SHORT COMMUNICATION

Defective formation of IgA memory B cells, Th1 and Th17 cells in symptomatic patients with selective IgA deficiency

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Abstract

Objective. Selective IgA deficiency (sIgAD) is the most common primary immunodeficiency in Western countries. Patients can suffer from recurrent infections and autoimmune diseases because of a largely unknown aetiology. To increase insights into the pathophysiology of the disease, we studied memory B and T cells and cytokine concentrations in peripheral blood.

Methods. We analysed 30 sIgAD patients (12 children, 18 adults) through detailed phenotyping of peripheral B-cell, CD8+ T-cell and CD4+ T-cell subsets, sequence analysis of IGA and IGG transcripts, in vitro B-cell activation and blood cytokine measurements.

Results. All patients had significantly decreased numbers of T-cell-dependent (TD; CD27+) and T-cell-independent (TI; CD27−) IgA memory B cells and increased CD21low B-cell numbers. IGA and IGG transcripts contained normal SHM levels. In sIgAD children, IGA transcripts more frequently used IGA2 than controls (58.5% vs. 25.1%), but not in adult patients. B-cell activation after in vitro stimulation was normal. However, adult sIgAD patients exhibited increased blood levels of TGF-β1, BAFF and APRIL, whereas they had decreased Th1 and Th17 cell numbers.

Conclusion. Impaired IgA memory formation in sIgAD patients is not due to a B-cell activation defect. Instead, decreased Th1 and Th17 cell numbers and high blood levels of BAFF, APRIL and TGF-β1 might reflect disturbed regulation of IgA responses in vivo. These insights into B-cell extrinsic immune defects suggest the need for a broader.

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INTRODUCTION

Selective IgA deficiency (sIgAD), the most common primary immunodeficiency in Western countries, is defined by very low to absent serum levels of IgA with normal IgG and IgM. The prevalence differs between countries and ranges between 1:328 and 1:3040. The majority of the individuals with between countries and ranges between 1:328 and 1:3040. The majority of the individuals with normal IgA are asymptomatic and identified by coincidental findings. Still, a number of patients are prone to suffer from recurrent infections and to develop autoimmune diseases and/or allergies. Altogether, the clinical symptoms of immunodeficiency and immune dysregulation are much higher in sIgAD than in the normal population. This concerns autoimmunity with a prevalence of 25–31% and includes systemic lupus erythematosus, rheumatoid arthritis and type 1 diabetes. In addition, between 18% and 56% of sIgAD patients suffer from one or more allergies. The pathogenesis of the disease is unknown.

We and others have shown that IgA memory B cells can derive from T-cell-dependent (TD) and T-cell-independent (TI) responses (Figure 1a). In human peripheral blood, most IgA memory B cells express CD27 and originate from CD40L-mediated T-cell help in germinal centres. In contrast, CD27-negative IgA memory B cells originate from TI responses in the intestinal tract and are characterised by high IgA2 usage and increased reactivity to intestinal bacteria. This CD40L-independent pathway involves the binding of the cytokine A proliferation-inducing ligand (APRIL) to its receptor transmembrane activator and CAML interactor (TACI), resulting in the expression of activation-induced cytidine deaminase (AID). Additional cytokines, such as transforming growth factor-beta (TGF-β), vasoactive intestinal peptide (VIP) and IL-10, induce germline IGA transcripts leading to IGA class switching. Previous studies described reduced numbers of Ig class-switched memory B cells and CD19+IgA+ B cells in sIgAD patients. Recently, Blanco et al. grouped sIgAD and other antibody deficiency patients based on the immunological focus on genomics and functional analyses to unravel the pathogenesis of sIgAD.

Keywords: B-cell memory, cytokine concentration, IgA, selective IgA deficiency, Th1 cells, Th17 cells
analysis of IGA transcripts in children and adults with slgAD. We also measured cytokine concentrations in blood samples of adult IgAD patients.

Here, we show that both TD- and TI-derived IgA memory B cells are reduced or absent in slgAD patients. Interestingly, molecular maturation and B-cell activation were not impaired, but Th1 and Th17 numbers were decreased in adult slgAD patients. In contrast, we observed increased cytokine concentrations in B-cell activating factor (BAFF), APRIL and TGF-β1. Together, these results show that SlgAD patients do not exhibit class switch abnormalities, but a defect in the formation of IgA memory B cells, and Th1 and Th17 cells.

