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Clinical significance of autoantibodies in a large cohort of patients with chronic graft-versus-host disease defined by NIH criteria

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Abstract

There is an unmet need for identifying new clinical biomarkers in chronic Graft-versus-Host-disease (cGVHD) suitable for diagnosis and disease monitoring. Circulating autoantibodies represent an ongoing immune response and suggest a pathogenic role for B cells in cGVHD. Autoantibodies could be useful markers of cGVHD disease activity, severity, or organ specificity; however, their clinical utility is not established. The focus of this study was to determine the incidence and associations of a broad array of clinical autoantibodies with cGVHD manifestations in a large patient cohort characterized by NIH criteria. A panel of 21 circulating antibodies commonly used in clinical medicine was tested in 280 cGVHD patients (70% severe) enrolled in a cross-sectional prospective natural history study. Median cGVHD duration was two years. Patients with circulating autoantibodies (62%) had significantly higher levels of IgM ($P < 0.0001$), IgG ($P < 0.0001$), and IgA ($P = 0.001$), elevated uric acid ($P = 0.008$) and total protein ($P = 0.0004$), and higher numbers of CD31 ($P = 0.002$), CD41 ($P = 0.001$), CD81 ($P = 0.023$) T cells, and CD191 B cells ($P < 0.0001$). Multiple antibodies were detected in 35% of patients. Prior rituximab therapy ($n = 66$) was associated with reduced presence of autoantibodies (48 vs. 66% $P = 0.01$). Only oral

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Additional Supporting Information may be found in the online version of this article.

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cGVHD was significantly associated with presence of autoantibodies in this study ($P = 0.028$). No significant associations were found between cGVHD activity and severity, and presence of autoantibodies. Circulating autoantibodies are common in patients with advanced cGVHD. Their presence is associated with better quantitative immunologic reconstitution but does not have utility as a clinical biomarker of cGVHD.

Introduction

Chronic graft-versus-host disease (cGVHD) remains a serious late complication of allogeneic hematopoietic stem cell transplantation (HCT) [1–4]. The clinical presentations of cGVHD are similar to autoimmune disorders such as scleroderma, systemic lupus erythematosus (SLE), Sjogren's syndrome and rheumatoid arthritis [5–7]. These autoimmune disorders are significantly associated with antibody production leading to target tissue damage, immune complex formation and tissue deposition [8–10]. Both allo- and auto antibodies are commonly observed in cGVHD, but their role in the pathogenesis of cGVHD still remains unclear [11–14]. Antibodies may be present before first clinical presentation of cGVHD [15] similar to autoimmune diseases [16] and anti-HY allo antibodies have been significantly associated with the development of cGVHD [17]. Antibodies may also reflect the presence and intensity of the autoimmune response in cGVHD [18]. Nucleic acid components of DNA- and RNA- autoantigens are released due to tissue damage and apoptosis in graft-versus-host reactions. After binding to these antigens antibodies may serve as a stimulus for activation of autoreactive B cells, complement fixation, immune complex formation and engagement of Fc and Toll like receptors (TLR) [19]. In addition, deficient clearance of the damaged patterns (DAMPS) can lead to accumulation and chronic activation of the innate immunity [20]. Besides, the presence of circulating antibodies, a disturbance of B-cell homeostasis with prolonged reconstitution of B cells, accumulation of atypical B cells due to an excess of B-cell activation factor (BAFF) and over-activation of B-cells were described in patients with cGVHD [21–24]. Anti-CD20 B cell depletion has been tested in prophylaxis and treatment of cGVHD with mixed success [25–32].

There is a prominent unmet need for developing clinically useful biomarkers for cGVHD diagnosis and disease monitoring. However, in spite of evidence of their frequent detection in patients, the biological significance and role of autoantibodies in cGVHD is not defined [33]. The 2005 NIH consensus project provided new classification of cGVHD diagnosis and staging [2,34]. This classification leads to better disease characterization, stricter diagnostic definitions of cGVHD, and separation from acute GVHD. Using these standardized and more detailed cGVHD criteria may enhance the ability to detect significant associations between circulating auto antibodies and disease manifestations. Here, we investigated a broad spectrum of autoantibodies for their potential utility in defining cGVHD activity, severity and organ specificity in a large cohort of cGVHD patients with wide spectrum of organ involvement described by NIH criteria.

