaluated using criteria and guidelines of the National Institute of Health [14]. For the analysis, active cGVHD was defined as the presence of at least one diagnostic clinical sign of cGVHD and exclusion of other possible diagnosis, while resolved cGVHD was defined as the complete resolution of all organ manifestation, excluding ocular symptoms, as cGVHD may irreversibly damage the lacrimal gland. A total of 52 patients had active cGVHD [median day 977; mild ($n = 15$, median day 700, range 177–2445), moderate ($n = 19$, median day 1005, range 208–2313), severe ($n = 18$, median day 1224, range 224–2773)], 28 patients had resolved cGVHD (median day 1207, range 147–2849), and 18 patients had never experienced cGVHD (median day 1015, range 124–2655).

Table 1. Patient characteristics.

	Without cGVHD ($n = 18$)	Active cGVHD ($n = 52$)	Inactive cGVHD ($n = 28$)
Diagnosis			
Acute myeloid leukemia	6	24	7
Acute lymphoid leukemia	0	5	4
Chronic myeloid leukemia	4	5	2
Chronic myelomonocytic leukemia	1	0	0
Chronic lymphoid leukemia	2	4	0
Non Hodgkin lymphoma	1	9	7
Myelodysplastic syndrome	1	0	3
Osteomyelofibrosis	1	1	1
Multiple myeloma	1	1	4
Severe aplastic anemia	1	0	0
Hodgkin's disease	0	3	0
Patient age (mean, range)	50 (27–64)	46 (16–68)	42 (19–66)
Source ($P = 0.004$)			
Bone marrow	6	3	2
Peripheral blood stem cells	12	49	26
Days after transplantation (median; range) ($P = 0.720$)	901 (124–2655)	742 (177–2773)	799 (147–2849)
Donor ($P = 0.358$)			
matched related donor	10	22	15
matched unrelated donor	5	26	9
mismatched unrelated donor	3	4	4
Donor lymphocyte infusion ($P = 0.74$)	4	12	8
Intensity of immunosuppression ($P < 0.001$)			
none	18	10	10
low	0	10	6
moderate	0	23	12
high	0	9	0

cGVHD, chronic graft-versus host disease.

The centers' policy on first- and second-line treatment of cGVHD was based on the recently published recommendations of the consensus conference on clinical practice in cGVHD [15,16]. In the majority of patients, first-line treatment of cGVHD consisted of prednisolone in combination with a calcineurin-inhibitor. The intensity of current immunosuppression was classified as described by Mitchell [17]: low intensity: treatment with prednisone alone at dose of <0.5 mg/kg/day, moderate intensity: single agent prednisone ≥ 0.5 mg/kg/day and/or any other single agent or modality, high intensity: prednisone ≥ 0.5 mg/kg/day and two or more agents or modalities. Topical agents were not captured. The difference of intensity of immunosuppression between patients with resolved and active cGVHD (Table 1) was significant ($P = 0.024$). None of the patients received treatment with rituximab before analysis of lymphocyte subsets.

The patients with cGVHD received antiviral (e.g. aciclovir), antibiotic (e.g. azithromycin in the presence of bronchiolitis obliterans), and anti-fungal prophylaxis in accordance with local guidelines. Yearly vaccination against seasonal influenza was recommended for all patients. Vaccination against pneumococci was usually performed 12 months after HSCT with a polysaccharide-based vaccine. Based on hospital records dating from day 100 after HSCT until study entry, a retrospective analysis was performed to document the occurrence of infections as described by Cordonnier [18]. As the hospital database may not contain all grade 1 infections (infectious episodes treated as outpatient by the local practitioner), we decided to analyze only grade 3 (e.g. bacteremia, aspergillosis, fungemia with severe sepsis) and grade 2 infections (all other infections requiring intravenous treatment but not requiring transfer to the intensive care unit). The grade 2 and grade 3 infections were defined as severe infections.

Twenty-four out of 98 patients received donor lymphocyte infusion (DLI) because of minimal residual disease or high risk of disease recurrence. No difference between the groups (active versus resolved versus no GVHD) was observed with respect to the number of DLI per patient who received DLI (mean 2.2, range 1–6, $P = 0.74$) as well as the time point between analysis of blood samples and last DLI (mean 743 days, range 98–2099 days, $P = 0.64$).

