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Allogeneic Hematopoietic Stem Cell Transplantation Recipients Have Defects of Both Switched and IgM Memory B Cells

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Allogeneic hematopoietic stem cell transplant (HSCT) recipients were assessed to elucidate memory B cell defects underlying their increased susceptibility to infections, particularly by encapsulated bacteria. Circulating IgM memory B cells ($CD19^+$, $CD27^+$, IgM^+) and switched memory B cells ($CD19^+$, $CD27^+$, IgM^-) were enumerated in allogeneic HSCT recipients (n = 37) and healthy controls (n = 35). T lymphocyte subpopulations and serum levels of immunoglobulins, including IgG subclasses, and antibodies to pneumococcal polysaccharides were also assayed. Allogeneic HSCT recipients were deficient in both switched memory and IgM memory B cells compared to healthy controls (both $P < .0001$), irrespective of time post-HSCT. Switched memory B cell deficiency correlated with $CD4^+$ T cell deficiency, and both correlated with serum levels of IgG1 ($P < .0001$), possibly reflecting impaired B cell isotype switching in germinal centres. "Steady-state" serum levels of antibodies to pneumococcal polysaccharides did not correlate with circulating memory B cells. Graft-versus-host disease (GVHD) was associated with lower IgM memory B cell counts and lower serum levels of IgG2, IgG4, IgA, and pneumococcal antibodies. The increased susceptibility of allogeneic HSCT patients to infection may reflect a combination of memory B cell defects, which are most common in patients with a history of GVHD.

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KEY WORDS: HSCT, IgM memory and switched memory B cells, $CD4^+$ T cells, Pneumococcal antibodies, GVHD

INTRODUCTION

Individuals who have previously received a Hematopoietic stem cell transplant (HSCT) have an increased risk of acquiring infections by bacteria, particularly encapsulated bacteria. This is associated with a deficiency of circulating memory B lymphocytes (B cells) and serum immunoglobulins and impaired production of antibodies to polysaccharide antigens that may persist for many years. Affected individuals can therefore be considered to have an acquired antibody deficiency disorder [1-3]. Further elucidation of the abnormalities of B cell numbers and function underlying this disorder may result in improved diagnostic methods and treatments.

Streptococcus pneumoniae (pneumococcus) is the most frequent pathogen in allogeneic HSCT patients, and may cause severe and sometimes fatal invasive infections months or years following transplantation, with an incidence of up to 27% in long-term survivors and frequent recurrences [4-6]. Pneumococcal infections following allogeneic HSCT often occur relatively late and are 3 times more frequent than in autologous HSCT recipients [7]. Risk factors for invasive pneumococcal disease postallogeneic HSCT include graft-versus-host disease (GVHD), splenectomy, and the use of antithymocyte globulin (ATG) or total-body irradiation (TBI) for conditioning [2,4,6,7]. However, the immunologic defects associated with pneumococcal

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disease in these patients have not been completely defined.

Antibody responses to capsular polysaccharide antigens are the most impaired. For example, Nordoy et al. [8] found that only a minority of patients responded to vaccination with pneumococcal polysaccharides even 4-10 years post-HSCT, despite all patients maintaining "protective" serum levels of tetanus toxoid antibody [8]. Others have shown that "protective" levels of antibodies against pneumococcal antigens decrease during the first year after transplantation, regardless of serum immunoglobulin levels [3]. The opsonophagocytic activity of antibodies to pneumoccoal polysaccharides is also impaired [9].

In addition to pneumococci, other pathogens also cause disease with increased frequency or severity in HSCT recipients [10]. This has been associated with immune defects that include low serum levels of immunoglobulins, particularly IgG2, IgG4, and IgA, impaired antibody responses and hyposplenism, which are well-documented acquired immune defects in adult and pediatric allogeneic HSCT recipients [1,3,6,8,11,12].

Early studies revealed that the majority of circulating B cells 1 year after HSCT are naïve IgD^+ B cells [13], and that such B cells lack somatic mutations in the immunoglobulin heavy chain variable region, indicating a maturational arrest [14]. Human naïve B cells exported from the bone marrow, have a $CD19^+CD27^-IgM^{low}IgD^+$ immunophenotype, and pass through a transitional B cell immunophenotype ($CD38^+$) prior to becoming $CD27^+$ memory B cells [15]. The CD27 molecule has been recognized as a cell-surface marker that identifies somatically mutated memory B cells, which are critical for the production of protective antibody responses [16-18]. The human memory B cell compartment ($CD19^+$, $CD27^+$) contains 2 subpopulations; (1) IgM memory B cells (IgM^+), which possess a prediversified IgM antigen receptor and are capable of responding immediately to the antigens of encapsulated bacteria in a T cell-independent fashion [17-19]; and (2) switched memory B cells (IgM^-), which require T cell costimulation to produce high affinity IgG and other isotypes of antibody within germinal centres of lymphoid tissue [20]. Abnormal T cell-dependent B cell responses after allogeneic-HSCT might reflect impaired germinal center formation and B cell isotype switching [21], although information is incomplete.