Methods

Study conduct

Patients enrolled in the natural history study of clinical and biological factors determining outcomes in cGVHD (NCI protocol clinicaltrials.gov identifier: NCT00331968) from October 2004 to May 2013 were included in this analysis. This protocol provides a one-time week-long evaluation during which all patients undergo comprehensive assessment of cGVHD by the multidisciplinary team at the NIH Clinical Center. Peripheral blood samples were analyzed for presence of a panel of autoantibodies which are commonly used in clinical medicine. Patients were subdivided in two groups—autoantibody positive and autoantibody negative, based on absolute values or titers. Activity of cGVHD was defined as clinician decision to intensify systemic therapy and recently validated by this group, as reported by Grkovic et al. [35]. Intensity of systemic therapy at study entry was defined as no therapy, mild (single agent prednisone 0.5 mg/kg/day), moderate (prednisone ≥ 0.5 mg/kg/day and/or any single agent/modality), and high (2 or more agents/modalities ± prednisone ≥ 0.5 mg/kg/day) [36]. The study protocol was approved by the NCI IRB and all patients signed written informed consent.

Laboratory assessments

Blood samples were submitted to the Department of Laboratory Medicine, Clinical Center, NIH. Evaluation of immune status was performed by detecting CD3+, CD4+, CD8+ T cells, CD19+ B cells, and NK cells (CD56+CD3−). Serum levels of IgG, IgM, and IgA immunoglobulins were quantified by nephelometry using Beckman Coulter IMMAGE (Beckman Coulter Inc., Brea, CA) and Siemens Dimension Vista (Siemens Diagnostics, Tarrytown, NY). The patient's serum was screened for the 21 autoantibodies listed in Supporting Information Table I with details of methodologies for antibody assessment. Antibody testing was performed by ELISA and/or immunoflouresence. For immunoassay-based methods used to screen antibodies patient values were compared to the relevant reference interval as provided by the manufacturer. In house ELISA antibody testing was performed on the DSX ELISA processing system. (Dynex Technologies, Chantilly, VA). Patients positive for ENA were further screened for anti-SmRNP, Smith, SSA, and SSB autoantibodies.

Statistical analysis

Factors which were reported as a continuous parameter, or which could be essentially considered as if continuous, were compared between two groups, positive and negative for autoantibodies, using a Wilcoxon rank sum test. Ordered categorical parameters were compared between the two groups using a Cochran-Armitage test for trend. Dichotomous parameters were compared between the two groups using Fisher's exact test. Unordered categorical parameters were compared between two groups using Mehta's modification to Fisher's exact test.

For univariate analysis the association of different autoantibodies with cGVHD characteristics such as activity, severity, organ specificity, duration of cGVHD with time after HCT, and intensity of immunosuppression were selected. Following an initial screening

by univariate methods, multivariable logistic regression analysis was used to identify a set of factors which could jointly impact the particular parameter under consideration. Covariates with a *P*-value <0.05 were entered into the multivariate analysis. All *P*-values reported are two tailed, and presented without any formal adjustment for multiple comparisons. In view of the number of tests performed, *P*-values such that $P < 0.005$ could be considered statistically significant while $0.005 < P < 0.05$ would represent strong trends. Titers of autoantibodies are shown in medians. Analyses were performed using R, SPSS 20.0 (IBM Company, Chicago, IL), and SAS version 9.3 (SAS Institute, Cary, NC).

Results

Patient characteristics

Two-hundred eighty patients with cGVHD, median age of 46 years (range, 4–70) at a median of 36 (range, 4–297) months after HCT were included in the study. The clinical characteristics of the patients are shown in Table I. Patients were subdivided in two groups, positive with autoantibodies ($n = 174$) and negative, without antibodies ($n = 106$). No significant differences in transplant characteristics were observed between these two groups. Chronic GVHD assessment was conducted according to the NIH consensus criteria, cGVHD patient characteristics are shown in Table II. All patients were in remission of their original hematologic disease. The majority of patients (70%) had severe cGVHD. 33% had *de novo* onset cGVHD. Median time to onset of cGVHD was 7 months. The most frequent organ manifestations of cGVHD at study entry were skin (78%), eyes (77%), and lung involvement (75%), and severe disease (NIH scores 2–3) was present in 61%, 43%, and 35% of these organs, respectively. Over ninety percent of patients had multiorgan involvement, identified as more than two organs. The median duration of cGVHD was 2 years (range, 0.4–222 months) (Table II).

