

Memory B Cells and Pneumococcal Antibody After Splenectomy¹

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Splenectomized patients are susceptible to bloodstream infections with encapsulated bacteria, potentially due to loss of blood filtering but also defective production of anticarbohydrate Ab. Recent studies propose that a lack of Ab is related to reduced numbers of IgM⁺ CD27⁺ memory B cells found after splenectomy. To test this, we analyzed CD27⁺ memory B cell subsets, IgG, and IgM pneumococcal Ab responses in 26 vaccinated splenectomized subjects in comparison to memory B cell subsets and Ab responses in healthy controls. As shown previously, the splenectomized autoimmune subjects had fewer total, isotype switched, and IgM⁺ CD27⁺ memory B cells as compared with controls, but there was no difference in memory B cells subsets between controls and splenectomized subjects with spherocytosis. There was no difference between the geometric mean IgG Ab response between normal controls and splenectomized subjects ($p = 0.51$; $p = 0.81$). Control subjects produced more IgM Ab than splenectomized autoimmune subjects ($p = 0.01$) but the same levels as subjects with spherocytosis ($p = 0.15$.) There was no correlation between memory B cell subsets and IgG or IgM Ab responses for controls or splenectomized subjects. These data suggest that splenectomy alone may not be the sole reason for loss of memory B cells and reduced IgM antipneumococcal Ab. Because subjects with autoimmunity had splenectomy at a significantly older age than participants with spherocytosis, these data suggest that an age-related loss of extra splenic sites necessary for the maintenance or function of memory B cells may lead to impaired immunity in these subjects. *The Journal of Immunology*, 2008, 181: 3684–3689.

Splenectomized patients are susceptible to overwhelming bloodstream infections, especially those due to encapsulated bacteria (1, 2). For more than three decades, this has been attributed to the role of the spleen in the production of Ab to encapsulated organisms as an impaired Ab response to pneumococcal vaccine was demonstrated after splenectomy (3–7). Because *Streptococcus pneumoniae* accounts for the majority of post splenectomy infections, pneumococcal vaccination before splenectomy, to override the potential defect after surgery, is a standard preoperative strategy (8). As for other vaccines, the initial response to immunization is Ag-specific IgM Abs but long-term protection, as for other administered vaccines, is attributed to serotype-type specific IgG antipneumococcal Abs (9, 10). However, the Ab responses of splenectomized subjects, which were examined in the older studies, did not incorporate a preabsorption step using cell wall polysaccharide, now considered essential in such assays. In fact, using newer assays, studies have shown that splenectomized patients actually may have post vaccination IgG antipneumococcal titers comparable to normal subjects (11, 12) leaving room for additional explanations for post splenectomy infections.

Recent studies have focused on the biological role of human CD27⁺ memory B cells, especially IgM⁺ memory B cells. Cell sur-

face markers and gene expression analyses have identified circulating blood IgM⁺ IgD⁺ CD27⁺ B cells as the potential counterpart of B cells in the marginal zone compartment of the spleen (13, 14). In the case of the hyper IgM syndrome due to mutations of CD40 or CD40L, this population of cells is the only memory B cell subset, due to lack of normal isotype switch (15). A current view is that these B cells may be generated independently from germinal centers, undergo somatic hypermutation of Ig variable regions (but not isotype switch) and are exclusively involved in Ab production to T independent Ags, such as pneumococcal carbohydrates (13, 14). This is consistent with the observation that a clone of IgM⁺CD27⁺ B cells with somatic mutations and specificity for pneumococcal Ags was found in the spleen, and later in the circulation, of a subject after immunization (16). In concert with this view, IgM⁺ memory B cells and switched memory B cells, were found to be significantly reduced in the peripheral circulation of asplenic or splenectomized subjects (17). Based on these collective observations, it has been proposed that the spleen is critical for the production and/or survival of IgM⁺ CD27⁺ B cells, and that the loss of this B cell population after splenectomy might explain impaired carbohydrate-specific Ab production, predisposing to systemic infections (17, 18). However, as Ab production after carbohydrate Ag vaccination has not been investigated in splenectomized subjects in parallel with B cell phenotype, we analyzed CD27⁺ B cell subsets and IgG and IgM pneumococcal Ab responses in a large group of vaccinated splenectomized subjects as compared with healthy controls.

