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Altered B-cell subsets and functional B-cell defects in selective IgM deficiency

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ABSTRACT

Primary selective IgM deficiency (sIgM) is characterized by diminished serum IgM, infections and autoimmunity. Although there is some evidence of B-cell defects the pathogenesis of sIgM is poorly understood. We determined peripheral B-cell subsets and IgM-expression levels in 31 adult sIgM patients by flow cytometry. In a subset of patients B-cell subset alterations and antibody-secreting cells were determined by flow cytometry and ELISpot assay after *in vitro* differentiation.

Patients had significantly increased transitional, decreased IgM only, switched and non-switched memory B cells and decreased membrane IgM-expression levels on memory B-cell subsets compared to healthy controls. A strongly diminished B-cell differentiation and expansion capacity was observed in 5/6 investigated patients. Severely reduced IgM-secreting capacity was detected in 2/6 patients.

Taken together, our results show altered B-cell subsets and severe functional B-cell defects in sIgM. This may provide a diagnostic tool and basis for subclassification of patients to study the pathogenetic background.

1. Introduction

Selective IgM deficiency (sIgM) represents a poorly characterized dysgammaglobulinemia, defined by isolated low IgM level in serum and susceptibility to infections according to ESID criteria. IgM levels can range from absent to levels slightly below two SD of age-adjusted means in children and adults [1–5]. IgG subclass deficiencies were observed in some patients [2–4].

Patients with sIgM suffer from moderate to severe infections. Most common are respiratory tract infections but also gastroenteritis, sepsis and meningitis were reported [2,5,7]. Furthermore, sIgM is associated with autoimmunity [5].

While the innate immunity appears normal with regard to numbers of innate immune cells, levels of complement proteins and neutrophil functions [4,8,9], some abnormalities of T and B cells were reported. Normal numbers of peripheral T and B cells including membrane IgM-expressing B cells were described in the majority of patients [2,3,10–14]. Some patients had decreased peripheral B cells and some an altered CD4/CD8 ratio [2–4,12,13]. B-cell subset distribution has been poorly analyzed thus far [4,8,15]. A detailed analysis of B-cell subsets in adult sIgM patients is missing. T-cell subsets within the $CD4^+$ and $CD8^+$ T-cell compartment have not been investigated yet.

Functionally, both T-cell and B-cell defects were suggested as pathogenic causes for sIgM. Increased IgM-specific T-cell suppressor functions and defects in T helper function that impair the B-cell differentiation into antibody-secreting cells (ASC) after *in vitro* stimulation were described in some patients [3,9,12]. Other studies observed B-cell intrinsic defects [10,12,13,16]. In these experiments mixed co-cultures of T and B cells from patients and healthy controls in presence of *pokeweed mitogen* (PWM), *Staphylococcus aureus cowan 1* (SAC) and/or concanavalin A were performed, followed by immunoglobulin quantification with ELISA or quantification of ASC *e.g.* with the hemolytic plaque assay [17]. ELISpot analysis, which quantifies the number of ASC on single cell level, has not been applied so far. Furthermore, B-cell subset expansion and distribution after *in vitro* stimulation was not yet investigated.

Within this study, we provide a comprehensive immunological characterization of adult sIgM patients, who were diagnosed at our

Abbrevations: ASC, Antibody-secreting cells; DN, $CD27^-IgD^-$ double negative; ELISpot, Enzyme linked immuno spot assay; HC, Healthy controls; MAC, *Mycobacterium avium complex*; MZ-like, Marginal zone-like; PBMC, Peripheral blood mononuclear cells; PWM, *Pokeweed mitogen*; SAC, *Staphylococcus aureus cowan I*; sIgM, Selective IgM deficiency; TT, Tetanus toxoid.

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immunodeficiency outpatient clinic for adults. We investigated peripheral blood B- and T-cell subsets by flow cytometry *ex vivo* and B-cell subsets after *in vitro* differentiation and proliferation. ELISpot analysis was applied to determine antibody secreting capacity of single B cells after *in vitro* differentiation. Our study revealed a severe deficiency of B-cell expansion in most patients accompanied by diminished IgM secretion capacity in a subset of patients. B-cell subset alterations and diminished membrane IgM expression levels on B-cell subsets, which can be determined by flow cytometry may provide a diagnostic tool to more accurately diagnose sIgM deficiency.