RESULTS

We included 30 slgAD patients (12 children, 18 adults) with IgA serum concentrations < 0.07 g L⁻¹ (patient details in Supplementary table 1). To obtain more insights into the pathogenesis of slgAD that might be useful to develop potential treatment strategies, we included mainly symptomatic patients. Twenty-six out of 30 patients suffered from recurrent respiratory tract infections, 11/25 patients experienced one or more allergies, and 5/27 patients had autoimmune complications. Two adult patients carried heterozygous mutations in TNFRSF13B.

Decreased CD27⁻ and CD27⁺ IgA⁺ memory B cells in slgAD patients

To investigate whether IgA memory B-cell formation was affected, we analysed peripheral blood B cells in patients (children n = 11, adults n = 14) and compared them with healthy controls (children n = 67, adults n = 29; Figure 1a). We found that both children and adult patients exhibited decreased IgA memory B cells (Figure 1b). Furthermore, IgA memory B cells were undetectable in 11 out of 14 adult patients. In children with slgAD, we found increased numbers
of naive and CD21\textsuperscript{low} B cells, but low IgM ‘IgD’ memory B-cell numbers (Figure 1c). In contrast, in adult patients, only CD21\textsuperscript{low} B cells were increased. IgG memory B cells were studied only in adults, and these were similar to controls (Figure 1d).

Interestingly, some of these abnormalities in peripheral B cells of sIgAD patients showed similarities with B-cell abnormalities observed in CVID patients, such as reduced memory B cells and increased numbers of CD21\textsuperscript{low} B cells.\textsuperscript{28} Still, CVID patients are low in serum IgG, and the different subgroups (as described previously by Driessen \textit{et al.}\textsuperscript{26}) showed additional defects in naive and memory B-cell subsets that we did not observe in sIgAD patients (Supplementary figure 2). Thus, sIgAD patients showed signs of chronic inflammation as observed by increased CD21\textsuperscript{low} B cells, and these were explicitly defective in IgA B-cell memory.

\textbf{Ig class switching and SHM in transcripts of sIgAD patients}

Similar to others,\textsuperscript{24} we detected low numbers of rearranged \textit{IGA} transcripts in PBMCs of patients with sIgAD. We analysed unique sequences to determine Ig subclasses and compared their relative distribution to sequence analysis in controls. \textit{IGA} transcripts from paediatric patients consisted of \textit{IGA2} significantly more often (58.5\%) than controls (25.1\%) (Figure 2b), whereas in adult patients, \textit{IGA2} transcripts were used less frequently (18.1\%) than in controls (50\%; Figure 2b). Despite B cells of adult sIgAD patients having reduced usage of \textit{IGA2}, distal \textit{IGG2} subclass usage was normal (Supplementary figure 3a).

Somatic hypermutation (SHM) frequencies in \textit{IGHV} regions in \textit{IGA} transcripts from patients were analysed. We observed that these were similar to controls (Figure 1c). Moreover, we did not observe differences in the selection for replacement mutations in the complementarity determining regions (CDR) (Figure 1d). Likewise, SHM frequencies and selection for replacement mutations in \textit{IGHV} of adult patients’ \textit{IGG} transcripts were similar to controls (Supplementary figure 3b and c). Thus, the reduction in \textit{IGA2} usage in sIgAD adults is not due to a general defect in SHM or class switch recombination, but rather a selective defect in IgA responses and memory B-cell formation.

\textbf{Normal B-cell activation of adult sIgAD patients’ naive B cells}

B cells from sIgAD patients have previously been shown to produce IgA following \textit{in vitro} stimulation with CD40L and IL-21.\textsuperscript{29} However, it is currently unknown whether sIgAD patients show differences in B-cell activation after short-term stimulation with TD or TI stimuli. To investigate this, we stimulated peripheral blood B cells from adult patients (patient numbers: 14, 22 and 25) with several TD (anti-CD40) and TI (anti-IgM, CpG or APRIL plus TGF-\textit{b}) stimuli for 48 h. Cultures with either stimulus resulted in the upregulation of CD80, CD86, CD69 and CD95 on peripheral blood B cells of adult patients and controls (Figure 3). Thus, in symptomatic sIgAD adults, B-cell activation did not appear to be defective upon stimulation with TD or TI stimuli.