HSCT has been associated with a deficiency of $CD27^+$ memory B cells, particularly in recipients with GVHD [22,23], although it has been recently shown that memory B cells transferred by allogeneic bone marrow transplantation can contribute to the antibody repertoire of the recipient [24]. Deficiency of IgM memory B cells in asplenic and aged individuals has been associated with an increased susceptibility to invasive pneumococcal disease [17]. Many other studies have demonstrated that abnormalities of circulating memory B cell numbers also exist in a variety of other disorders [25-28].

The aim of this study was to further characterize the nature of the memory B cell deficiency in allogeneic HSCT recipients. In particular, we sought to determine if such individuals have a deficiency of IgM or switched memory B cells that might account for their increased susceptibility to invasive pneumococcal disease, and to determine if serum levels of IgM, IgA, total IgG, and IgG subclasses and "steady-state" IgG and IgG2 antibodies to pneumococcal polysaccharides are related to circulating memory B cell numbers. We also investigated the relationship between numbers of circulating memory B cells and the presence of GVHD, previous use of ATG and TBI, time since transplantation, and the current circulating T cell counts.

MATERIAL AND METHODS

Patients and Controls

We undertook a cross-sectional study of 37 patients who had previously received an allogeneic HSCT and 35 healthy controls. Informed consent was obtained from all patients and controls. Allogeneic HSCT recipients were at least 6 months posttransplantation and in remission at the time of assessment. Patient information collected included time since transplantation, type of allogeneic transplantation (sibling or matched unrelated), previous autologous transplantation, history of acute and/or chronic GVHD (aGVHD, cGVHD), previous ATG or TBI, and splenectomy. All subjects were >18 years of age. Demographic characteristics of patients and controls are given in Table 1. Laboratory assessments were performed on a single occasion between July 2005 and August 2007.

Analysis of Memory B Cells by Flow Cytometry

Leukocytes were enriched from a 10-mL peripheral blood sample anticoagulated with ethylenediaminetetraacetic acid (EDTA) by collecting a phosphate-buffered saline (PBS)-washed buffy coat and resuspending it in approximately 1 mL of PBS Flow Buffer containing 2% fetal calf serum (FCS). The following antibodies coupled with fluoroscein isothiocyanate (FITC), R-phycoerythrin (PE), or the tandem dye R-phycoeythrin_cyanin 5.1 (PC5) were used for flow cytometry; anti-CD19-PC5 (clone J4.119 Immunotech, Marseille, France), anti-CD27-PE (clone IA4-CD27 Immunotech), anti-IgM-FITC (polyclonal fab′2 Dako, Glostrup, Denmark), isotype control-PE (clone 679.1Mc7 Immunotech), isotype control-FITC (fab′2 Dako). A total of 100 μL of diluted

Table 1. Median (IQR) Values of Total Lymphocytes and Lymphocyte Subpopulations Across Control and HSCT Groups