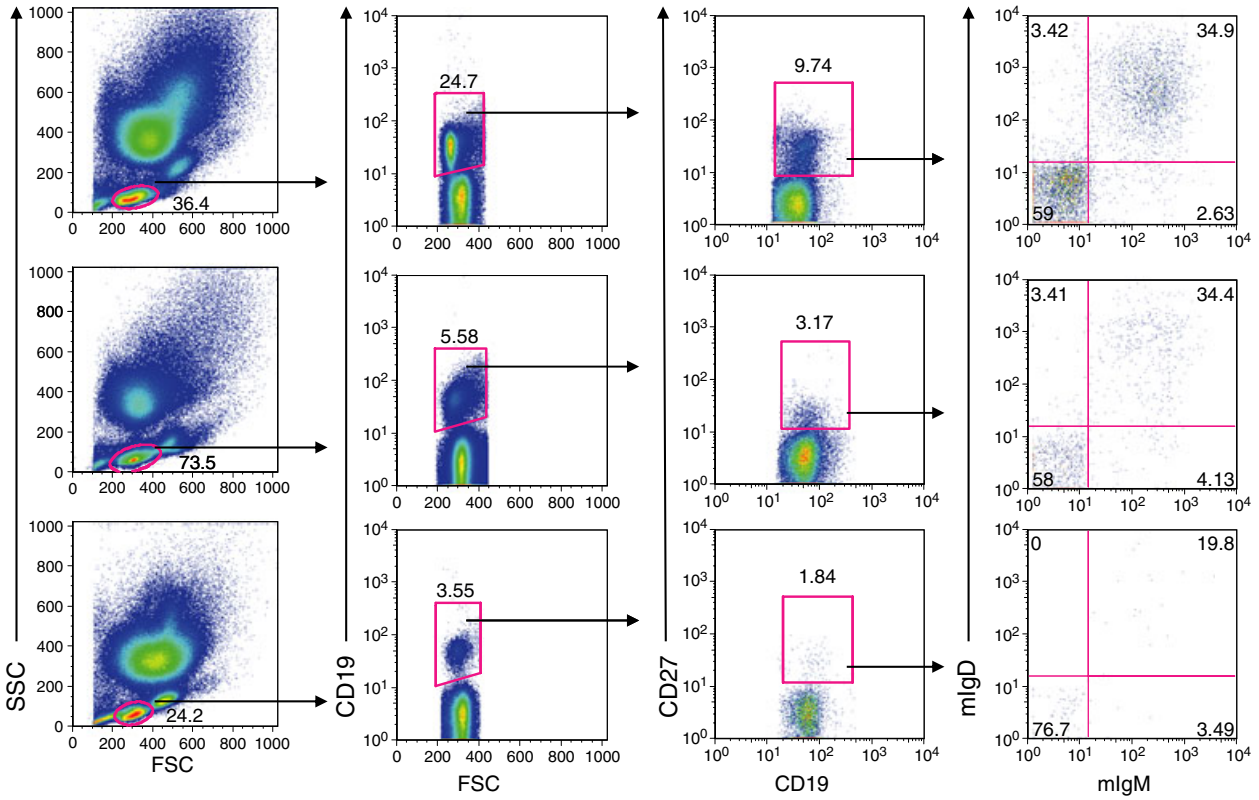

Figure 1 Representative FACS analyses of peripheral lymphocytes for patients without chronic graft-versus host disease (cGVHD) (upper row), with resolved cGVHD (middle row), and with active cGVHD (lower row). The blots depict gated lymphocytes stained for the B cell specific marker CD19, the marker for memory B cells CD27 as well as IgD and IgM to divide between IgD^- IgM^- class switched and IgD^+ IgM^+ memory B cells. Percentages of cells in the respective quadrants are provided.