Materials and Methods

Study populations

Nonsplenectomized healthy controls (12) and 26 splenectomized subjects, 19 who had had immune thrombocytopenic purpura (ITP),³ 1 who had autoimmune hemolytic anemia, and 6 who have hereditary spherocytosis (HS), were enrolled at the Mount Sinai School of Medicine or the Weill

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³ Abbreviations used in this paper: ITP, immune thrombocytopenic purpura; HS, hereditary spherocytosis; AI, autoimmune disease.

Table I. *Subjects*

	Controls	Splenectomized Subjects	
		Autoimmune	HS
Number of Participants (<i>n</i>)	12	20	6
ITP ^a		19	
AIHA		1	
HS			6
Gender			
Male	4 (33.3%)	5 (25.0%)	2 (33.3%)
Female	8 (66.7%)	15 (75.0%)	4 (66.6%)
Age (years)	40.2 (26–64)	48.5 (15–80)	33.2 (10–47)
Age at Splenectomy (years)		35.6 (8–70)	5.4 (3–9)
Time Since Splenectomy (years)		12.9 (2.0–50)	25.6 (7–42)
Previously Vaccinated	0	18 (90.0%)	5 (83.3%)
Time Since Last Vaccination (years)		8.8y (2–25)	7.2y (3–13)
Number of Prior Vaccinations		1.1	1.8
Never Vaccinated		1	0
Unknown Vaccination History		1	1

^a AIHA = Autoimmune Hemolytic Anemia.

Medical College of Cornell University in New York City, New York. Splenectomized subjects who had been immunized with a pneumococcal vaccination within 2 years of enrollment, or had received steroids, Ig, rituximab, or any other immunosuppressive medications within six months of enrollment were excluded. Control participants were healthy adults of age 26–64 who had not received a previous pneumococcal vaccination and were not taking any immunosuppressive medications. Complete blood counts including lymphocyte counts and absolute lymphocyte counts were determined before and after immunization for splenectomized subjects. The clinical characteristics (gender, age, indication for splenectomy, time since splenectomy, vaccination history, and history of significant infections) were determined. Blood smears were examined for Howell-Jolly bodies, to clinically confirm absence of splenic function (19). The protocol and informed consent were approved by Internal Review Boards of both hospitals.

Serum Ig and Abs

Serum Ig levels IgG, IgA, IgM, and IgG subclasses were measured at enrollment. All subjects were immunized with a 0.5 ml i.m. injection of pneumococcal vaccine (Pneumovax 23; Merck), which contains 25 µg of each of the following capsular polysaccharides: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Pre-immunization and 4 to 7 wk later, postvaccination serotype-specific IgG and IgM Ab concentrations to the 23 pneumococcal serotypes were measured by a cell wall polysaccharide-adsorbed ELISA for each serotype (20). For IgG, each sample was compared with the pneumococcal standard reference serum 89SF (United States Food and Drug Administration, Bethesda, MD) to express serotype-specific IgG concentrations, as µg/ml (21). A clinically protective Ab titer was defined as a serotype-specific IgG concentration ≥1.3 µg/ml (22–24). Because quantitation curves for IgM Ab to pneumococcal serotypes have not been standardized, test samples were run six times with the 89SF standard reference serum to confirm consistency, establish quantitation curves, and to interpolate serotype-specific IgM concentrations, expressed as µg/ml for each.