			HSCT Patients			
			GVHD		Years since Transplant	
	Controls N = 35	HSCT N = 37	no N = 11	yes N = 26	≤1* N = 22	>1 N = 14
Male (%)	35%	54%	64%	50%	64%	43%
Age (years)	45 (27-54)	41 (33-51)	36 (31-50)	43 (36-52)	39 (33-51)	42 (35-49)
Lymphocytes (x10^9/L)	**1.9** (1.6,2.3)	**1.4** (0.9,2.2)	1.1 (1.0,2.0)	1.4 (0.9,2.2)	**1.0** (0.7,1.7)	**2.2** (1.3,2.7)
B cells (x10^6/L)						
Total memory	**45.8** (30.7,69.3)	**7.6** (3.2,22.2)	21.0 (6.4,53.0)	6.7 (2.6,19.6)	**6.4** (2.6,14.3)	**21.7** (5.7,62.9)
IgM memory	**18.0** (8.2,32.9)	**3.2** (1.1,10.1)	**10.1** (3.1,18.4)	**2.4** (1.0,5.8)	**2.1** (1.0,4.0)	**8.7** (3.5,20.5)
Switched memory	**23.6** (16.4,40.7)	**5.3** (1.5,13.1)	8.0 (3.2,31.0)	4.6 (1.3,11.8)	**4.2** (1.3,6.0)	**13.0** (1.9,37.9)
B cells (% B cells)						
Total memory	**19.0** (13.2,26.2)	**5.0** (2.0,9.5)	7.1 (4.0,16.6)	4.1 (1.9,7.9)	6.0 (2.1,11.8)	4.1 (2.3,7.8)
IgM memory	**6.6** (3.7,12.9)	**2.2** (0.9,3.7)	3.2 (1.4,4.0)	2.0 (0.7,3.0)	2.3 (0.7,4.2)	2.0 (0.9,2.6)
Switched memory	**10.4** (6.9,17.4)	**2.0** (0.9,5.5)	2.7 (1.8,9.9)	1.7 (0.7,5.4)	2.2 (1.3,5.5)	2.4 (0.8,5.2)
CD4 T cells (x10^6/L)	**870** (725,1082)	**320** (255,483)	407 (305,450)	298 (251,515)	316 (175,448)	385 (289,859)
CD4 T cells (% lymphocytes)	**48** (42,54)	**26** (21,30)	27 (26,34)	25 (15,29)	26 (23,30)	26 (20,31)
CD8 T cells (x10^6/L)	462 (321,658)	360 (198,616)	352 (189,542)	383 (203,702)	320 (159,474)	566 (347,699)
NK cells (x10^6/L)	**143** (109,242)	**224** (174,291)	203 (178,244)	234 (167,324)	228 (185,301)	214 (120,261)

NK indicates natural killer; GVHD, graft-versus-host disease; GVH, graft versus host; BMT, bone marrow transplantation; HSCT, hematopoietic stem cell transplantation.

Median value pairs in bold indicate significant differences ($P < .05$, Mann-Whitney *U*-test) when comparing either (a) HSCT patients versus controls; or (b) among HSCT patients: (1) presence versus absence of GVH disease, or (2) sample obtained more than 1 versus <1 year since transplant.

*Five of 11 patients without GVHD and 17/26 patients with GVHD were within 1 year posttransplantation.

buffy-coat was incubated for 10 minutes at room temperature with appropriate amounts (5 μL or 10 μL) of the following antibodies: CD19-PC5, CD27-PE, IgM-FITC in 1 tube, and CD19-PC5, Isotype control-PE, Isotype control-FITC in a second tube. Following incubation, the cell/antibody mixture was processed in a Beckman Coulter Multi-Q_Prep instrument (Fullerton, CA) to lyse the red blood cells and to stabilize and fix the lymphocytes prior to acquisition and analysis on the flow cytometer. Three-color data acquisition was performed using a Beckman Coulter XL-MCL flow cytometer with System II analysis software or a Beckman Coulter Elite-ESP flow cytometer with Elite analysis software. Validation data was obtained on replicate samples from both instruments to ensure reliability and comparability of data acquired from either cytometer. B cell subpopulations were expressed as a proportion of total B cells and as a count that was derived using the total lymphocyte count also measured on Beckman Coulter flow cytometer.

Assays of Lymphocyte Subsets and Serum Levels of Immunoglobulins and Pneumococcal Polysaccharide Antibodies

Flow cytometry was used to quantify T cell populations. Natural killer (NK) cells were defined as CD16/CD56 dual-positive cells. Serum levels of total IgG, IgM, IgA, and IgG subclasses were assayed by nephelometry in a Core Services Laboratory accredited by the National Association of Testing Authorities (NATA), Australia. Serum levels of total IgG and IgG2 antibodies to pneumococcal polysaccharides were assayed by ELISA (Binding Site, Evolis, BioRad, Hercules, CA).

Statistical Analyses

Analyses presented here consider IgM memory and switched memory B cells as both counts and proportions of total B cells. As all lymphocyte subpopulation counts were derived from the total lymphocyte count, statistically significant correlations between subpopulation counts were only considered further if there was also a correlation between proportions of those subpopulations. The median and interquartile range (IQR) are used as summary statistics. Comparative analyses are nonparametric and include the Mann-Whitney *U*-test and Spearman's rank correlation test. A value of $P < .05$ is considered to be significant. Analyses were carried out using S-PLUS 7.0 for Windows (Insightful Corp., Seattle, WA).