\textbf{Reduced Th1 and Th17 cell numbers and increased serum TGF-\textit{b}1, APRIL and BAFF levels}

To address a potential B-cell extrinsic defect in sIgAD, we phenotyped CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell subsets and measured peripheral blood concentrations of several cytokines implicated in IgA class switching: TGF-\textit{b}1, APRIL and BAFF.\textsuperscript{11,30} We found that IgA-deficient children had higher numbers of total CD8\textsuperscript{+} T cells, naive CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, as well as CD4\textsuperscript{+} central memory T cells than controls (Supplementary figure 4a). In sIgAD adults, naive CD4\textsuperscript{+} and CD8\textsuperscript{+}, memory CD4\textsuperscript{+}, Th2, follicular helper (Tfh) and regulatory (Treg) cell numbers were similar to controls. In contrast, we observed higher numbers of CD8\textsuperscript{+} central memory T cells (Figure 4b) and lower numbers of Th1 and Th17 cells (Figure 4a and b).

Soluble TGF-\textit{b}1, BAFF and APRIL were readily detectable in patients’ blood and were significantly higher in adult patients than in controls (Figure 4c). Hence, the defect in IgA class switching in our sIgAD patients does not seem to be related to decreased blood concentrations of these cytokines.

\textbf{DISCUSSION}

In the present study, we searched for underlying B-cell intrinsic and extrinsic defects in children and adults with sIgAD by characterising the peripheral blood B-cell and T-cell compartments, \textit{IGA}
transcripts and cytokine concentrations. We observed a reduction in TI-derived CD27+CD27− IgA+ and TD-derived CD27+ IgA+ memory B cells in all patients. We did not find an in vivo class switch defect to downstream IgG and IgA subclasses, and affinity maturation of IgA transcripts of sIgAD patients was similar to controls. However, patients had reduced Th1 and Th17 cells and increased blood concentrations of TGF-β1, BAFF and APRIL. Together, these results indicate that sIgAD patients have defective regulation of the IgA response.

Nine out of 12 children and 17 out of 18 adults from our cohort with sIgAD suffered from recurrent infections, and several exhibited atopy and autoimmunity. However, the four asymptomatic patients did not differ immunologically from symptomatic patients. Therefore, symptomatic patients will benefit from a better understanding of the pathophysiology of sIgAD that lead to new treatment strategies. Recently, Blanco et al. showed that the detection of IgA+ memory is not related to the presence of symptoms in sIgAD patients. The majority of adult patients in our cohort had undetectable peripheral IgA+ memory B cells, which is higher than in the study of Blanco et al., in which IgA+ memory B cells were undetectable in 50% of the patients. The authors defined two sIgAD groups: individuals in group 1 had a normal to a mild decrease in number of IgA+ memory B cells. In contrast, in group 2, individuals had

![Diagram of IgA subclass distribution in children and adults](image-url)
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The percentages of symptomatic patients did not differ between the two groups. However, sIgAD patients from group 2 experienced more frequently respiratory tract infections and autoimmunity. Therefore, adult patients in our study may be more comparable to the sIgAD patients of group 2 than group 1.

The generation of IgA2+ memory B cells is mainly TI at mucosal sites such as the lamina propria in the intestine. Our results showing a deficiency of TI- and TD-derived IgA1+ memory B cells in our patients are in line with the presence of both IgA1+ and IgA2+ memory B cells in sIgAD patients.

