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Original Article

Analysis of subsets of B cells, Breg, CD4Treg and CD8Treg cells in adult patients with primary selective IgM deficiency

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Abstract: Primary selective IgM deficiency (SIGMD) is a rare and recently IUIS-recognized primary immunodeficiency disease with increased susceptibility to infections, allergy, and autoimmune diseases. The pathogenesis of selective IgM remains unclear. The objective of the study was to understand the pathogenesis of selective IgM deficiency via a comprehensive analysis of subsets of B cells, naïve and memory subsets of CD4+ and CD8+ T cells, and Breg, CD4Treg, and CD8Treg cells. Twenty adult patients with SIGMD (serum IgM 4 mg/dl-32 mg/dl) and age-and gender-matched healthy controls were studied. Naïve B cells, transitional B cells, marginal zone B cells, germinal center B cells, IgM memory B cells, switched memory B cells, plasmablasts, $CD21^{low}$ B cells, B1 cells, CXCR3+ naive and memory B cells; naïve, central memory, and effector memory subsets of CD4+ and CD8+ T cells, and CD4Treg, CD8Treg and Breg were phenotypically analyzed using multicolor flow cytometry. A significant increase in $CD21^{low}$, IgM memory B cells, Breg and CD8Treg, and a significant decreased in germinal center B cells, and CXCR3+ naïve and memory B cells were observed in SIGMD. These alterations in subsets of B cells, and Breg and CD8Treg cells may play a role in the pathogenesis of SIGMD.

Keywords: Breg, CD4Treg, CD8Treg, CXCR3+ B cells, B1 cells, $CD21^{lo}$, memory B cells, germinal center B cells, transitional B cells, marginal zone B cells, memory T cells

Introduction

Immunoglobulin M (IgM) comes in two flavors; the nature IgM and innate IgM. The innate IgM provides the initial response to foreign antigen and plays a regulatory role in subsequent immune response development, accelerating the production of high-affinity IgG antibodies. Though selective IgM deficiency (SIGMD) was described more than 45 years ago in children with fulminant meningococcal septicemia [1], it has been largely an ignored primary immunodeficiency [2], and was not included in IUIS classification of primary immunodeficiencies until most recent classification in 2014 [3]. It appears to be more common than originally realized. Primary selective IgM deficiency is observed in both children and adults with no gender bias [4-6].

The most common clinical manifestations of SIGMD are infections with extracellular and intracellular bacteria, viruses, and fungi; there is an increased prevalence of allergic and autoimmune diseases [3-16]. The pathogenesis of SIGMD remains unclear. Previously we have reported that adults with SIGMD have normal T cell numbers and functions and 50% display impaired specific anti-polysaccharide IgG antibody response [6]. Similar impaired IgG specific antibody responses have been observed in mice deficient in secreted IgM [17].

Both CD4+ and CD8+ T cells have been divided into naïve, central memory, and effector memory cells, which differ in their capacity to proliferate, secrete cytokines, and susceptibility to apoptosis, and are identified by a group of cell surface markers [18-21].

Peripheral B cells are also divided into various subsets and are identified by phenotypic array of surface markers. These include transitional B cells, follicular B cells, germinal center (GC) B

cells, and IgM and switched memory B cells, and constitute conventional B cells, which react to new antigens to produce antibodies by differentiating into plasmablasts and plasma cells [22-24]. Separate from these conventional B cells are marginal zone (MZ) B cells that specialize in response to blood borne pathogens and may represent a type of memory B cells [25], and B1 cells that constitutively and spontaneously produce natural antibodies, which are predominantly of IgM isotypes [26, 27]. Furthermore, regulatory lymphocytes (CD4Treg, Breg, and more recently rediscovered CD8Treg) regulate immune responses [26-32]. The purpose of this investigation was to perform an extensive immunological analysis of various subsets of B cells, naïve and memory subsets of CD4+ and CD8+, and Breg, CD4Treg, and CD8Treg in adult patients with SIGMD.

Our data show that adult patients with SIGMD display significantly increased in $CD21^{low}$, IgM memory B cells, Breg, CD8Treg, and a significant decreased in GC B cells and CXCR3+ naïve and memory B cells, which may play a role in the pathogenesis of SIGMD.

Methods

Patients

Twenty patients (age: 24 years-56 years; F:M ratio 1.1:1.0) with SIgMD deficiency (serum IgM range 4 mg/dl to 32 mg/dl; reference range 65 mg/dl-263 mg/dl) and 20 age and gender-matched healthy controls were studied. The Institutional Review Board (Human) of the University of California, Irvine approved this study.