2. Material and methods

2.1. Patients and control samples

Citrate blood was drawn from 12 adult sIgM patients and 12 healthy controls and immediately processed for flow cytometry analysis. ELISpot analysis was performed for six patients and six controls only. B-cell subsets were further retrospectively evaluated in a second cohort of 19 adult sIgM patients. The study was approved by the institutional ethics committee of the Charité-Universitätsmedizin Berlin. Patients gave informed consent.

2.2. Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation. For $CD3^+$ T-cell depletion the RosetteSep™Human-CD3 Depletion Cocktail was applied according to manufacturer's instructions (Stemcell Technologies, France). PBMC were stained immediately or after *in vitro* stimulation with fluorochrom-conjugated anti-human monoclonal antibodies (see Supplementary Table 1). Cells were analyzed with a LSRII Fortessa Flow Cytometer (Becton Dickinson, USA). Dead cells were excluded by propidium iodide staining. Data evaluation was done with the FlowJo 9.5.2 software (TreeStar, USA). Differential blood counts were used to calculate absolute cell numbers.

2.3. Cell stimulation

T-cell depleted and whole PBMC were left unstimulated or stimulated for seven days 37 °C in IMDM medium ($2–3 \times 10^6$ cells/ml) supplemented with 10% FCS, ITS, CpG, SAC, PWM, CD40L, IL-2, IL-10 and IL-21. For reagents and concentrations used, see Supplementary Table 2.

2.4. ELISpot

Stimulated cells were added to 96-well MultiscreenHTS-IP filter plates coated with IgM, IgG and IgA antibodies. Secreted antibodies were detected with biotin-conjugated IgM-, IgG- and IgA-antibodies and HRP-conjugated streptavidin. Spots were quantified with the ImmunoSpot 5.1 and ImmunoCapture 6.4 Software at an ImmunoSpot®Analyzer (CTL Europe GmbH, Germany). For reagents and concentrations used, see Supplementary Table 2.

2.5. Statistics

The two-sided Mann–Whitney U test was applied. Values were expressed as median and interquartile range. Statistical analysis was performed with the GraphPad Prism v5.0 Software (USA). p-Values of $p \leq 0.05$ were considered as statistically significant.

3. Results

3.1. Patients and clinical findings

Twelve adult patients (five male, seven female; median age, range: 42, 22–57) with recurrent infections and serum IgM-level of <40 mg/dl but normal IgG and IgA level at time of diagnosis were included in the first study. Patients' clinical characteristics are summarized in Table 1. The median IgM level (range) at time of diagnosis was 32 (23–39) mg/dl (reference: 40–230 mg/dl; Fig. 1A). IgM deficiency was confirmed by repeated measurements within 1–4 years (Fig. 1B). Serum IgG and IgA levels were normal with 929 (735–1194) mg/dl and 212 (113–373) mg/dl, respectively (reference: 700–1600 mg/dl IgG, 70–400 mg/dl IgA; Fig. 1C). Two patients had reduced levels of the IgG3 subclass, other IgG subclasses were normal for all patients (see individual values for patients in Supplementary Table 3).

All patients suffered from recurrent infections. Patients' main complaints were infections including upper respiratory tract infections (83%) with recurrent sinusitis, pharyngitis and/or otitis, and lower respiratory tract infections (25%). Four patients suffered from atypical infections: one patient had an atypical pneumonia with *mycobacterium avium complex* (MAC), one patient had recurrent enteritis with *Giardia lamblia*, and two patients had recurrent mucosal candidosis. Three patients suffered from recurrent labial herpes virus infections, one had herpes zoster at the age of 19.

Table 1
Patients'characteristics.

Patient	1	2	3	4	5	6	7	8	9	10	11	12
Recurrent infections	URT LRT	URT	URT	URT	URT LRT	URT LRT		URT		URT	URT LRT	URT
	HSV					HSV						
					MAC							
							EGL MC		MC			
Allergic/ atopic disease			POLL CAT	CAT DUST								
					NI/CH					NI/CH		
	PEN											
Autoimmunity	HT				HT PSOR							
Others	FAT	FAT			FAT							
	LPA JO						LPA JO					

URT, upper respiratory tract; LRT, lower respiratory tract; HSV, herpes simplex virus; MAC, mycobacteriosis; EGL, enteritides *Gardia lamblia*; MC, mucosal candidosis; POLL, grass/tree pollen; CAT, cat; DUST, house dust; NI/CH, nickel/chrome; PEN, penicillin; HT, Hashimotos' thyroiditis; PSOR, psoriasis; FAT, fatigue; LPA, lipoprotein A hyperlipoproteinemia; JO, jaw osteonecrosis.