Prevalence of autoantibodies

Autoantibodies were identified in 62% ($n = 174$) of patients and multiple antibodies were detected in 35% of patients ($n = 61$) Fig. 1. According to onset of cGVHD, the distribution of autoantibodies was 30% in *de novo*, 28% in quiescent and 42% in progressive cGVHD (NS) (Table II). The most frequent antibodies were ANA (29%) and RF (13%). Furthermore, 74% of patients who had antibodies exhibited a longer duration of cGVHD (>1 year) (NS). Patients with circulating autoantibodies had significantly higher levels of IgM, IgG, and IgA, elevated uric acid and total protein, and higher numbers of CD3+, CD4+, CD8+ T cells, and CD19+ B cells (Table III).

Autoantibodies according to NIH severity and activity

According to the cGVHD NIH global severity score, autoantibodies were present in 60% with severe (117/196) and in 68% (54/80) with moderate cGVHD ($P = 0.27$) (Table II). We further compared overall antibody incidence, individual autoantibodies, and their titers to severity of cGVHD. Among those with autoantibodies, no association between overall incidence of autoantibodies and severity of cGVHD was detected (Supporting Information Table II). Active cGVHD was present in 43% ($n = 119/280$) of patients and within this subset, circulating autoantibodies were present in 60% ($n = 71/119$), (Table II). No

significant relationship between overall incidence of autoantibodies and activity of cGVHD was observed (Supporting Information Table III).

Circulating autoantibodies in cGVHD patients with different organ manifestations

Next, we studied individual organ involvements with the aim to identify organ-specific antibodies. Only oral cGVHD showed significantly higher frequency of overall autoantibodies ($P = 0.028$) mainly ANA (79% vs. 60%, $P = 0.0023$, ANA titer 1.3U vs 0.7U, $P = 0.0008$). No significant association between overall incidence of autoantibodies or titers with skin, liver, lungs or eye cGVHD was observed. Significantly higher titer of RF was seen in patients ($n = 108$) with moderate to severe joints and fascia involvement (20 vs. 15 IU/ml, $P = 0.003$).

Presence of autoantibodies according to duration and therapy of cGVHD

When short (≤1 year) and longer (>1 year) cGVHD durations were compared, a trend towards higher incidence of ACA G titer (2.6 vs. 0.74 GPL, $P = 0.028$) and RF (15% vs. 6%, $P = 0.07$) were detected in the longer duration cGVHD group. When separated by intensity of systemic therapy (0–4), 74% of patients without any systemic therapy at the time of evaluation had one or more antibodies, compared to 26% of those on some form of systemic treatment for cGVHD ($P = 0.06$). No difference was seen between previous lines of immunosuppressive therapy for cGVHD; however, patients who received prior anti-CD20 antibody therapy for cGVHD had significantly lower incidence of autoantibodies (48% vs. 66%, $P = 0.01$), as seen in Fig. 2.

Multivariable analysis

The following variables (uric acid, total protein, IgG, IgM, IgA, C4, CD3+, CD4+, CD8+ T cells, CD19+ B cells, cGVHD of oral mucosa, liver, GI, lung involvement, and intensity of immunosuppression) were considered for inclusion in a multivariable model for predicting whether a patient had any positive antibodies or not. The above mentioned laboratory parameters were significantly different in univariate analysis (Table III) with a $P < 0.05$. The final multivariable model showed that higher levels of IgG and IgM, and less GI and lung cGVHD involvement were independently associated with the presence of autoantibodies (Table IV).

Discussion

The primary focus of this study was to determine the prevalence and potential role of autoantibodies as disease markers in a large cohort of patients with cGVHD characterized by NIH criteria. The dysregulated humoral immunity in cGVHD can be comparable to autoimmune diseases (AID), where the pathogenic role of autoantibodies has been confirmed. Furthermore, higher titers of autoantibodies may be useful for monitoring diseases activity in AID [37].

A substantial proportion of cGVHD patients demonstrated autoantibodies (62%) but no significant association with severity, activity, and clinical characteristics of cGVHD was found. In our study, transplant characteristics and duration of cGVHD were similar between

autoantibody positive and autoantibody negative groups. In accordance with our analysis, where patients with long-lasting cGVHD have a trend towards higher incidence of autoantibodies, Fujii et al. reported increased frequency of anti-dsDNA antibody and ANA in patients with late (≥ 9 months after HCT) compared with early onset (<9) cGVHD, suggesting a predominant immune activation and accumulation of autoantibodies in the later stage of the disease [18]. Additionally in his analysis, BAFF level was elevated, which is known to rescue autoreactive B-cells resulting in expansion of anti-DNA antibodies [38]. In this study patients with autoantibodies had a significantly better immune reconstitution regarding overall higher T-, B cells and serum immunoglobulin numbers.