Materials

Methods: All patients were studied prior to administration of intravenous immunoglobulin therapy (in 50% of patients with specific anti-polysaccharide antibody deficiency). Analyses of T cells, B cells, various subsets of B cells (naïve, IgM and switched memory, transitional B cells, MZ B cells, GC B cells, $CD21^{lo}$ B cells, B1 cells, CXCR3+ naïve and memory B cells, and plasmablasts), several subsets of CD4+ and CD8+ T cells (naive, central memory, effector memory), and CD4Treg, CD8Treg, and Breg cells were performed by multicolor flow cytometry, using various monoclonal antibodies and isotype controls. Data were analyzed by Flow Jo software.

Antibodies and reagents

B cell subsets: The following anti-human antibodies were used to identify various subsets of B cells: CD19 PerCP, CD27 FITC, CD38 FITC, CD21 PE, CD70 PE, CD27 APC, CD24 FITC, CD38 PE, CD183 PE, anti-IgM APC, and anti-IgD PE; all from BD Pharmingen, San Jose, California. CD43 APC was purchased from Biolegand, San Diego, California.

T cell subsets: The following monoclonal antibodies and isotype controls were used for the analysis of subsets of CD4+ and CD8+ T cells: CD4 PerCP, CD8 PerCP, CD45RA APC, CCR7 FITC, CD3 PerCP, and CD278 (ICOS) PE. All antibodies were purchased from BD Parmingen, San Jose, California.

Antibody panel for 4-color B cell Phenotype:

Panel	FITC	PE	PerCP	APC
1	CD27	Anti-IgD	CD19	Anti-IgM
2	CD38	CD21	CD19	Anti-IgM
3	CD27	CD70	CD20	CD43
4	CD24	CD38	CD19	
5	CD38	IgD	CD19	CD27
6	CD27	CD183	CD19	

Antibody panel for T cell Phenotype:

Panel	FITC	PerCP	APC	PE
1	CCR7	CD4	CD45RA	
2	CCR7	CD8	CD45RA	CD183 (CXCR3)
3		CD3		CD278 (ICOS)

Immunophenotyping: One ml of whole blood was added to 3 ml of phosphate buffered saline (PBS), vortexes and then centrifuged at 200 g for 5 min. The supernatants were aspirated, cells resuspended in 3 ml PBS and washed twice. After the final centrifugation and aspiration, 1 ml PBS was added to the cell pellet and analyzed for cell surface staining. Following staining, blood was lysed by 1x lysing solution (BD Pharmingen, SanJose, California), and washed with PBS and analyzed. Flow cytometry was performed using FACSCalibur (Becton-Dickinson, San Jose, CA) equipped with argon ion laser emitting at 488 nm (for FITC, PE and PerCP excitation) and a spatially separate diode