Flow cytometry

Whole blood was drawn into standard ethylenediaminetetraacetic acid (EDTA) containing collection tubes and erythrocytes were lysed before staining of the peripheral blood cells for 15 min at 4 °C with a mixture of the following antibodies at optimal concentrations: anti-CD27-FITC (clone M-T271), anti-IgD-PE (clone IA6-2), anti-CD19-PECy7 (clone J3-119), anti-IgM-APC (clone G20-127); anti-CD20-FITC (clone L27), anti-CD38-PE (clone HIT2), anti-IgM-APC; anti-CD10-PE (ALB1) (BD Bioscience, San Jose, California with the exception of anti CD10 and anti-CD19 antibodies which were from Beckman Coulter, Brea, California). Four color data acquisition was performed using a FACSCalibur and data were analyzed with the help of the CELLQUEST software (BD Bioscience). Representative FACS analyses of peripheral lymphocytes are shown in Fig. 1. The absolute number of cell populations was calculated by the use of differential blood counts performed in parallel to the flow cytometry.

Measurement of immunoglobulin class and subclass levels

In 70 of 98 patients, levels of the immunoglobulins (Ig) A, G, M and IgG subclasses were measured. Total Ig levels were assayed in lithium-heparine blood samples by turbidimetric immunoassay. IgG subclass levels were measured in serum samples by nephelometry. Patients receiving immunoglobulin substitution were excluded from analysis of immunoglobulin levels.

Statistical analysis

All data were stored and analyzed using the SPSS statistical package 17.0 (SPSS Inc., Chicago, Illinois, USA). Descriptive statistics were computed for continuous and categorical variables. Medians and ranges were used to describe continuous variables, frequencies, and relative frequencies for categorical variables.

For continuous variables, determination of significant differences between the groups was realized by the analysis of variance (ANOVA) or by the Kruskal–Wallis test and Mann–Whitney *U*-tests, respectively, as nonparametric tests for comparisons of independent samples. Test selection was based on evaluating the variables for normal distribution employing the Kolmogorov–Smirnov test. Comparisons among the groups for categorical variables were done using the chi-square test or the Fisher's exact test. All *P*-values resulted from the two-sided statistical tests and in general, values of $P < 0.05$ were considered to be statistically significant. Adjustments of alpha levels were carried out using the Bonferroni correction, i.e. the level of significance was lowered to 0.05/3 = 0.017 for comparisons of control group with the other three groups.

Results

Patients with active cGVHD show reduced B cell numbers and a complete absence of the IgM^+ memory B cell pool

Absolute $CD19^+$ B cell counts were significantly lower in patients with active cGVHD (30.0×10^6/l) than in patients with resolved cGVHD (140.0×10^6/l; $P = 0.019$), in those without cGVHD (175.0×10^6/l; $P = 0.002$), or in healthy controls (140.0×10^6/l; $P = 0.003$). Absolute $CD20^+$ B cell counts were also lower in patients with active cGVHD than in patients with resolved or without cGVHD (data not shown). In addition, significant differences in absolute numbers of the $CD27^-$ B cell compartment were observed between the groups: active cGVHD (14.6×10^6/l) vs. resolved cGVHD (90.0×10^6/l) or vs. without cGVHD (158.0×10^6/l; both $P < 0.001$) or vs. control (87.0×10^6/l; $P < 0.001$) (Table 2).

Comparing patients with active cGVHD receiving immunosuppression or before start of immunosuppressive therapy indicated significant differences for absolute $CD19^+$ ($P = 0.003$), $CD19^+$ $CD27^-$ ($P = 0.002$), and $CD19^+$ $CD27^+$ B cells ($P = 0.007$), while no difference was observed for nonclass switched IgM^+ memory ($CD19^+$ $CD27^+$ IgM^+ IgD^+) ($P = 0.357$) and class switched memory ($CD19^+$ $CD27^+$ IgM^- IgD^-) ($P = 0.465$) B cells (Table 3).

A subset-analysis of the $CD27^-$ B cell pool performed in 75 patients revealed a lack of immature B cells ($CD10^+$ $CD38^{++}$ $CD20^+$ IgM^{++}) in all patients (0×10^6/l; range 0–29.6×10^6/l), while counts for transitional B cells ($CD10^-$ $CD38^{++}$ $CD20^+$ IgM^{++}) were lower in patients with active cGVHD (0×10^6/l; range 0–25.6×10^6/l; $n = 34$) than in patients with resolved ($n = 24$) or without ($n = 17$) cGVHD (both 0.9×10^6/l) (data not shown).