RESULTS

Circulating Memory B Cells Were Depleted in Allogeneic HSCT Recipients

We first determined if memory B cell deficiency was evident in the HSCT recipients and if this was because of deficiency of a particular subpopulation. Both the counts and proportions of circulating total memory ($CD27^+$) B cells were low in allogeneic HSCT recipients compared with healthy controls ($P < .0001$) (Figure 1a). This depletion reflected decreased counts and proportions of both IgM memory

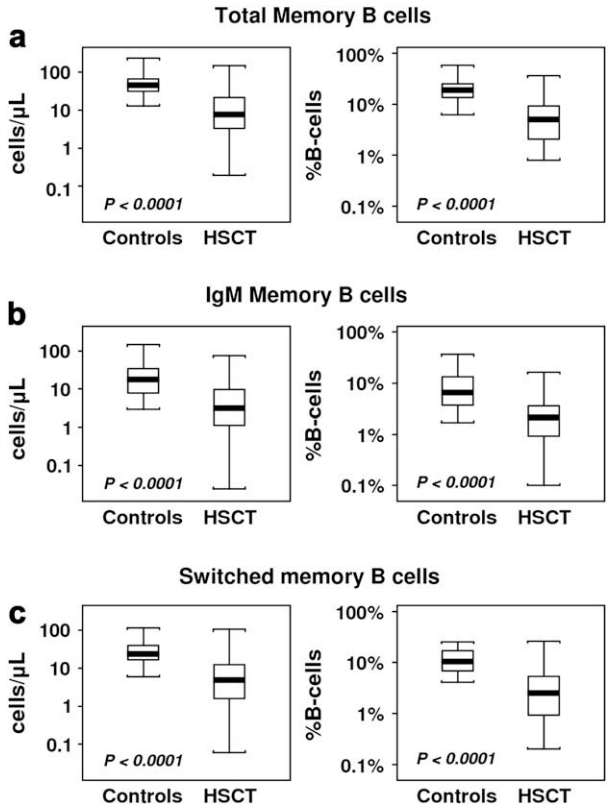

Figure 1. Allogeneic HSCT patients (n = 37) are deficient of total, IgM, and switched memory B cells when compared with controls (n = 35). The plots indicate the median (thick line), interquartile range (box), and range (whiskers) of the respective data samples.

(Figure 1b) and switched memory B cells (Figure 1c) (all $P < .0001$). Deficiency of memory B cell subpopulations persisted in a subanalysis restricted to those recipients greater than 1 year post-HSCT ($P = .05$, switched memory B cells; $P = .09$, IgM memory B cells).

Memory B Cell Subpopulations Correlated with Serum Levels of IgG Subclasses and IgA But Not Antibodies to Pneumococcal Polysaccharides

As memory B cell subpopulations were deficient in HSCT recipients we next examined the correlation between total memory, IgM memory, and switched memory B cells and serum levels of IgM, IgA, total IgG, and IgG subclasses and antibodies to pneumococcal polysaccharides. Switched memory B cell counts and proportions correlated strongly with serum levels of total IgG (r = .69, $P < .0001$ and r = .6, $P = .0007$, respectively) and IgG1 (r = .73, $P < .0001$ and r = .61, $P = .0009$) (Figure 2) and less strongly with serum levels of IgG2 (r = .43, $P = .03$ and r = .51, $P = .008$), IgG3 (r = .44, $P = .02$ and r = .34, $P = .09$) and IgG4 (r = .49, $P = .01$ and $r = .55$, $P = .004$). Switched memory B cell counts and proportions also correlated with serum levels of IgA (r = .50, $P = .008$ and r = .46, $P = .02$) but not IgM (Table 2).

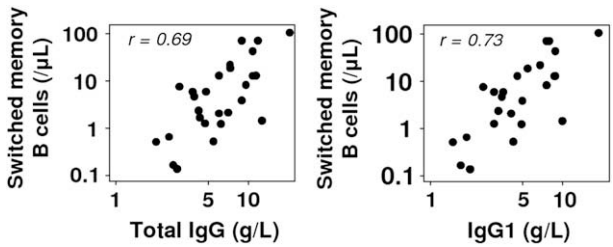

Figure 2. Switched memory B cells correlate strongly with serum levels of total IgG and IgG1 ($P < .0001$) in allogeneic HSCT patients.

IgM memory B cell counts but not proportions correlated with serum levels of total IgG, IgG1, and IgA. Neither total IgM nor switched memory B cell counts or proportions correlated with serum levels of IgG or IgG2 antibodies to pneumococcal polysaccharides.