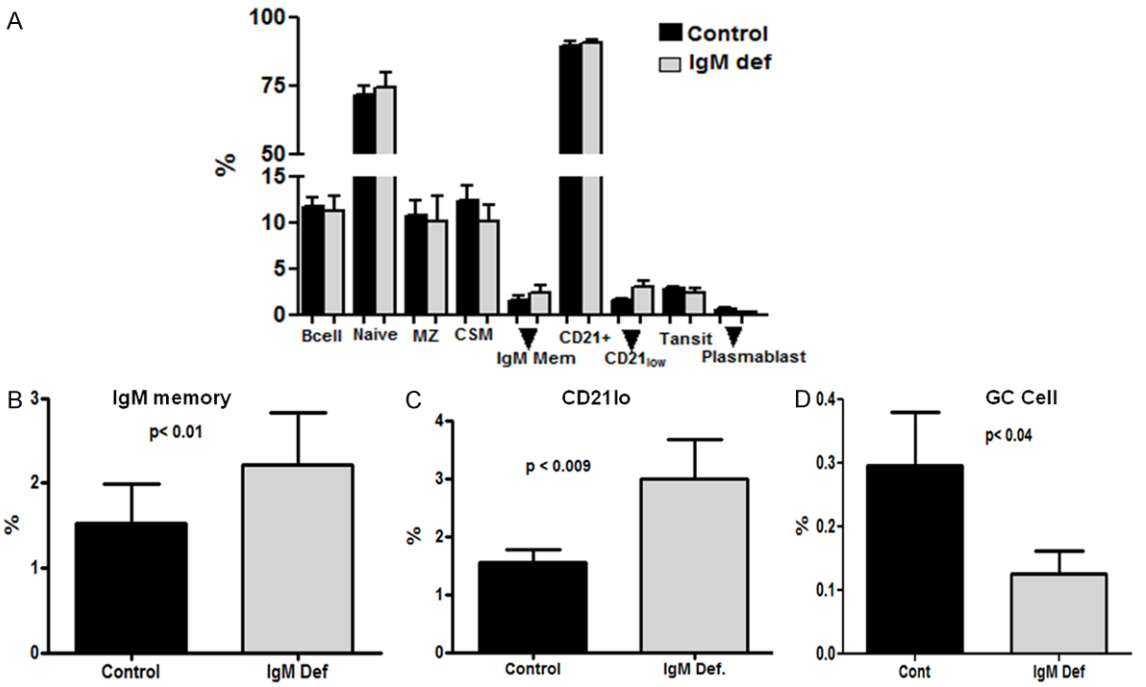

Figure 1. B cell subsets in SIGMD. PBMNC were stained with specific monoclonal antibodies defining various subsets of B cells and isotype controls and analyzed by multicolor flow cytometry using FACSCalibur. A shows all subsets of B cells as % of total lymphocytes. IgM memory B cells (B) and $CD21^{lo}$ B cells (C) were significantly increased; germinal center (GC) were significantly decreased (D).

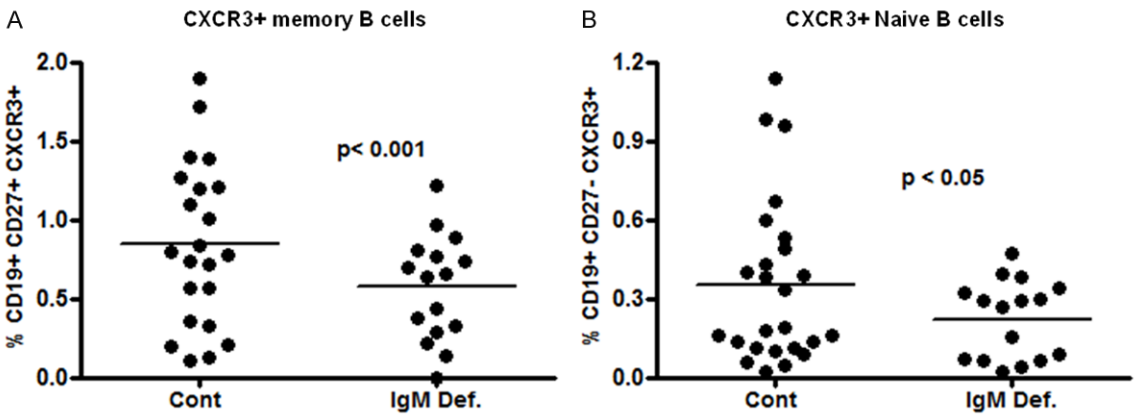

Figure 2. CXCR3 positive naïve and memory B cells in SIGMD. PBMNC were stained with monoclonal antibodies specific for CD19, CD27, and CXCR3, and isotype controls. CXCR3+ naïve and memory B cells were analyzed by multicolor flow cytometry. Both CXCR3+ naïve B cells (A) and CXCR3+ memory B cells (B) were significantly decreased in SIGMD.

laser emitting at 631 nm (for APC excitation). Forward and side scatters were used to gate and exclude cellular debris. Ten thousand cells were acquired and analyzed using Flowjo software (Tree star Inc., Ashland, Oregon).

B cell and B cell subsets were identified by following cell surface markers: naïve B cells-CD19+/CD27-/IgD+/IgM+, transitional B cells-CD19+/CD38+/IgM++, MZ B cells-CD19+/CD27+/IgD+/IgM+, IgM memory-CD19+/CD27+/IgM+, GC B cells-CD19+/IgD-/CD27+/CD38+, Class switch memory-CD19+/CD27+/IgD-/IgM, plasmabalst-CD19+/CD38++/IgM-, mature B cell- $CD21^{high}$/CD19+/CD38-, $CD21^{Low}$ cells-CD19+/CD38-/$CD21^{low}$, B1 cells-CD20+/CD70

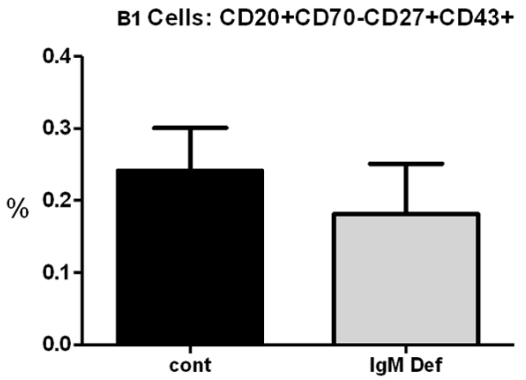

Figure 3. B1 cells in SIGMD. PBMNC were stained with monoclonal antibodies against CD20, CD70, CD27, CD43 and isotype controls, and analyzed by FACSCalibur. There was a trend towards decreased B1 cells in SIGMD; however, difference was not significant (P>0.6).