Comparing patients with mild/moderate/severe cGVHD, the analysis revealed no significant differences in absolute $CD19^+$, $CD19^+$ $CD27^-$, $CD19^+$ $CD27^+$, and nonclass switched IgM^+ memory B cells, but transitional B cell counts ($0.9/0.1/0 \times 10^6$/l) and class switched B cell counts ($1.8/0.79/0.01 \times 10^6$/l) were highest in patients with mild cGVHD. The difference between moderate and severe cGVHD was significant for class switched B cells ($P = 0.042$) (data not shown).

The intensity of immunosuppression was not associated with the counts of B cells in patients with resolved cGVHD, while increased immunosuppression resulted in significantly lower counts for $CD19^+$ ($P = 0.003$), $CD19^+$ $CD27^-$ ($P = 0.002$), and $CD19^+$ $CD27^+$ ($P = 0.007$) in the active cGVHD group (Table 3). In addition, lower

Table 2. Analysis in patients with and without cGVHD.

	Control (n = 16)	Without cGVHD (n = 18)	Active cGVHD (n = 52)	Inactive cGVHD (n = 28)	
B cells					
$CD19^+$ (10^6/l)	140.0* (91–271)	175.0† (200–553)	30.0 (0–259)	140.0‡ (1–856)	P = 0.003
$CD19^+$ $CD27^-$ $CD38^{++}$ $CD10^+$ $CD20^+$ IgM^+ & $CD19^+$ $CD27^-$ $CD38^{++}$ $CD10^-$ $CD20^+$ IgM^+ (10^6/l)	87.0* (15–169)	158.0† (20–520)	14.6 (0–499)	90.0‡ (0–667)	P < 0.001
all $CD19^+$ $CD27^+$ ($CD19^+$ $CD27^+$ IgD^+ IgM^+ & $CD19^+$ $CD27^+$ IgD^- IgM^- & $CD19^+$ $CD38^+$ $CD20^-$) (10^6/l)	39.0 (19–111)	16.0 (0–200)	3.0 (0–128)	12.0‡ (0–80)	P < 0.001
$CD19^+$ $CD27^+$ IgD^+ IgM^+ (10^6/l)	2.3* (0–14)	1.7† (0–10)	0 (0–1)	0.4‡ (0–18)	P < 0.001
$CD19^+$ $CD27^+$ IgD^- IgM^- (10^6/l)	27.1* (12–82)	7.4† (0–57)	0.1 (0–96)	3.4‡ (0–70)	P = 0.005
Immunoglobulins					
IgA (0.7–4 g/l)	ND	1.15†	0.64	0.97‡	P = 0.002
IgG (7–14 g/l)	ND	9.90†	5.30	7.05	P = 0.007
IgM (0.4–2.3 g/l)	ND	0.65	0.49	0.35	P = 0.478
IgG1 (3.82–9.29 g/l)	ND	6.37†	4.32	4.81	P = 0.266
IgG2 (2.42–7.00 g/l)	ND	2.27	1.40	1.84	P = 0.679
IgG3 (0.28–1.76 g/l)	ND	0.63†	0.31	0.62‡	P = 0.035
IgG4 (0.04–0.86 g/l)	ND	0.08	0.05	0.09	P = 0.512

cGVHD, chronic graft-versus host disease.
Significant differences marked as follow: *active cGVHD versus control, †active versus without cGVHD, ‡active versus inactive cGVHD.
The P-values refer to the comparison between the three groups of patients.

counts of class switched memory B cell were observed in patients with active cGVHD (0.1) compared with patients with resolved cGVHD (3.4×10^6/l; P = 0.032), without cGVHD (7.4×10^6/l; P = 0.003), or vs. the control group (27.1×10^6/l; P < 0.001) (Table 2).