Deficiency of Switched Memory B Cells and Serum IgG Subclasses Correlated with $CD4^+$ T Cell Deficiency

Because switched memory B cell production is dependent on $CD4^+$ T cell function we next determined if memory B cell numbers correlated with the $CD4^+$ T cell counts. Allogeneic HSCT patients had lower median circulating $CD4^+$ T cell counts and proportions than healthy controls (both $P < .0001$), but there was no difference in $CD8^+$ T cell counts ($P = .2$) (Figure 3a). $CD4^+$ T cell deficiency was less pronounced but still evident in patients >1 year post-HSCT ($P = .005$). $CD4^+$ T cell counts were positively correlated with switched memory B cell counts (r = .66, $P < .0001$) and IgM memory B cell counts (r = .44, $P = .006$) (Figure 3b), a finding that was substantiated by positive correlations between $CD4^+$ T cell proportions and both switched memory B cell proportions (r = .51, $P = .001$) and IgM memory B cell proportions (r = .41, $P = .01$) (data not shown).

$CD4^+$ T cell counts correlated with serum levels of total IgG (r = .64, $P = .0003$) and IgG1 (r = .66, $P = .0002$) (Figure 3c), and less strongly with IgG2 (r = .45, $P = .02$), IgG3 (r = .54, $P = .005$) and IgG4 (r = .46, $P = .02$). In contrast, $CD4^+$ T cell counts did not significantly correlate with serum levels of IgM, IgA or pneumococcal antibodies (Table 2).

A History of GVHD Was Associated with Lower Serum Levels of IgG2, IgG4, IgA, and Pneumococcal Antibodies and Lower IgM Memory B Cells

Allogeneic HSCT recipients with GVHD carry the highest risk of acquiring pneumococcal sepsis. Therefore, memory B cell subpopulations and serum levels of immunoglobulins and antibodies were compared in patients with and without a history of GVHD. Patients with a history of GVHD had lower

Table 2. Spearman Correlations between Total Lymphocytes, Lymphocyte Subpopulations, and Serum IgG Levels and Antipneumococcal Antibodies, Among HSCT Patients

	Lymphocytes	CD4 T Cells	CD4 T Cells (% Lymphocytes)	CD8 T Cells	B Cells (x10^6/L) Total Memory	IgM Memory	Switched Memory	B Cells (% B Cells) Total Memory	IgM Memory	Switched Memory
Memory B cells (x10^6/L)										
Total	*0.69*	*0.61*	0.20	*0.36*						
IgM	*0.53*	*0.44*	0.17	0.15	*0.89*					
Switched	*0.73*	*0.66*	0.21	*0.46*	*0.94*	*0.74*				
Memory B cells (%B cells)										
Total	−0.01	0.23	*0.54*	−0.02	*0.47*	*0.38*	*0.46*			
IgM	−0.21	−0.01	*0.41*	−0.32	*0.36*	*0.49*	0.21	*0.83*		
Switched	0.22	*0.40*	*0.51*	0.28	*0.49*	0.25	*0.62*	*0.85*	*0.48*	
Total serum IgG (g/L)	*0.50*	*0.60*	*0.45*	0.16	*0.62*	*0.48*	*0.69*	*0.45*	0.24	*0.60*
IgG1	*0.55*	*0.62*	*0.46*	0.19	*0.63*	*0.50*	*0.73*	*0.47*	0.23	*0.61*
IgG2	0.19	0.39	*0.46*	−0.07	0.36	0.26	*0.43*	0.38	0.21	*0.51*
IgG3	0.39	*0.51*	*0.43*	0.18	0.38	0.23	*0.44*	0.25	0.01	0.34
IgG4	0.29	*0.44*	*0.52*	0.10	*0.41*	0.26	*0.49*	*0.49*	0.26	*0.55*
Serum IgA	0.32	0.31	0.32	0.12	0.52	*0.44*	*0.50*	*0.48*	0.37	*0.46*
Serum IgM	0.11	0.16	0.13	0.03	0.28	0.20	0.25	0.43	0.36	0.28
Pneum. IgG antibodies	0.23	0.30	0.26	−0.03	0.25	0.28	0.23	0.04	−0.03	0.14
Pneum. IgG2 antibodies	0.13	0.20	0.10	−0.05	0.21	0.28	0.16	0.03	0.03	0.07

Italic values indicate *P*-values < .05.

serum levels of IgG2 ($P = .001$) and IgG4 ($P = .01$) as well as IgA ($P = .02$) (Figure 4) and lower IgM memory B cell counts ($P = .05$) than patients without GVHD. Furthermore, a history of GVHD was also weakly associated with lower serum levels of IgG and IgG2 antibodies to pneumococcal polysaccharides ($P = .07$ and $P = .06$, respectively). Serum levels of IgG2 antibodies to pneumococcal polysaccharides correlated with serum IgG2 levels in all patients (r = .64, $P = .002$) and GVHD patients (r = .55, $P = .03$) (data not shown). A history of GVHD was not associated with lower switched memory B cell or $CD4^+$ T cell counts ($P = .1$ and .3, respectively) or serum levels of IgM ($P = .9$).