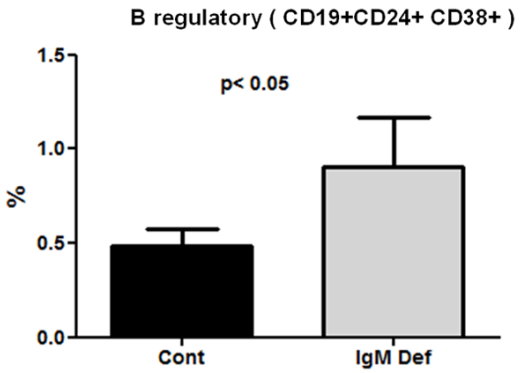

Figure 4. Regulatory B cells in SIGMD. PBMNC were stained with monoclonal antibodies against CD19, CD24, and CD38+ and corresponding isotype controls, and analyzed by multicolor flow cytometry. A significantly increased Breg (P<0.05) were present in SIGMD.

/CD27+/CD43+, CXCR3+ B cell-CD19+/CD27/CD183+, and Breg-CD19+/CD24+/CD38+.

Following cell surface phenotype identified subsets of CD4 T cells and CD8+T cells: naïve (T_N)- CD4+/CD8+CD45RA+CCR7+, central memory (T_{CM})- CD4+/CD8+CD45RA-CCR7+, effector memory (T_{EM})- CD4+/CD8+CD45RA-CCR7-, CD45RA+ effector memory (T_{EMRA}) or terminally differentiated effector memory- CD4+/CD8+CD45RA+CCR7-, CD8Treg-CD8+CD183+CCR7+CD45RA-.

For CD4Treg, cells were stained with PerCP-labeled anti-CD4 and FITC-labeled anti-CD25, according to manufacturer's protocol, followed by Foxp3 intracellular staining with APC-labeled anti-Foxp3 and isotype control (Mouse IgG1κ-APC). Staining procedures was performed according to the manufacturer's recommendation. In the population of CD4 cells, Treg cells were identified as CD4+CD25^{high} Foxp3+ cells.

Statistical analysis was performed by paired student t test.

Results

B cell subsets in SIGMD are altered

Total number of B cells is normal in patients with SIGMD. Alterations in B cell subsets have been reported in common variable deficiency [33, 34]. Therefore, we examined naïve B cells, mature B cells, transitional B cells, MZ B cells, GC B cells, IgM memory B cells, class switched memory B cells, CD21^{low} B cells and plasmablasts by multicolor flow cytometry, using various combinations of antibodies and isotype controls (**Figure 1A**). CD21^{low} B cells (**Figure 1C**) and IgM memory B cells (**Figure 1B**) were significantly increased in SIGMD as compared to controls, whereas switched memory B cells were comparable to controls. GC B cells were significantly decreased in patients with SIGMD (**Figure 1D**).

CXCR3+ B cells are reduced in SIGMD

CXCR3 is a G protein-couple receptor for three chemokines (CXCL9, CXCL10, CXCL11), whose role in the trafficking of CD8+ T effector cells has been well established [35, 36]. Recently a role of CXCR3 in B cell migration to inflammatory sites has been proposed [37]. Our data show that expression of CXCR3 is significantly greater in CD19+CD27+ memory B cells as compared to CD19+CD27- naïve B cells in both controls and patients (**Figure 2**). Furthermore, both CXCR3+ naïve B (**Figure 2B**) and memory B cells (**Figure 2A**) were significantly reduced (P<0.001, P<0.05 respectively) in patients with SIGMD as compared to control.

B1 cells in SIGMD

The principal and unique function of B1 cells is spontaneous and constitutive secretion of natural antibodies, which are predominantly IgM [27]. In our patients with SIGMD, there was a decrease in B1 cells as compared to controls