Table II. *Serum Ig levels^a*

	Serum Ig (mg/dL) ^b						
	IgG Total	IgG1	IgG2	IgG3	IgG4	IgM	IgA
Reference Range	600–1600	344–966	133–622	12–138	1–115	40–250	70–315
Controls (<i>n</i> = 12)	1154.3 ± 296.2	615.8 ± 234.2	447.4 ± 122.2	68.0 ± 37.8	35.1 ± 26.4	109.1 ± 46.7	196.1 ± 63.4
Autoimmune (<i>n</i> = 20)	1149.2 ± 266.2	613.7 ± 191.3	433.1 ± 169.8	67.5 ± 35.5	63.3 ± 93.5	122.4 ± 64.5	283.9 ± 106.6
HS (<i>n</i> = 6)	1001.5 ± 117.8	530.5 ± 123.1	332.7 ± 146.3	76.8 ± 35.4	88.6 ± 158.4	107.9 ± 68.5	228.3 ± 61.0

^a Ig ranges for adult subjects; ^b Ig mean ± 1 SD.

B cell analyses

PBMC were isolated from blood after centrifugation on a Ficoll-Hypaque density gradient (Beckman Coulter). PBMCs were washed and examined, using four-color flow cytometry analysis FACSCalibur (BD Biosciences) and CellQuest computer software (BD Biosciences). Based on the expression on CD27, IgD, and IgM, CD19⁺ B cells were divided in six well-characterized subsets, CD27[−] (naive), CD27⁺ memory, CD27⁺ IgM[−] IgD[−] (isotype switched memory), and CD27[−] IgM⁺, IgM memory B cells, as previously described (25, 26).

Statistical analyses

The percentage of circulating naive, IgM⁺, and switched memory B cells were compared between groups using one-way ANOVA. The Scheffé test was used for individual group comparisons to control for Type 1 error. For Ab studies, baseline and post vaccination responses were compared by evaluating the mean number of serotypes before and after vaccination at or above the estimated protective titer using one-way ANOVA. To compare Ab concentrations to the 23 serotypes between patient groups, the pre- and postvaccination Ab titers were log-converted and the geometric means calculated for IgG and IgM Ab. Univariate ANOVA was used to assess the effect the age, gender, time since splenectomy, and age at splenectomy on the outcome measures. Variables were considered covariants and controlled for in the correlation analyses if their two-tailed *t* test determined significance. Spearman rank correlation coefficients (*r*) were calculated for relationships between the various B cell populations, serum Ig, and Ab titers and the total number of serotypes to which protective Ab titers were achieved. Statistical analyses were performed using SPSS 12.0 software (SPSS).

Results

Splenectomized subjects and controls

The distribution of age and gender of the splenectomized and control subjects in each group were comparable (Table I). The HS patients had had a

Table III. Peripheral blood B and memory B cells

	Controls (n = 12)	Autoimmune (n = 20)	HS (n = 6)
Percent Lymphocytes	Normal Range (15–50)	27.11 ± 10.14	27.10 ± 9.87
Absolute Lymphocyte Count	Normal Range (1000–4500)	2243 ± 819	2802 ± 935
% B Cells (CD22 ⁺)*	8.10 ± 2.67	10.82 ± 7.64	10.55 ± 5.91
% Naive B Cells** (CD22 ⁺ CD27 ⁻)	62.08 ± 18.59 ^{a,b}	85.54 ± 12.22 ^a	79.56 ± 11.33 ^b
% Memory B Cells (CD22 ⁺ CD27 ⁺)	37.92 ± 18.59 ^{c,d}	14.46 ± 12.22 ^c	20.44 ± 11.33 ^d
% IgM Memory B Cells (CD22 ⁺ CD27 ⁺ IgM ⁺ IgD ⁺)	17.13 ± 11.90 ^{e,f}	5.75 ± 8.74 ^e	7.77 ± 5.28 ^f
% Switched Memory B Cells (CD22 ⁺ CD27 ⁺ IgM ⁻ IgD ⁻)	17.73 ± 12.19 ^{g,h}	6.47 ± 6.00 ^g	12.27 ± 11.73 ^h