Of interest, nonclass switched IgM^+ memory B cells are frequently lacking in patients with active cGVHD (0×10^6/l; range 0–1×10^6/l) in contrast to patients with resolved cGVHD (0.4×10^6/l; P < 0.001) or to patients without cGVHD (1.7×10^6/l; P < 0.001) or to the control group (2.3×10^6/l; P < 0.001). In addition, patients with resolved cGVHD receiving no immunosuppressive therapy had higher nonclass switched IgM^+ memory B cell counts (2.9×10^6/l) when compared with patients of the same group with mild (0.9×10^6/l) or moderate (0.2×10^6/l) immunosuppressive therapy (table 3). The difference between patients with and without immunosuppressive therapy did not reach statistical significance (P = 0.377). No correlation was observed for B cells with the age of the patient. In patients with cGVHD, no significant differences were observed with respect to B cell-counts before and after 1 year after HSCT (data not shown).

Serum levels of IgG are significantly reduced in patients with active GVHD

Data for serum Ig as well as IgG subclass analyses were available for 70 of the 98 patients (Table 2). Although no decline in mean levels of IgA (1.05 g/l), IgG (7.65 g/l), IgM (0.81 g/l), IgG1 (5.38 g/l), IgG2 (2.29 g/l), IgG3 (0.60 g/l), IgG4 (0.17 g/l) was measured for the whole population, significant differences were observed among the groups. Comparing patients without cGVHD with patients with active cGVHD revealed significantly higher levels of IgA (1.15 g/l vs. 0.64 g/l; P = 0.001), IgG (9.90 g/l vs. 5.3 g/l; P = 0.003), IgG1 (6.37 g/l vs. 4.32 g/l; P = 0.003), and IgG3 (0.63 g/l vs. 0.31 g/l; P = 0.008).

Patients with resolved cGVHD versus patients without cGVHD differed significantly with respect to IgG levels (7.05 g/l vs. 9.90 g/l; P = 0.029). In addition, we observed significant differences for IgG2 between patients with mild and severe cGVHD (2.17 g/l vs. 0.91 g/l; P = 0.006) as well as between patients with moderate cGVHD and severe cGVHD (1.71 g/l vs. 0.91 g/l; P = 0.023). Of interest, IgG2 was the only Ig that correlated significantly with the onset of severe infections (P = 0.029), while it was not significant for IgA (P = 0.158), IgG (P = 0.229), IgM (P = 0.114), IgG1 (P = 0.643), IgG3 (P = 0.080), and IgG4 (P = 0.682).

Patients with active GVHD and immunosuppressive therapy seemed to have slightly lower Igs and IgG subclasses when compared with patients with active GVHD receiving no immunosuppression (Table 3).

The analysis of the relationship of Igs and IgG subclasses with B cells revealed a correlation between $CD19^+$ cells and IgA (P = 0.043), IgG2 (P = 0.003), IgG4 (P = 0.005)

Table 3. Intensity of immunosuppression.