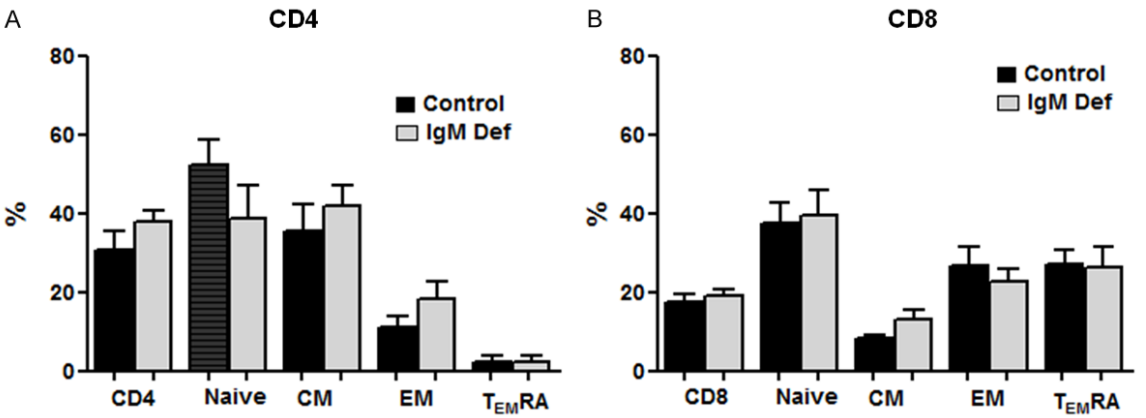

Figure 5. Naïve and memory subsets of CD4+ and CD8+ T cells in SIGMD. Naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) subsets of CD4+ and CD8+ were analyzed by multicolor flow cytometry using monoclonal antibodies against CCR7, CD45RA. All subsets of CD4+ (A) and CD8+ (B) T cells in SIGMD were comparable to healthy controls. CD4+ and CD8+ cells are % of lymphocytes. Subsets are % of total CD4+ and CD8+ T cells.

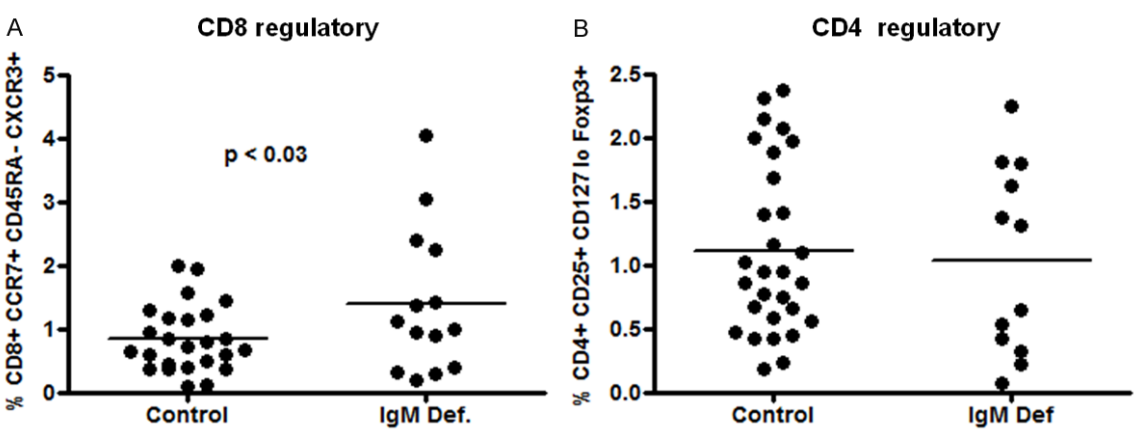

Figure 6. CD4Treg and CD8Treg in SIGMD. PBMC were stained with antibodies specific for CD8, CD183, CCR7, CD45RA and isotype controls for CD8Treg (A), and stained with antibodies specific for CD4, CD127low, and FoxP3 and isotype controls for CD4Treg (B), and analyzed by multicolor flow cytometry. CD8Treg were significantly increased in SIGMD.

(**Figure 3**); however, it did not reach statistical significance (P>0.6).

Breg are increased in SIGMD

B regulatory cells play an important role in regulating both innate and adaptive immune responses [28-30]. Breg were significantly (P< 0.05) increased in patients with SIGMD as compared to controls (**Figure 4**).

Subsets of CD4+ and CD8+ T cells were unchanged in SIGMD

CD4+ and CD8+ T cells are divided into naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and effector memory CD4+ and CD8+ T cells expressing CD45RA (T_{EMRA}). These subsets differ in homing, their capacity to proliferate in response to antigens, cytokine production, effector cytotoxic function, and susceptibility to apoptosis [18, 20, 21]. Therefore, we analyzed these subsets, using anti-CD8, anti-CD4, anti-CCR7, and CD45RA, and CD28 monoclonal antibodies and isotype controls. No significant difference was observed in any of the subsets of either CD4+ or CD8+ T cells between patients and controls (**Figure 5**).