* B cell percentage of the circulating lymphocyte population; ** percentage of B cells. Values are expressed as the mean ± 1 SD. ^ap = 0.0004, ^bp = 0.07, ^cp = 0.0004, ^dp = 0.07, ^ep = 0.009, ^fp = 0.11, ^gp = 0.008, ^hp = 0.2.

splenectomy at a significantly younger age (5.4 vs 35.6 years; $p = 0.001$) than the autoimmune disease (AI) patients; however, the time elapsed from surgery to study enrollment did not differ significantly between these two groups. Twenty-three of the splenectomized patients (88.5%) were known to have had at least one prior pneumococcal vaccination; six of these had been vaccinated twice (23.1%). The average time elapsed since the most recent pneumococcal immunization averaged 8.8 years for AI patients and 7.2 years for HS patients, not significantly different. Baseline Ig and IgG subclass levels were within the normal range for all but two ITP patients, whose IgM levels were within 10 mg/dl of the lower limit of the normal range (Table II).

Comparing memory B cells in splenectomized and control subjects

Controls and splenectomized subjects of both groups had similar percentages of both lymphocytes and numbers of circulating lymphocytes; all were in the normal range (Table III). Controls and splenectomized subjects also had similar numbers of B cells but the splenectomized AI cohort had more naive (CD22⁺CD27⁻) B cells ($p = 0.0004$) and significantly fewer total memory cells (CD27⁺, $p = 0.0004$; switched memory, CD27⁺IgM⁻IgD⁻, $p = 0.008$; and IgM⁺ memory B cells) than did control subjects ($p = 0.0009$) (Fig. 1 and Table III). Although all memory B cells were somewhat lower overall in the HS subjects as compared with controls, there was no significant difference between the numbers of naive, total memory, or IgM memory or isotype switched memory B cells for controls and HS subjects (Fig. 1). Age or gender were not related to any B cell subset. The prior use of steroids or rituximab (four subjects) were not correlated to any B cell subset for AI subjects.

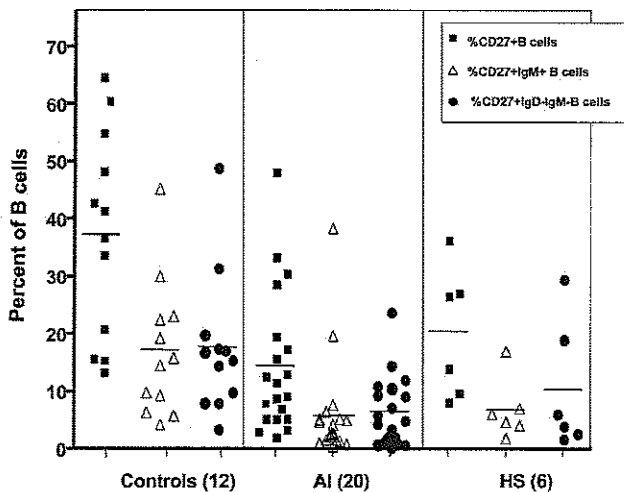


FIGURE 1. Circulating memory B cell populations (% CD27⁺ B cells, CD27⁺ IgM⁺, and CD27⁺ IgD⁻IgM⁻) were examined for healthy controls and splenectomized subjects with previous AI or HS. Although four AI subjects had received rituximab therapy 6–15 mo before enrollment, these subjects had similar percentages of memory B cells of all subsets as compared with other AI subjects. The data are expressed as median (middle horizontal bar) and all data points for each group.

Pneumococcal Ab responses

Sera of splenectomized patients tended to have higher concentrations of IgG Ab to a greater number of pneumococcal serotypes at baseline than controls, presumably as a result of prior vaccination (Fig. 2). However, following immunization, controls and all splenectomized subjects developed comparable overall IgG Ab responses, resulting in protective levels of Ab (1.3 µg/ml or more) to 17.5, 16, and 18.0 serotypes of the 23 serotypes in the vaccine. There was no significant difference between the geometric mean of IgG Ab response between normal controls and subjects with AI or HS ($p = 0.36$ and $p = 0.82$, respectively.) Because controls had less IgG at baseline compared with the splenectomized subjects and similar levels of IgG antipneumococcal Ab after immunization, a greater fold increase was observed for nonsplenectomized controls (geometric mean, post/pre = 11.7) as compared with AI or HS subjects (5.2- and 3.8-fold increase, respectively).