	None		Low intensity		Moderate intensity		High intensity		
	Inactive GVHD	Active GVHD	Inactive GVHD	Active GVHD	Inactive GVHD	Active GVHD	Inactive GVHD	Active GVHD	
B cells									
$CD19^+$ (10^6/l)	145 (0–856)	163 (20–1040)	140 (4–556)	32 (0–140)	111 (3–710)	11 (0–2590)	none	23 (0–480)	$P = 0.003$
$CD19^+$ $CD27^-$ $CD38^{++}$ $CD10^+$ $CD20^+$ IgM^+ & $CD19^+$ $CD27^-$ $CD38^{++}$ $CD10^-$ $CD20^+$ IgM^+ (10^6/l)	*130* (0–406)	55 (0–120)	66 (2–390)	15 (0–130)	44 (2–667	8 (0–500)	none	4 (0–380)	$P = 0.002$
all $CD19^+$ $CD27^+$ ($CD19^+$ $CD27^+$ IgD^+ IgM^+ & $CD19^+$ $CD27^+$ IgD^- IgM^- & $CD19^+$ $CD38^+$ $CD20^-$) (10^6/l)	10 (0–34)	10 (0–70)	20 (1–80)	3 (1–10)	10 (1–43)	2 (0–60)	none	2 (0–130)	$P = 0.007$
$CD19^+$ $CD27^+$ IgD^+ IgM^+ (10^6/l)	2.9 (0–13)	0 (0–1,4)	0.9 (0–13)	0 (0–0,7)	0.2 (0–17)	0 (0–2,6)	none	*0* (0–0)	$P = 0.357$
$CD19^+$ $CD27^+$ IgD^- IgM^- (10^6/l)	2.9 (0–29)	0.04 (0–5)	3.4 (1–69)	0.91 (0–9)	4.1 (0–19)	0.20 (0–37)	none	*0.01* (0–96)	$P = 0.465$
Immunoglobulines									
IgA (0.7–4 g/l)	1.41	1.37	1.30	0.99	0.90	0.40	none	0.10	$P = 0.001$
IgG (7–14 g/l)	9.25	11.7	7.8	4.0	6.4	5.07	none	3.17	$P = 0.001$
IgM (0.4–2.3 g/l)	0.35	0.90	0.5	0.49	0.45	0.3	none	0.3	$P = 0.968$
IgG1 (3.82–9.29 g/l)	5.09	7.94	5.13	2.57	4.76	3.43	none	1.54	$P = 0.002$
IgG2 (2.42–7.00 g/l)	1.51	2.33	2.30	1.54	1.94	1.57	none	1.02	$P = 0.089$
IgG3 (0.28–1.76 g/l)	0.70	0.91	0.43	0.29	0.73	0.14	none	0.38	$P = 0.198$
IgG4 (0.04–0.86 g/l)	0.07	0.16	0.10	0.05	0.09	0.05	none	0.05	$P = 0.007$

GVHD, graft-versus host disease.
The *P*-values refer to the comparison between active cGVHD groups without ($n = 10$) and with ($n = 42$) immunosuppressive therapy.

as well as between $CD27^-$ B cells and IgG ($P < 0.001$) and IgG2 ($P = 0.020$). No correlation was observed for serum Ig and IgG subclasses with the age of the patient or source of stem cells (data not shown).

The rate of severe infections is associated with the complete absence of the IgM^+ memory B cell pool

Severe infections were observed in only 5.6% (1/18) of patients who never experienced cGVHD, while 51% (41/80) of patients with a history of cGVHD suffered from severe infections, which was statistically significant ($P < 0.001$). The rate of severe infections increased with severity of cGVHD as follows: mild cGVHD 33.3% (5/15), moderate cGVHD 52.6% (10/19), severe cGVHD 61.1% (11/18), and resolved cGVHD 53.5% (15/28) ($P = 0.004$).

Severe bacterial infections were observed in 34.6% (33/98) of all HSCT recipients, followed by viral infections in 14.2% (14/98) and fungal infections in 3.1% (3/98). Respiratory tract infections including pneumonia were the most frequent bacterial infections in the cohort of cGVHD patients. Significant correlations were observed between the occurrence of severe infections and B cells counts: $CD19^+$ ($P = 0.003$), the $CD27^-$ B cell compartment ($P < 0.001$), memory B cells ($P = 0.002$), $CD27^+$ IgD^+ IgM^+ ($P = 0.037$), and class switched memory B cell ($CD\ 27^+\ IgD^-\ IgM^-$) ($P = 0.014$) as well as between severe infections and IgG2 ($P = 0.029$) or the intensity of immunosuppression ($P = 0.009$). Of note, the complete absence of the IgM^+ memory B cell pool correlated significantly with the occurrence of severe infections ($P = 0.039$).

Discussion

Chronic graft-versus host disease is a complex multi-organ disease [19]. B cells and their role in cGVHD are currently receiving renewed interest [1].

B cell deficiency after HSCT is associated with late infections [10,13] and is a well-recognized feature of cGVHD that could be a result of GVHD itself or its treatment [20,21]. The rise of B cell counts is faster in patients without than in those with cGVHD [22,23]. Low numbers of B cell progenitors in patients with cGVHD were reported in the first year after HSCT [21,24]. In contrast, higher number of B cells in patients without cGVHD compared with healthy controls with focus on $CD19^+$ $CD27^-$ B cells was observed which can be explained by the course of immunoregeneration after HSCT recapitulating ontogeny during infancy [23].