The detection of autoantibodies was more frequently found in patients with oral cGVHD involvement and less in GI and lung cGVHD involvement. The latter may suggest direct pathogenic role of autoantibodies with germinal center formation and immunoglobulin deposition in affected tissues as recently described by Flynn et al. [39]. Of note, ANA was the primary autoantibody detected in patients with oral cGVHD. Positive ANA was described previously in six studies including 293 cGVHD patients that did not apply NIH diagnosis and staging criteria. In these studies the median prevalence of ANA positivity was 38% (range 22% to 82%) [5,13,40–43] compared with 29% in our cGVHD cohort. However, ANA positivity can be seen in 15% of healthy adults and 8% of children, usually at a low titer [44]. Also, ANA is more frequent in individuals >60 years and women (25%). In contrast to published data on 102 patients [13,40,43], the incidence of ds-DNA in our study was only 3% compared to 15% (range, 3–31%). Anti-phospholipid antibodies (ACA M and G) are found in patients with localized systemic sclerosis and in SLE [45], in our study ACA IgM was significantly elevated in patients with moderate cGVHD, whereas ACA IgG was only found in patients with GI cGVHD. Anti-SSA (Ro) and anti-SSB (La) are highly specific for *Sjögren's syndrome* and are seen in approximately one-third of patients with systemic sclerosis predominantly with sicca manifestations, and up to 60% in patients with SLE. In our cGVHD cohort, the majority of patients positive for SAA had sicca. RF can be found in a wide variety of clinical settings and, in rheumatoid arthritis, high titer of RF correlate with severe erosive joint disease [46]. From 34 previously reported cGVHD patients, 9% (range, 8–10) were positive for RF what is similar to our results [13,40,43]. In our cohort, other autoantibodies were much less common.

Here we tested a large number of well characterized cGVHD patients using NIH criteria for diagnosis and staging who had a wide variety of disease manifestations [35]. However, this study does have limitations. The absence of longitudinal analysis limits further interpretation of the association of the antibodies and cGVHD. Autoantibodies can be also present prior to the development of autoimmune diseases [16] and are described in post-allogeneic patients without cGVHD [15]. Our antibody panel excluded alloantibodies which have been associated with development of cGVHD [12,17]. Finally, it is challenging to draw conclusions on the role of single autoantibody higher titers due to their overall low incidence.

In conclusion, autoantibodies are very common in cGVHD reflecting the dysregulated humoral immunity in these patients and their levels correlate with better quantitative immunological reconstitution. However, autoantibodies show no relationship to activity,