Baseline serum Ab concentrations of the IgM isotype were similar in all splenectomized subjects and controls (Fig. 3). Comparing geometric mean responses after vaccination, control subjects (while varied) produced more IgM Ab than AI subjects ($p = 0.01$) but not HS subjects ($p = 0.37$). As was seen for the IgG response, the titer-fold geometric mean increase in IgM Ab level for controls (5.4), was greater than for subjects with ITP or HS subjects (1.7 and 3.8, respectively), but this difference was not significant for HS subjects. There was no significant correlation between IgG or IgM Ab titers, age at time of study, age at splenectomy, time since splenectomy, number of immunizations with pneumococcal vaccine, or (for AI subjects) any medical treatment received. Because all HS subjects had splenectomy as children, the three AI subjects who had splenectomy in childhood (at ages 8, 9, and 10) are indicated (Figs. 2 and 3, a–c). These data were obtained 52, 14, and 5 years after splenectomy. One of these had a high IgG Ab, and two had

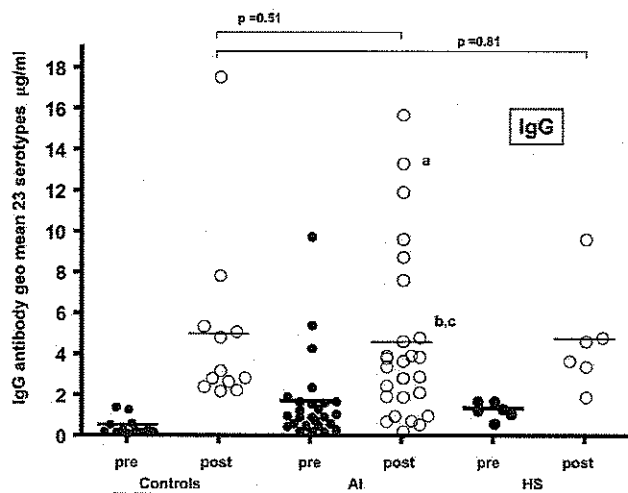


FIGURE 2. The geometric mean IgG Ab responses (µg/ml) to 23 serotypes before (●) and after (○) pneumococcal vaccination, are shown for healthy controls and splenectomized subjects with previous AI or HS. Three of the AI subjects were splenectomized under age 21 (ages 8, 9, and 10 years). These data were obtained at ages 60, 25, and 15, respectively; these subjects are indicated in a, b, and c from this cohort.

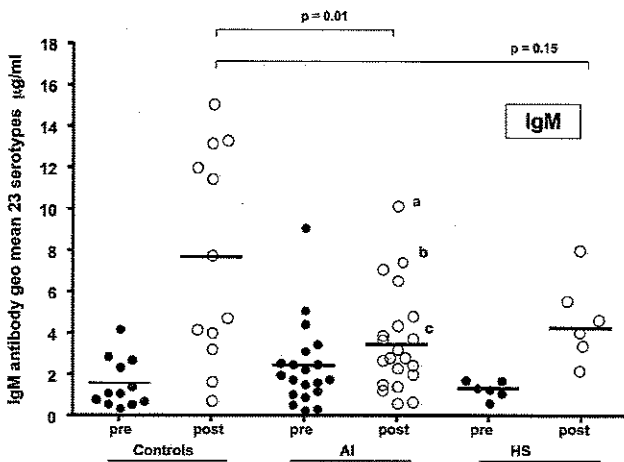


FIGURE 3. The geometric mean IgM Ab responses to 23 serotypes ($\mu\text{g/ml}$) after pneumococcal vaccination are shown for healthy controls and splenectomized subjects with previous AI or HS. Three of the AI subjects were splenectomized under age 21 (ages, 8, 9, and 10 years). These data were obtained at ages 60, 25, and 15, respectively; these subjects are indicated in *a*, *b*, and *c*.

the highest levels of IgM Ab. There were no relationships between IgG or IgM pneumococcal Ab responses and the numbers of $\text{CD27}^+ \text{IgM}^+$ memory B cells, or isotype switched memory B cells for either splenectomized subjects or controls (Table IV).