In this study, the absolute $CD19^+$ B cell counts in patients with active cGVHD were lower as compared to patients with resolved or without cGVHD. In comparison to previous studies, however, the B cell counts in our cohort tended to be lower, which may be explained by the exclusion of patients with undetectable low B cell number in previous studies [8,25] and different patient populations. B cell counts are influenced by several factors such as the type of stem cell graft [24], the time after transplantation, the intensity of immunosuppression, and possibly the grade of cGVHD. However, the intensity of immunosuppression was not graded in previous studies. Therefore, the comparison with other studies is tenuous. In our analysis, B cell counts correlated significantly with intensity of immunosuppression and time of sample assessment. Kuzmina *et al.* [25] reported on a median number of 350×10^6/l $CD19^+$ B cells at a median of 46 months, while our analysis was performed at a median of 34 months showing a median B cell count of 96×10^6/l. Moreover, we observed no correlation between the severity of cGVHD and the number of $CD19^+$, $CD19^+$ $CD27^-$, $CD19^+$ $CD27^+$, and non-class switched IgM^+ memory B cells. In contrast to the impact of cGVHD on B cells, no correlation was detectable between CD4+ T cells counts including naïve, memory and regulatory CD4+ T cells and severity of cGVHD or intensity of immunosuppression (data not shown).

Despite the fact of lower B cell counts in patients with cGVHD, beneficial effects of rituximab in treating cGVHD were reported [2–4]. The mechanisms of action of rituximab are not completely understood, but depletion of autoantibody producing B cells appears to be an important effect as demonstrated previously [26]. Moreover, a small population of T cells with immune-regulatory capacities coexpressing CD20 is described in healthy individuals as well as in patients with rheumatoid arthritis, which were eliminated along with B cells during rituximab treatment [27], which may be another mode of action of rituximab in cGVHD too.

Two main subpopulations of $CD19^+$ B cells can be distinguished: $CD27^-$ and $CD27^+$ B cells. CD27 is a marker of memory B cells and a hallmark of the direct precursors of Ig secreting cells [28]. In contrast, $CD27^-$ B cells do not express somatically mutated Ig V regions and have thus been coined ''naïve mature.'' In patients with extensive cGVHD decreased memory B cell counts were reported [10]. Moreover, memory B cells can be further subdivided in ''IgM^+ memory'' (IgM^+ IgD^+) B cells and switched memory (IgM^- IgD^-) B cells. IgM^+ memory B cells are described as highly specific, long-lived cells, generated in response to infectious agents or vaccines in a T cell-independent fashion, while switched memory B cells require T cell costimulation to produce high affinity IgG and other isotypes of antibody within germinal centers of lymphoid tissues [29]. Greinix *et al.* [8] demonstrated that patients with active cGVHD had significantly lower

numbers of nonclass switched ($CD19^+$ $CD27^+$ IgD^+) as well as class-switched ($CD19^+$ $CD27^+$ IgD^-) memory B cells as compared with patients who never experienced cGVHD. D'Orsogna [30] reported on defects of both, switched and nonswitched memory B cells in HSCT recipients. With respect to total memory (6.7 vs. 21.0) and switched memory (4.6 vs 8.0) B cell counts (in 10^6/l) in patients with and without cGVHD, our results (as shown in Table 2) are comparable with those reported by D'Orsogna. In addition to the reported analysis [30], we further subdivided the group of cGVHD patients into those with active and those with resolved GVHD, which revealed significantly lower counts of class-switched memory B cells in patients with active cGVHD.

Non-switched IgM^+ memory B cells seem to play a key role in the T cell-independent response to bacterial polysaccharides as poor antibody responses have been reported after vaccination with a polysaccharide-based vaccine in HSCT recipients, especially in patients with cGVHD [31–33]. The analysis of nonswitched IgM^+ memory B cells in our cohort revealed a notable lack of these cells specifically in patients with active cGVHD but not in those with resolved cGVHD nor in those without disease or in the control group. In addition, patients with resolved cGVHD receiving no immunosuppressive therapy had significantly higher nonswitched IgM^+ memory B cell counts when compared with patients of the same group with mild or moderate immunosuppression. Patients with active cGVHD receiving exclusively systemic immunosuppression lacked these subsets of B cells which impedes the differentiation of the effect of immunosuppression versus cGVHD.