Effect of TBI/ATG or Splenectomy

There was no association between previous treatment with ATG or TBI and memory B cell subpopulations, CD4 T cells, serum immunoglobulin levels, or pneumococcal antibodies (data not shown). Only 1 HSCT recipient had a previous splenectomy.

DISCUSSION

We have shown that allogeneic HSCT recipients have long-term total memory B cell deficiency, confirming the observations of others [13,22,23], and show for the first time that this is because of long-term depletion of both circulating IgM memory and switched memory B cells. We have also shown that circulating switched memory B cell counts correlate with serum levels of IgG subclasses and IgA, but not IgM, and that serum levels of IgG2, IgG4, IgA, and pneumococcal antibodies are lower in patients with GVHD. Furthermore, switched memory B cell counts and serum IgG subclass levels correlated with $CD4^+$ T cell counts. Thus, we show that HSCT patients have immune defects that impair the reconstitution and/or generation of memory B cells, and that these abnormalities also affect $CD4^+$ T cell counts and the maintenance of serum IgG subclass and IgA levels.

The 2 subpopulations of memory B cells have distinct functions. IgM memory B cells are equivalent to marginal zone B cells of the spleen [18], which bind to microbial cell wall polysaccharides in the circulation and transport them to spleen germinal centers as well as initiating an "early" low-affinity IgM antibody response against the microbe. Switched memory B cells are produced in germinal centers of lymphoid tissues under the influence of $CD4^+$ T cells, particularly follicular helper T cells (T_{FH} cells), and migrate to various regions of the body, including the bone marrow, where they differentiate into plasma cells producing high-affinity antibodies of all isotypes [16,29]. The number of circulating IgM and switched

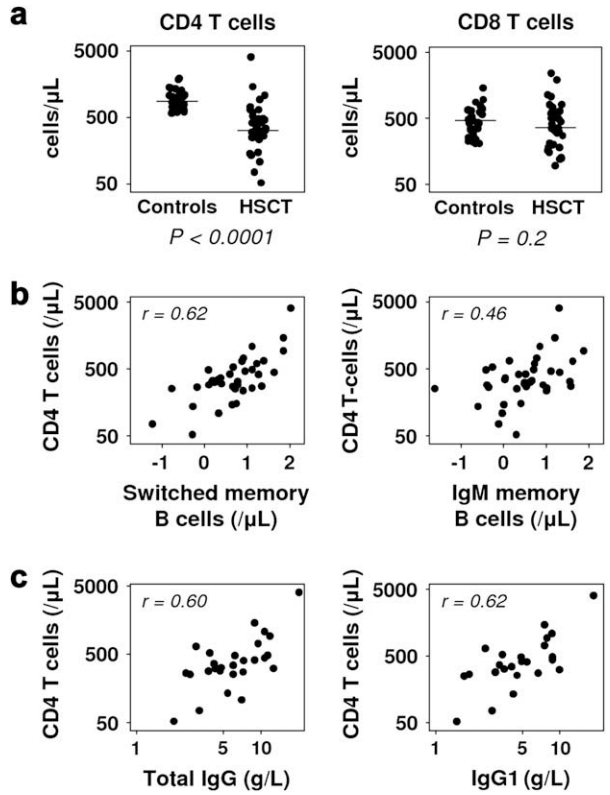

Figure 3. Memory B cell and IgG deficiency correlates with the CD4 T cell deficiency.

memory B cells can therefore be considered as an indicator of the density and/or function of B cell marginal zones and germinal centers, respectively, as has been argued for primary antibody deficiency disorders [30].

Our demonstration that circulating switched memory B cell numbers are low in HSCT patients may therefore reflect a reduced germinal center density and/or function and impaired B cell immunoglobulin isotype switching. Supporting evidence is found in

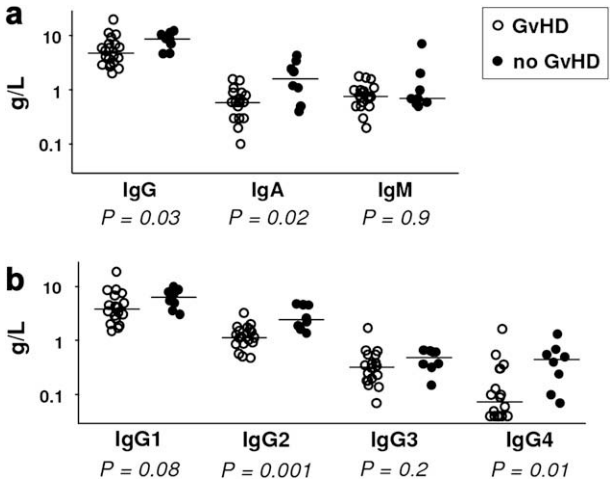

Figure 4. Serum levels of IgA, IgG, and IgG subclasses are lower in allogeneic HSCT patients with GVHD.