Discussion

Splenectomized patients have a predilection for systemic pneumococcal infections. Hosea et al. (3) reported in 1981 that nine adult splenectomized patients had a poor response to the pneumococcal vaccine, although it should be noted that seven of the subjects in this report had autoimmune disease and five were receiving immune suppressants (prednisone or cyclophosphamide or both) at the time of study (4). However, based on this report, and the incidence of systemic infections after splenectomy, the primary strategy for avoidance of post splenectomy sepsis has been the administration of a pneumococcal vaccine before surgery. This immunization practice has continued despite subsequent reports that showed the techniques used in early studies to measure Ab titers had also not accounted for nonspecific binding of cell wall polysaccharides, which interfere with quantitation of Ab titers (20, 27, 28). A number of more recent studies, which incorporate a cell wall polysaccharide pre-absorption step, suggest that splenectomized patients do make adequate IgG antipneumococcal and other bacterial Abs, although IgM responses have been variably reduced (11, 12, 29). The clinical significance of a reduced IgM Ab response is unclear because IgG Ab is considered the clinically protective isotype and the purpose of immunization (10, 24, 28, 30, 31).

Table IV. Antibody responses and memory B cells^a

	Pneumococcal Ab Response (Isotype)	$\text{CD27}^+ \text{IgM}^+$ B Cells (<i>p</i> Values)	$\text{CD27}^+ \text{IgM}^- \text{IgD}^-$ B Cells (<i>p</i> Values)
Controls	IgG	0.07	0.65
	IgM	0.18	0.66
AI	IgG	0.14	0.49
	IgM	0.71	0.97
HS	IgG	0.67	0.68
	IgM	0.71	1.00

^a Correlation analyses between Ab titers and memory B cell subsets for these groups of subjects was performed using the Spearman rank correlation coefficient; the *p* value was determined based on the numbers in each data set.

The clearance of encapsulated organisms is believed mediated by opsono-phagocytosis in the liver and spleen, although the spleen is considered primary in this regard as in asplenic states, bacteria proliferate rapidly (3, 32). The puzzling role of the spleen in protection against encapsulated organisms has been re-explored, as splenectomized subjects were shown to have reduced numbers of circulating $\text{IgM}^+ \text{CD27}^+$ memory B cells (16, 17). Generally, $\text{IgM}^+ \text{CD27}^+$ memory B cells are viewed as able to thrive and undergo somatic hypermutation independently from germinal centers. Because IgM memory B cells have been shown to produce Ig with specificity for polysaccharide Ags (16) and B cells of this phenotype are found in marginal zone of the spleen and after splenectomy are depleted from the peripheral blood (17), the possibility that these cells are the equivalent of T independent, B-1a cells in the mouse (33) has been raised (16). These cells in mice produce natural Abs of the IgM isotype, including those with specificity for pneumococci (34, 35), and contribute to early defense against invasive bacterial disease in both animals and possibly in man although this point has not been settled (35). Congenitally asplenic mice, or splenectomy of wild type mice is associated with loss or depletion of these cells, and an inability to produce Abs to streptococcal polysaccharides (33). Although the origin and role of these cells in humans is far from clear and alternative scenarios have been proposed (36), the reduction of $\text{IgM}^+ \text{CD27}^+$ cells has been suggested as reasons for severe bacterial infections in splenectomized subjects (16, 17) and for a higher risk of infections in both infants and patients with common variable immune deficiency, because both groups have reduced numbers of memory B cells (25, 37, 38).