Kruetzmann [34] demonstrated that nonswitched IgM^+ memory B cells are absent in congenital asplenics and severely depleted following splenectomy. Thus, nonswitched IgM^+ memory B cells are dependent upon a functional spleen for their generation and/or survival. In HSCT recipients a reduction in spleen size was reported, which correlated with the presence of cGVHD and the risk of pneumococcal sepsis [35]. As cGVHD is associated with functional asplenia [35–38], which occurs in up to 15% of HSCT recipients [36] and contributes to the high susceptibility to bacterial infections in patients with cGVHD [25,33,37], the depletion of nonswitched IgM^+ memory B cells in patients with active cGVHD may be the consequence of functional asplenia. In our cohort, 51% of the patients with cGVHD developed severe infections and required hospitalization. This was significantly (by 10-fold) higher when compared to patients without cGVHD. Two patients died from sepsis. Furthermore, a significant correlation was observed for the occurrence of severe infections and the complete absence of the IgM^+ memory B cell pool. The spectrum of infections included mainly bacterial infections, which is in line with reported results [39].

Defects of humoral immunity together with hypogammaglobulinaemia are well documented [13,40–42]. IgG serum levels can be taken as surrogate markers for B cell function. However, it is important to note that IgG levels may, in part, reflect residual host plasma cell production, as host plasma cells may persist for up to 2 years after HSCT [43]. In cGVHD patients with hypogammaglobulinaemia, decreased total ($CD19^+$) B cell counts as well as nonclass-switched and class-switched memory B cells were reported as compared with the patients with normo- or hypergammaglobulinaemia [25]. In line with these results we found significantly decreased lgG serum levels in cGVHD patients and significant differences in IgG2 levels between patients with severe versus mild or moderate cGVHD. The impaired ability to produce IgG2 and lower levels for IgG2 antibodies to pneumococcal polysaccharides in cGVHD patients [30] contribute to the observed increased susceptibility to severe infections in our cohort of patients with cGVHD, because (1) a significant correlation was observed between IgG2 and the onset of severe infection, (2) IgG2 antibodies play a key role in the antibody response against pneumococcal polysaccharides, and (3) unresponsiveness to capsular polysaccharide vaccine was reported in IgG2 deficient patients [44], which underlines the need to use the recommended pneumococcal conjugated vaccine, especially in cGVHD patients [45].

In addition, patients with active cGVHD had significantly lower IgA serum levels as compared to patients without cGVHD. Low IgA serum levels correlated with decreased number of B cells which is in line with the results of other study groups [13,30,43]. As higher rates of recurrent infections in patients with primary IgA deficiency and concomitant immune defects were reported [46] and IgA-deficiency is associated with autoimmunity [47], our findings may be of interest. However, the possible role of IgA deficiency in the pathogenesis of cGVHD needs to be determined in future studies.

In summary, our data underline a close association of diminished B cell counts with cGVHD as well as the rate of severe infections. The lack of nonclass-switched IgM^+ memory B cells in patients with cGVHD may indicate functional asplenia resulting in immune compromise leading to an increased rate of severe, mostly bacterial infections in cGVHD patients. Analysis of B cell subsets can provide a diagnostic tool for monitoring cGVHD activity but requires prospective evaluation.

Authorship

IH: study concept, performed study, data collection, analysis and interpretation, wrote and approved the manuscript. BMH: study design, collected and analyzed data, final approval of manuscript. GK: analyzed data, final

approval of manuscript. EH: provision of study materials and patients, final approval of manuscript. PH: data collection, analysis and interpretation, final approval of manuscript. ME: provision of study materials, final approval of manuscript. MF: provision of study materials and patients, final approval of manuscript. DW: study concept and design, performed study, data collection, analysis and interpretation, wrote and approved the manuscript.

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