severity of cGVHD and have very limited organ specificity, so at this stage they should not be used as clinically useful markers in cGVHD patients. Further research should elucidate the biological role of these antibodies in cGVHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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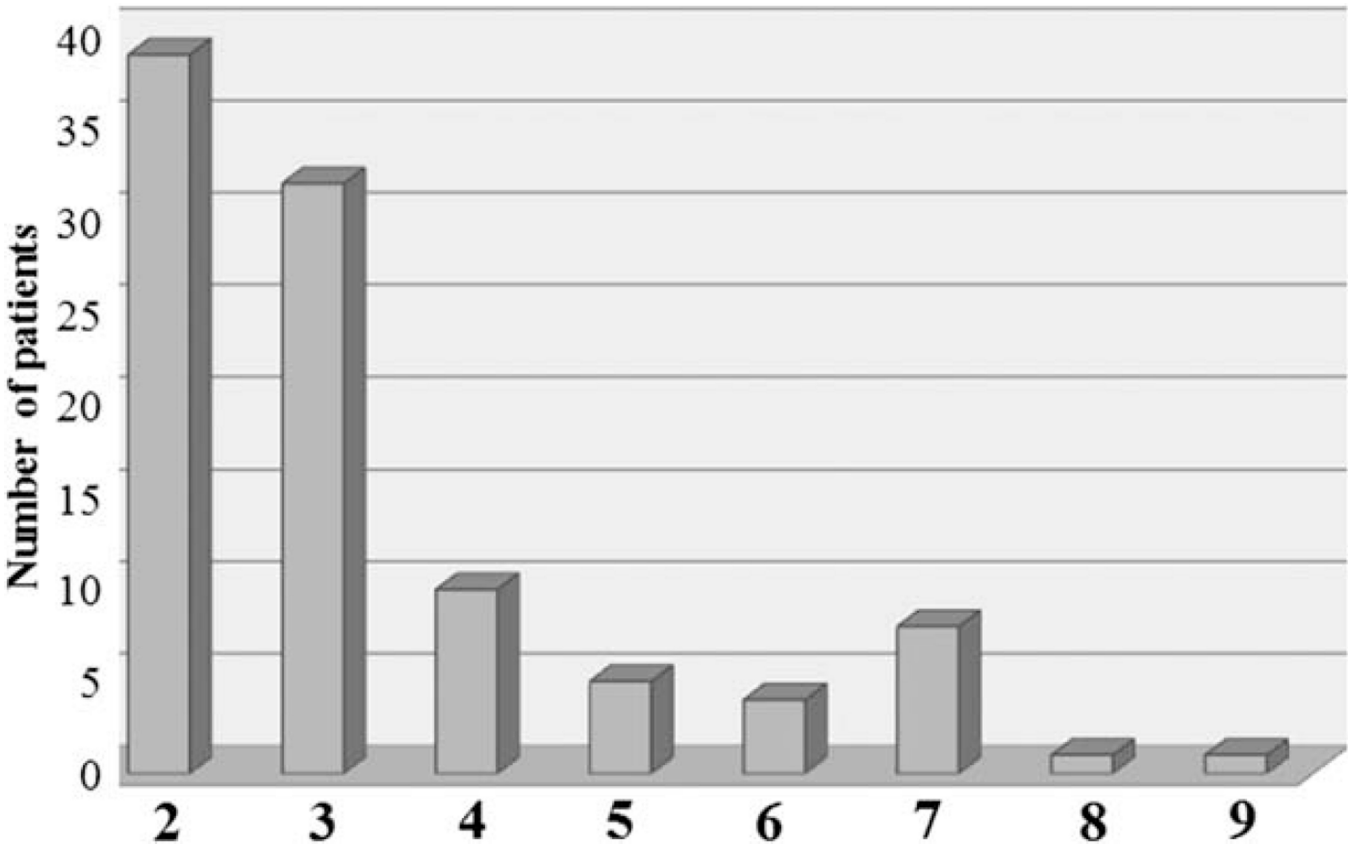

Figure 1.
Distribution of multiple autoantibodies in patients with cGVHD. Each bar represents the number of patients with numbers of autoantibodies (≥ 2).