In this study, we examined the peripheral B cell phenotype and IgG and IgM Ab production after pneumococcal vaccination, in a group of splenectomized subjects as compared with healthy controls. As Kreuzmann et al. noted (17), some of the splenectomized subjects, the group who had had autoimmune disease as a precursor to splenectomy, had reduced numbers of circulating IgM^+ memory B cells, as well as reduced numbers of total memory and isotype switched memory B cells. In contrast, there was no significant difference in peripheral B memory cell phenotypes between controls and subjects with splenectomy due to HS. Although these data may indicate that the lack of a spleen may not be the sole reason for the reduced numbers of memory B cell numbers in the AI group, a smaller number of HS subjects were available for study than AI subjects. Thus, we cannot exclude the possibility that deficits of memory B cells might emerge with a larger cohort of HS subjects. Although there was a wide variability in the geometric mean responses to pneumococcal vaccination between subjects in each group, and control subjects had less antipneumococcal Ab before immunization, after immunization, all splenectomized subjects, as also shown in previous studies (11, 12, 29), produced protective responses to a similar number of serotypes, and had geometric mean IgG responses similar to that of controls. Control subjects had higher post vaccination IgM responses than the AI subjects but not the HS subjects, showing that the lack of a spleen may not be the only reason for these differences. Although we cannot exclude the possibility that the subjects with autoimmunity may have intrinsic or residual immunologic differences in Ab function when compared with subjects with HS, a notable difference between the HS and AI patients was the age at splenectomy, 5.4 vs 35.6 years. Aside from the differences in the numbers of subjects in each group, this highly significant age difference at splenectomy may explain the reduction in memory B cells and possibly IgM Ab responses between these groups. Potentially in agreement with this possibility, Weller et al. noted that while splenectomized adults had reduced numbers of CD27^+ B

cells (both isotype switched and IgM⁺IgD⁺CD27⁺ cells, as shown by Kreuzmann et al. (17)), their group of four younger asplenic patients studied did not show any significant alteration in either numbers or mutational status of these memory B cells (16). These data and our observations suggest that, unlike the mouse, the younger human immune system might retain a plasticity of B cell development due to the availability of other sites which can provide essential margin-zone like influences (16). One-year-old infants have well developed and mutated circulating IgM⁺IgD⁺CD27⁺ B cells, although at this age, the marginal zone of the spleen is not yet mature, suggesting that extra splenic sites such as Peyer patches, tonsils (16), or perhaps bone marrow (39) may be involved in these maturational processes.

In the mouse, natural IgM Ab is important in the control of microbial infections (40) and after splenectomy, mice lack B-1a B cells and have reduced serum IgM levels (33). However, the function of serum IgM in humans is much less clear, although natural IgM Abs with specificity for polysaccharide Ags can be found after immunization (35), and in supernatants of human B cells cultured with ligands for TLR9 (41). Serum IgG and presumably IgM levels are predominantly sustained by plasma cells in the marrow which also allows for the maintenance of humoral memory to previously encountered Ags (42, 43). Although it has been suggested that IgM Ab responses to polysaccharide Ags are correlated with the number of circulating IgM memory B cells (17, 38), we could find no relationship between post immunization serum IgG or IgM Ab responses to pneumococcal vaccine (geometric mean of 23 serotypes or serotypes taken individually), and the number of B cells, CD27⁺ memory B cells, IgM⁺ memory B cells, switched memory B cells, or serum IgG or IgM, in the blood of either controls, AI, or HS splenectomized subjects.

Our data affirm that splenectomy is associated with diminished numbers of peripheral memory B cells and reduced IgM Ab production to T cell independent polysaccharide Ags for adult subjects who had autoimmune disease. However, as a group, the HS subjects who were splenectomized as children appeared to have both no loss of memory B cells and normal vaccination responses. IgG Ab responses to a pneumococcal vaccine were preserved in all subjects, suggesting that additional work to understand the role and maintenance of memory B cells, as well as means to prevent post splenectomy infection (44), is warranted.

Disclosures

The authors have no financial conflict of interest.

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