Regulatory CD4+ and CD8+ T cells in SIGMD

A role of CD4Treg in T cell tolerance is well established [31]. Recently, CD8+CCR7+CXC-

R3+ T cells (CD8Treg) have shown to display regulatory activity against autologous CD4+ and CD8+ T cells, and appear to play a role in tolerance [32]. Since patients with SIGMD have increased prevalence of autoimmune diseases, we analyzed both CD4Treg and CD8Treg in our patients with SIGMD and healthy controls. Interestingly CD8Treg were significantly increased ($P<0.05$) in SIGMD as compared to controls **Figure 6A**); however, no significant difference was observed in CD4Treg between patients and controls (**Figure 6B**).

Discussion

Selective IgM deficiency is defined as serum levels below 2 SD of the mean, which is usually less then 30 mg/dl in adults and 20 mg/dl in children [9]. However, some patients have complete absence of serum IgM; four of 20 patients in the present study had complete absence of serum IgM. In 1967, Hobbs et al [1] described IgM deficiency in 2 male children with fulminant meningococcal septicemia. Since then SIGMD has been reported in both children and adults [2, 4-6]. Serum IgG and IgA levels are normal. However, associated IgG subclass deficiency has been reported in few cases of SIGMD [2, 6] Patients with SIGMD are more prone to allergic and autoimmune diseases [2, 6, 7, 12, 14-16].

CD3+, CD4+, and CD8+ T cell numbers and T cell functions are generally preserved in majority of patients with selective IgM deficiency [6, 38-40], except in a syndrome of SIGMD with severe T cell lymphopenia (Gupta syndrome) that is associated with *Mycobacterium avium complex infections* [41, 42].

CD4+ and CD8+ T cells have been further classified into naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}), and have been characterized extensively for phenotype and functions [18-21]. Naïve T cells (T_N) upon exposure to an antigen undergo a clonal expansion of effector cells, which after clearing the antigen, undergo a phase of contraction when antigen-specific T cells undergo apoptosis, and a small number of antigen-specific T cells stabilizes and retained as memory T cells [18-21]. These memory T cells differentially express adhesion molecules and chemokine receptors, which allow them to home in peripheral blood lymphoid and extralymphoid tissues. Based upon the expression or lack of them, memory CD4+ and CD8+ T cells migrate to lymph nodes and spleen (central memory, T_{CM}) or to extralymphoid tissue like lung and liver (effector memory; T_{EM}). A small subpopulation of T_{EM} cells that re-acquires CD45RA and termed as T_{EMRA} or terminally differentiated and memory or exhausted T effector cells. T_{EM} and T_{EMRA} T cells T cells display poor proliferation, decreased telomere length, and are resistance to apoptosis. We did not observe significant difference in any of the subpopulations of CD4+ and CD8+ T cells in SIGMD.

B cell development initiates in the bone marrow from common lymphoid progenitors and progresses through sequential developmental stages [43]. Cells that have successfully recombined their immunoglobulin genes (immature B cells), express functional B cell receptor (BCR) leave the bone marrow, and are termed transitional B cells. Transitional cells represent a crucial step in the differentiation and selection of the mature B cell compartment. Only a small proportion of mature naïve B cells are activated by antigen, which leads to clonal expansion and differentiation. Antigen binding to the BCR activates B cells in the lymphoid follicle signaling to leave the follicle. After extralymphoid proliferation, short-lived plasma cells are formed producing antibodies predominantly of IgM class. Antigen-activated B cells that interact with follicular helper T cells enter the follicle, where they proliferate and form germinal centers (GCs). Here, they undergo class switch recombination (IgG, IgA, IgE) and somatic hypermutation (affinity maturation). Subsequently cells leave GCs to differentiate into long-lived plasma cells homing into the bone marrow to produce secreted antibodies of different isotypes for extended period, and a small population of GC B cells leaves the GCs to become memory B cells.

In the majority of patients with SIGMD, surface IgM+ B cells (sIgM+), CD19+ B cells, and CD20+ mature B cells are normal [4-6, 38-40]. In the present study, proportions of mature B cells were also comparable to controls, including in patients who had complete lack of serum IgM.

More recently, human transitional B cells have been subdivided into several subsets, which may important insights into human B cell development [44]. Transitional B cells mature across a developmental continuum with gradu-

al up-regulation of mature markers, concomitant loss of immature markers, and increased responsiveness to BCR cross-linking in terms of proliferation, calcium flux, and survival [45]. We did not observe any significant difference in transitional B cells in our patients with SIGMD. However, Mensen et al [46] reported increased transitional B cells in a subset of patients with SIGMD. The reason for this discrepancy may due to difference in the severity of SIGMD, and heterogeneity of SIGMD. Our patients had more severe SIGMD, including 4 patients had complete absence of IgM (range 4 mg/dl-32 mg/dl; normal reference range 65-263 mg/dl), as compared to Mensen's patient group who appears to have borderline low serum IgM levels (32-39 mg/dl; normal reference range 40-230 mg/dl) and the diagnosis of SIGMD may be questionable in some of these patients.