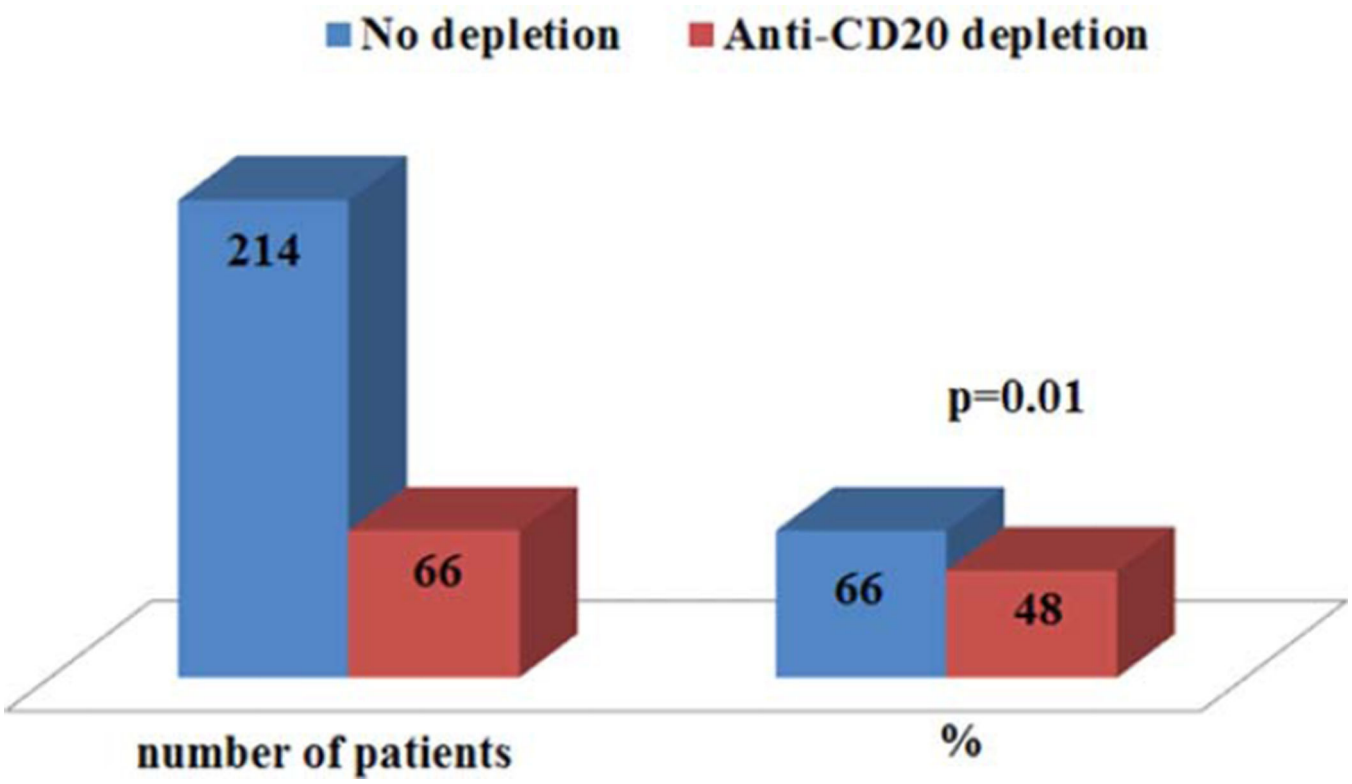

Figure 2.

Presence of autoantibodies according to B cell depletion therapy. The left blue bar represent patients without B cell depletion, in red are patients who received anti-CD20 therapy. On the right side the percentages of patients positive for autoantibodies according to the B cell depletions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I

Patient Characteristics

	Patients *n* = 280 (%)	Antibody positive *n* = 174 (%)	Antibody negative *n* = 106 (%)
Median age in years, range	46 (4–70)	46 (4–69)	45 (5–70)
Patient			
Male	158 (56)	100 (57)	58 (55)
Female	122 (44)	74 (43)	48 (45)
Gender match[a] F/M	56 (20)	30 (17)	26 (25)
Diagnosis[a]			
Acute leukemia	127 (45)	78 (45)	49 (46)
CML	38 (14)	26 (15)	12 (11)
Malignant lymphoma	81 (29)	46 (26)	35 (33)
MM	15 (5)	12 (6)	3 (3)
Nonmalignant	16 (6)	10 (6)	6 (6)
Other[b]	3 (1)	2 (1)	1 (1)
Conditioning regimen			
Myeloablative	153 (55)	94 (54)	59 (56)
RIC	127 (45)	80 (46)	47 (44)
TBI	105 (38)	59 (34)	46 (46)
Stem cell donors			
Related	173 (62)	110 (63)	63 (59)
Unrelated	107 (38)	64 (37)	43 (41)
HLA-identical	228 (81)	143 (82)	85 (80)
HLA-mismatched[a]	45 (16)	27 (16)	18 (17)
Stem cell source			
Bone marrow	52 (19)	29 (17)	23 (22)
PBSC	218 (78)	138 (79)	80 (75)
Cord blood	10 (4)	7 (4)	3 (3)
Median time form HSCT, months, range	36 (4–297)	36 (4–258)	36 (5–297)

N, number; GVHD, graft-versus-host disease; TBI, total body irradiation; RIC, reduced intensity conditioning; PBCS, peripheral blood stem cells.

[a]Missing values.

[b]Other diseases include sarcoma, CML-chronic myeloid leukemia, MM, multiple myeloma.

None of the comparisons were significant different.