severe combined immune deficiency (SCID) mice that have received an allogeneic bone marrow transplant where suboptimal antibody responses to T cell-dependent vaccines are associated with reduced germinal centre formation [21]. The effect of germinal center density on memory B cell numbers has not been directly studied in human allogeneic HSCT recipients. In view of our demonstration that switched memory B cell counts correlate with $CD4^+$ T cell counts, 1 possible cause of impaired germinal center function might be a deficiency of T_{FH} cells. These cells are present within germinal centers, and express CD4, CCR5, and ICOS (but not CCR7) [31,32] and produce IL-21 [31]. T cell-dependent and long-lived antibody responses are highly dependent on the functional properties and activity of T_{FH} [32], and their dysregulation most likely contributes to the pathogenesis of several immunodeficiency diseases [33]. Of note, IL-21 is a switch factor for B cells that differentiate into plasma cells producing IgG1 [34]. Reduced IL-21 production by T_{FH} cells might therefore be an explanation for the strong correlation between serum levels of IgG1 and switched memory B cell counts demonstrated in this study (see Figure 2).

The strong correlation of switched memory B cell counts and $CD4^+$ T cell counts with serum IgG1 and IgA levels suggests that HSCT recipients have a generalized impairment of T cell-dependent B cell antibody isotype switching, in addition to the well-documented defects of T-independent antibody responses. These immune defects might contribute to the increased susceptibility to opportunistic infections with various bacterial, viral, fungal, protozoan, and helminth pathogens in allogeneic-HSCT recipients [10].

Our finding that IgM memory B cell numbers correlate with total IgG1 and IgA was unexpected, as IgM memory B cells are T cell-independent and do not class switch. It has been shown in mice that IgM memory B cells shuttle between the marginal zone and follicular areas of lymphoid tissue, and may function as a link between innate and adaptive immune responses [35,36]. This capture and follicular delivery of blood-borne antigens is essential to the initiation of high affinity switched memory B cell responses within the germinal centre. Separately, it has been shown that $CD4^+$ T cell responses against pneumococcal antigens may contribute to long-term protection against pneumococcal colonization [37]. Therefore, circulating IgM memory B cell numbers might be linked to $CD4^+$ T cell-dependent antibody responses via their role in delivery of antigen to follicular dendritic cells, as suggested by others [36].

Depletion of IgM memory B cells might be a consequence of hyposplenism, which occurs in up to 15% of HSCT patients [38]. Functional hyposplenism is associated with cGVHD after allogeneic HSCT and may occur in the absence of features of hyposplenism

on blood films [38]. Furthermore, a reduction in spleen size post-HSCT can be detected by ultrasound examination and correlates with the presence of GVHD and risk of pneumococcal sepsis [12]. Finally, Nakayama [39] found that marginal zone reconstitution within the splenic white pulp is delayed after human bone marrow transplantation, and concluded that this could be responsible for the functional asplenia of long-term survivors.

GVHD has long been associated with an increased risk of pneumococcal infection in HSCT patients [4,7], but the reasons for this are unclear. The adverse effect of GVHD on recovery of naïve T cells is well documented [40,41], and is in part related to failure to regenerate a thymus [42]. Reduced density and/or function of germinal centres and marginal zones might also be a consequence of GVHD, as well as the effects of pretransplant conditioning [21,43]. Recipient-derived dendritic cells (DCs) that persist and contribute to host immunity following allogeneic BMT are a target of GVHD [44,45], and GVHD is associated with delayed reconstitution of follicular DCs, decreased $CD22^+$ B cells, and an inverted T cell CD4:CD8 ratio in lymph nodes of HSCT recipients [43]. Perivascular clusters of DCs within the bone marrow also provide critical survival signals to early B cells, and ablation of these bone marrow DCs causes a loss of recirculating B cells [46]. Therefore, the memory B cell deficiency we have demonstrated could also be secondary to DC dysfunction within the BM as well as in the germinal centers of peripheral secondary lymphoid organs. Corticosteroid and immunosuppressant therapy for GVHD may also be a factor in the pathogenesis of B cell depletion, as patients treated with immunosuppressant drugs (cyclosporine, corticosteroids) exhibit additional cytokine defects compared to non-treated patients [47].