A major population of transitional B cells migrates and differentiates in to mature follicular B cells and a minor population into mature MZ B cells. Marginal zone B cells in human, unlike mice, are present in the lymph nodes, tonsils, Payer's patches of intestine, and also in the circulating blood. After interacting with antigens exposed on antigen-presenting cells, MZ B cells differentiate into plasmablasts that produce large amounts of IgM and IgG and IgA via class switch recombination [25, 47]. We did not observe any difference in marginal zone B cells or plasmablasts in patients with SIgMD. This would be consistent with normal serum IgG and IgA in patients with SIgMD. Mensen et al [46] reported deceased MZ B cells in 1 of 12 patients with SIgMD.

Germinal centers are considered a special microenvironment where B cells proliferate rapidly, undergo isotype switching, and somatic hypermutation; the later critical for selecting B cells with increased B cell receptor or antibody affinity [48, 49]. B cells encounter antigen and interact with follicular helper T cells and follicular dendritic cells in the GCs light zone (LZ) and then migrate to the dark zone where they proliferate and undergo somatic mutation before cycling back to the LZ for further rounds of selection. We have observed significantly reduced number of GC B cells in patients with SIGMD. This is in agreement with impaired germinal center formation in secretory IgM deficient and FcμR mutant mice (lack secretory IgM) [50-52]. These mice, similar to humans with SIgMD have impaired specific IgG antibody response. Recently, decreased GC B cells have also been reported in a syndrome of SIGMD and T cell lymphopenia [41]. The mechanisms of decreased GC B cells in SIGMD are unclear. It is believed that changes in DNA methylation are required for the formation of GCs. Dominguez et al [53] have demonstrated a role of activation-induced cytidine deaminase (AID) in DNA demethylation. Recently, Yajima and colleagues [54] reported that the loss of IL-21 is considered to be involved in the disappearance of Bcl-6 and leads to atrophied germinal centers in selective IgM deficiency in multicentric Castleman's disease. Therefore, an impaired DNA methylation and/or a deficiency of IL-21 might be responsible for decreased GC B cells in SIGMD.

Activated and differentiated B cells further differentiate into memory or plasma cells. Marginal zone B cells generally differentiate into short-lived plasma cells, whereas GC B cells differentiate into long-lived memory cells, and plasma cells that migrate to the bone marrow [55]. We have observed increased number of IgM memory B cells but comparable number of switched memory B cells. Mensen et al [46], reported decreased switched memory B cells in 3 of 12 patients and decreased IgM memory B cells in patients with SIGMD. However, a decrease in switched memory B cells would be inconsistent with normal IgG and IgA in SIGMD. Recently, an increase in IgM memory B cells was also reported in a patient with CVID with *ITPKB* mutation [56]. Furthermore, regulatory B cells (Breg) are enriched in IgM memory B cells [57]. An increased in IgM memory B cells will also be consistent with increased Breg in our patients with SIGMD. The significance of increased IgM memory is unclear. It is also possible that there is a block in the differentiation of IgM B cells to plasma cells and therefore directs the differentiation to IgM memory B cells.

CD21 (complement receptor 2; CR2) is a type I membrane glycoprotein forms a complex with CD19 and CD81 to act as a B cell co-receptor. This population of B cell is distinct from other B cell subpopulation that resembles innate like B cells [58]. $CD21^{low}$ are increased in patients with CVID with autoimmunity, and systemic

lupus erythematosus [59, 60]. Impaired polysaccharide response in early life is believed to be secondary low expression of CD21 on B cells [61]. Patients with SIGMD as well as mice lacking secretory IgM respond poorly to polysaccharide antigens, and develop autoimmunity and autoimmune diseases [2, 6, 7, 12, 14-16, 50-52]. In our patients, we observed significantly increased proportions of $CD21^{low}$ B cells, which may explain both autoimmunity and poor anti-polysaccharide antibody responses in SIGMD [2, 6].