TABLE II

Chronic Graft-Versus-Host Disease Characteristics

	All patients *n* = 280 (%)	Positive *n* = 174 (%)	Negative *n* = 106 (%)
Severity at enrollment			
Mild	4 (1)	3 (2)	1 (1)
Moderate	80 (29)	54 (31)	26 (24)
Severe	196 (70)	117 (67)	79 (75)
Onset type of cGVHD			
de novo	91 (33)	53 (30)	38 (36)
Quiescent	79 (28)	48 (28)	31 (29)
Progressive	110 (39)	73 (42)	37 (35)
Organ involvement			
Skin	217 (78)	134 (77)	83 (78)
Score 2–3	172 (61)	105 (60)	67 (63)
Eyes	216 (77)	132 (76)	84 (79)
Score 2–3	121 (43)	74 (43)	47 (44)
Oral mucosa	184 (66)	123 (71)[b]	61 (58)
Score 2–3	40 (14)	30 (17)	10 (9)
Liver	135 (48)	91 (52)	44 (42)
Score 2–3	46 (16)	30 (17)	16 (15)
Lungs	210 (75)	122 (70)	88 (83)
Score 2–3	99 (35)	54 (31)	45 (42)
GI	122 (44)	66 (38)	56 (35)
Score 2–3	33 (12)	20 (11)	13 (12)
Genital	80 (29)	49 (28)	31 (29)
Score 2–3	61 (22)	38 (22)	23 (22)
Joint and Fascia	168 (60)	102 (59)	66 (62)
Score 2–3	108 (39)	61 (35)	47 (44)
≤ 2 organs	25 (9)	17 (10)	8 (8)
> 2 organs	255 (91)	157 (90)	98 (92)
Activity of cGVHD[a]			
Active	119 (42)	71 (41)	48 (45)
Nonactive	159 (58)	102 (59)	57 (54)
Treatment of cGVHD			
Lines for prior therapy	4 (0–9)	4 (0–9)	4 (0–9)
Intensity of IS[a]			
None	53 (19)	39 (22)	14 (13)
Mild	16 (6)	8 (5)	8 (8)
Moderate	99 (35)	66 (38)	33 (31)
High	110 (39)	60 (34)	50 (47)
Anti-CD20 for cGVHD	66 (24)	32 (18)	34 (32)
Median time to onset of cGVHD, months (range)	7 (1.6–267)	7 (1.6–144)	7 (1.7–267)

	All patients *n* = 280 (%)	**Positive *n* = 174 (%)**	**Negative *n* = 106 (%)**
Median duration of cGVHD in months (range)	24 (0.4–222)	23 (0.4–222)	26 (0.7–210)
Duration of cGVHD till study enrolment			
≤1year	78 (28)	45 (26)	33 (31)
>1year	202 (72)	129 (74)	73 (69)

N, number; GI, gastrointestinal; IS, immunosuppressive.

[a]Some patients with activity of cGVHD ($n = 2$) and intensity of immunosuppression ($n = 2$) have missing data.

[b]Statistical significance between the groups ($P < 0.05$).

TABLE III

Univariate Associations Between Laboratory Parameters and Autoantibodies, in Medians

Parameter (units)	Positive *n* = 174	Negative *n* = 106	*P*
Uric acid (mg/dl)	4.7	4.2	0.0082
Total protein (g/dl)	6.5	6.1	0.0004
IgM (mg/dl)	74	38	<0.0001
IgG (mg/dl)	762	514	<0.0001
IgA (mg/dl)	66	40	0.0012
C4 (mg/dl)	26	28	0.045
$CD3^{+} \times 10^{6}$	948	663	0.0023
$CD4^{+} \times 10^{6}$	454	222	0.0014
$CD8^{+} \times 10^{6}$	383	308	0.023
$CD19^{+} \times 10^{6}$	189	43	<0.0001
$CD56^{+} \times 10^{6}$	181	160	0.17
CRP	1.3	1.6	0.38
CPK	57	48	0.046
ESR	17.5	14	0.29
WBC	7.3	7.12	0.68
Eosinophils	0.09	0.07	0.1

CRP-C, reactive protein; CPK, creatine phosphokinase; ESR, erythrocyte sedimentation rate; WBC, white blood count; Ig, immunoglobulin; C4, complement 4.

TABLE IV

Multivariable Analysis of Parameters Associated with Presence of Circulating Autoantibodies

Factor	HR	95% CI	*P*
IgM	1.008	1.002–1.0013	0.0061
IgG	1.001	1.001–1.002	0.0001
Lung	0.428	0.251–0.728	0.0017
GI	0.434	0.254–0.742	0.0023

Ig, immunoglobulin M, G; GI, cGVHD of gastrointestinal tract; HR, hazard ratio; CI, confidence interval.