We have confirmed several previous observations that HSCT patients, particularly those with GVHD, have lower serum levels of IgG2, IgG4, and IgA [13,48,49], and also shown that serum IgG2 levels correlate with levels of IgG2 antibodies to pneumococcal polysaccharides. Although these deficiencies might reflect a "recapitulation of normal B cell ontogeny" [48], an alternative explanation is an acquired defect of B cell differentiation and class switch recombination in germinal centers. IgG2 antibodies play an important role in the opsonophagocytic antibody response against pneumococcal polysaccharides [50], and an impaired ability to produce IgG2 might contribute to the increased susceptibility to infection by pneumococci. However, it is unlikely that this is the sole cause because IgA-deficient patients who experience an increased susceptibility to infections have similar abnormalities [51], but rarely experience the overwhelming pneumococcal infections encountered by some HSCT patients. This suggests that there are additional immune defects. We have shown that patients with a history of GVHD also have fewer circulating IgM memory B cells than patients who have not experienced GVHD, which might compound the effects of impaired production of IgG2.

Serum levels of "protective" antipneumococcal antibodies decrease during the first year after HSCT [3]. This is not associated with changes in serum immunoglobulin levels or vaccination history. Here, we show that "steady-state" serum levels of total IgG or IgG2 antibodies to pneumococcal polysaccharides also show no correlation with circulating IgM memory or switched memory B cell numbers. This may be because these antibodies are produced by long-lived plasma cells [29]. Also, Lanzavecchia et al. [29] found that tetanus-specific memory B cells, but not total memory B cells, correlated with tetanus-specific antibody production in healthy controls. Therefore, it may be more informative to examine the relationship between pneumococcal polysaccharide-specific memory B cells and serum pneumococcal polysaccharide antibody levels in HSCT patients.

Our finding that switched memory B cells correlate with $CD4^+$ T cells suggest that germinal centre and dendritic cell function warrants further investigation in allogeneic HSCT patients. Delayed donor lymphocyte infusion is safe and effective at inducing graft-versus-leukemia (GVL) effect without inducing GVHD [52]. Furthermore, delayed infusion of donor derived antigen specific $CD4^+$ T cells can overcome an antigen specific T cell defect in the recipient [53]. Such infusions of donor-derived T cells may also allow correction of a specific antibody defect via recovery of memory B cell subpopulations. Similarly, there is now also experience in isolating and culturing donor derived DCs [54]. Allogeneic HSCT protocols that prevent GVHD may reduce the risk of pneumococcal sepsis by allowing better recovery of memory B cells. These hypotheses could also be tested in clinical trials.

We recognize several potential limitations to this study. Patients were sampled in a cross-sectional fashion only and memory B cell numbers could not be correlated with a clinical outcome of pneumococcal infection given the rarity of this condition in our allogeneic HSCT patient cohort, in which prophylactic antibiotic therapy is commonly used. Also, the flow cytometry antibody panel used to enumerate memory B cells did not include an antibody to IgD as is now recommended for the enumeration of circulating marginal zone B cells [30]. However, the large majority of circulating IgM^+, $CD27^+$ B cells have the characteristics of marginal zone B cells [18,55], allowing us to draw conclusions about marginal zone density and/or function in our patient cohort.

The current study focuses on the effect of allogeneic-HSCT on $CD27^+$ memory B cell subpopulations. Future studies could also examine the effect of

allogeneic-HSCT on transitional ($CD38^+$) B cells. If the abnormalities observed in our study are associated with defects in germinal centre function, as we hypothesize, allogeneic HSCT should be associated with an expansion of functionally immature transitional B cells, as has already been found in other human immunodeficiency disorders characterized by impaired humoral immunity [15].

In conclusion, we have shown that both IgM memory and switched memory B cells are decreased in allogeneic HSCT recipients compared to healthy controls. The depletion of switched memory B cells correlated with deficiency of $CD4^+$ T cells, serum IgG subclasses, particularly IgG1, and IgA. We suggest that this might be related to impaired B cell differentiation resulting from abnormal B and T cell interactions within germinal centers. Patients with a history of GVHD had lower numbers of IgM memory B cells as well as IgG2 deficiency and lower levels of antibody to pneumococcal polysaccharides, which might explain their particular susceptibility to pneumococcal infection. The enumeration of circulating memory B cell subpopulations in concert with the assay of serum levels of IgG subclasses, IgA and antibodies to polysaccharide antigens might have clinical utility in predicting susceptibility to infection by encapsulated bacteria. These findings also have important implications for vaccination strategies in allogeneic HSCT recipients.

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