B1 cells spontaneously secrete antibodies, predominantly of IgM isotype, of low affinity and are polyspecific [26, 27]. A major component of natural antibodies recognize phosphorylcholine, which is component of a number of bacterial pathogens, and apoptotic cell membrane [62, 63]. Therefore, natural antibodies have an important antimicrobial, role to remove apoptotic cells, and to regulate immune and inflammatory response [64-67]. Another component of natural antibodies recognize phosphatidylcholine, a key component of senescent red blood cells. Natural isohemagglutinins (IgM) are diminished in a subset of patients with SIGMD [23, 25]. We observed a trend towards decreased proportions of B1 cells in selective IgM deficiency however; difference did not reach statistical significance. Perhaps a study of a larger number of patients is needed. Recently, significantly decreased B1 cells have been reported in a patient with SIGMD and T cell lymphopenia [41]. It might be possible that B1 may not be significantly reduced in numbers but they may be functionally impaired in patients with SIGMD. Such a deficiency may be in part responsible for increased frequency of infections and autoimmunity secondary to impaired clearance of apoptotic cells, in patients with SIGMD.

CXCR3 is a G protein-couple receptor for three chemokines (CXCL9, CXCL10, CXCL11) that is mapped to chromosome X, is expressed on activated T cells, NK cells, myeloid and plasmacytoid dendritic cells, and B cells. Its role in the trafficking of T effector cells has been well established [33] CXCR3+ CD4+ T cells are accumulated in synovial tissue of patients with rheumatoid arthritis [35]. A role of CXCR3 in B cell is emerging. CXCR3 is expressed on B lymphocyte transition to plasma cells. Henneken et al [36] reported decreased number of CXCR3+ B in systemic lupus erythematosus. Since, patients with selective IgM are more susceptible to autoimmunity, we examined the expression of CXCR3 on naïve and memory B cell subsets. Both CXCR3+ naïve and memory B cells were significantly decreased in patients with SIGMD. It is possible that decreased expression of CXCR3 may result in impaired migration of B cells to elicit an effective immune response.

Recently, there has been increasing interest in understanding the role and mechanisms of Breg [28-30] and CD8Treg [32].

Breg regulate immune responses including inflammatory responses in a variety of autoimmune diseases [28], and more recently were shown to regulate the generation of peripheral CD4+Treg cells [68, 69]. In our cohort of patients with SIGMD, Breg are significantly increased. How increased Breg play a role in selective IgM deficiency is unclear. It is possible that Breg also regulate CD8Treg function of suppressing B cell differentiation to antibody-producing plasma cells or directly regulate function of non-regulatory effector B cells to differentiate in immunoglobulin producing plasma cells.

More recently, CD8Treg cells have been reported to play an important role in immune homeostasis [32]. A role of CD8Treg has been demonstrated in a number of animal models and autoimmune diseases in humans [70-73]. In our patients with SIGMD, CD8Treg cells (CD8+ CCR7+CD183+CD45RA-) were increased. We have observed that CD8Treg *in vitro* suppress B cell proliferation and immunoglobulin production (IgM>IgG>IgA; unpublished personal observation), therefore, increased CD8Treg may play a role in the suppression of IgM and the pathogenesis of SIGMD. We have also observed that CD8+Treg suppress CD4Treg (manuscript in preparation). In this study number of CD4Treg are normal; however, it is possible that they may be functionally impaired. Therefore, increased CD8Treg may possibly suppress regulatory function of CD4Treg without altering their numbers, and thereby play role in an increased susceptibility to autoimmunity in SIGMD.

The pathogenesis of SIGMD remains unclear. A number of mechanisms, based upon experiments on a small number of patients, have

been suggested. These include intrinsic defect of B cells, increased non-isotype specific suppressor T cells [39, 74], isotype-specific suppressor [75], decreased helper T cell functions [38], reduced secreted mu mRNA synthesis [76], and intrinsic B cell defect [40, 46]. Based upon our present studies, increased Breg and CD8Treg, and decreased GC B cells, and CXCR3+ B cells may also play a role in the pathogenesis of SIGMD. Since serum IgG, IgA, and IgE are normal there is no immunoglobulin isotype switch defect. Furthermore, surface IgM expression appears to be normal in most cases of SIGMD. Similar is the observation in mice with selective IgM deficiency [50-52]. Since assembly, degradation, and secretory pathways for membrane bound surface IgM and secreted IgM are different, it is possible that the defects in SIGMD might be in assembly, degradation, transport, and secretory pathway of secretory IgM at the level of endoplasmic reticulum [77]. We are currently investigating a role of certain ER transport genes in our cohort of patients with SIGMD.

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Disclosure of conflict of interest

None.

Authors' contribution

SG conceived the idea, interpreted data and edited the manuscript, AGL coordinated the study, acquired and analyzed the data, and wrote the manuscript, and SA performed flow cytometry.

Abbreviations

GC, germinal center; MZB, marginal zone B cells; PBMNC, peripheral blood mononuclear cells; T_N, Naïve T cells; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{EMRA}, CD45RA+ effector memory T cells or terminally differentiated effector memory T cells.

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