Although by definition, patients with sIgAD have normal IgG, some authors suggest that this disease may be part of a clinical spectrum of disorders such as CVID, based on the observation that some individuals initially diagnosed with sIgAD, progressed over time. Furthermore, genetic variants in TNFRSF13B are more prevalent in both disorders and can coexist within the same family. However, our two patients in which we

Figure 3. Normal B-cell activation in adult sIgAD patients. (a) Overlays of activation marker expression after 48-h stimulation of naive B cells from a healthy control. (b) MFI (median fluorescent intensity) of activation makers measured 48 h after in vitro stimulation of naive B cells from sIgAD adults (n = 3; patients 14, 22 and 25) and adult controls (n = 3). Technical replicates were not performed. Each experiment was performed on three biological samples per group.
Figure 4. Reduced Th1 and Th17 cell numbers and increased cytokine concentrations. (a) Gating strategy of CD4+ T cells to define Th1 (CD45RA−CC66−CXCR3−CCR4+), Th2 (CD45RA−CCR6−CXCR3−CCR4+), Th17 (CD45RA−CCR66−CXCR3−CCR4+), regulatory T cells (Treg; CD25−CD127−) and follicular helper T cells (Tfh; CD45RO−CXCR5+). (b) Quantification of T-helper cells in adult sIgAD patients and controls. (c) Cytokine concentrations in blood samples (dots represent plasma samples; triangles represent serum samples) of adult sIgAD patients and controls. (b, c) Red lines depict median values; statistics were calculated with the Mann–Whitney U-test; *P < 0.05, **P < 0.01, ***P < 0.001. Technical replicates were not performed. Numbers depict biological replicates.
found two previously reported TNFRSF13B variants associated with CVID did not show additional abnormalities in the B-cell compartment typical for CVID, such as decreased IgM or IgG memory B cells.

Interestingly, the SHM levels and degree of antigenic selection we identified were characteristics of normal affinity maturation of IGA and IGG in slgAD patients. In contrast, CVID patients frequently show an abnormal antigen-selected Ig gene repertoire with reduced SHM levels in IGA and IGG and abnormalities in subclass distribution such as decreased IGG2 usage.35,36 Our findings of increased IGA2 usage in children with slgAD and normal downstream usage of IGG subclasses in adults make a class switch defect in slgAD patients in our cohort unlikely. It remains unclear why IGA2 transcripts were enriched in children with slgAD, as this is a relative measure and could result from the overall reduction and a more severe reduction in IGA1. Importantly, patients were able to switch to both IgA subclasses in vivo, excluding an entire block in Ig switching to one or both IGA regions.

Previously, in vitro studies had focused on the production of IgA, differentiation into plasmablasts or upregulation of AID.29 Our short-term in vitro stimulations showed that TD and TI stimuli did not differ in terms of B-cell activation. Patients with predominantly antibody deficiency, including CVID, have reduced numbers of Th17 cells along with increased numbers of CD21low B cells.36-38 Interestingly, we found decreased Th17 and Th1 cell numbers in adult slgAD patients. Recently, Lemarquis et al.14 studied fifteen IgAD patients and did not find abnormalities in Th1 and Th17 cells. These contradictory findings could result because they used the proportion of T helper cells as a fraction of CD4+ T cells. Patients with congenital agammaglobulinemia, who lack mature B cells, exhibit decreased frequencies of Th17 cells.37 However, these authors questioned the role of B cells in the differentiation of Th17 cells as they showed that in healthy individuals, there is a negative correlation between frequencies of Th17 cells and class-switched memory B cells and serum concentrations of BAFF.37 We observed elevated concentrations of BAFF and APRIL in our adult SlgAD patients. This has also been described for children with slgAD.39 Since total B-cell numbers were normal in our adult patients, it remains unclear whether the increase in blood BAFF and APRIL levels results from increased production or decreased usage by target cells. In future studies, it would be essential to study dendritic cells as well as T helper cells and cytokine concentrations at local mucosal sites where IgA class switching occurs. Also, it would be interesting to elucidate whether Th17 and Th1 cells are also low in slgAD patients who have detectable IgA memory B cells, as well as comparisons of T helper cell numbers between slgAD patient groups 1 and 2, as recently defined.42 These studies might provide further insights into the role of Th17 cells in slgAD.

Another essential cytokine in the regulation of IgA class switching is TGF-β1. More than two decades ago, Muller et al.,40 using a cell bioassay, noted a moderate reduction in serum concentrations of TGF-β1 in slgAD patients, and these correlated negatively with the numbers of CD19+ B cells.38 In contrast, we found increased TGF-β1 in plasma in five out of 8 patients. Moreover, nearly all patients from our cohort exhibited normal numbers of B cells in peripheral blood. In mice, the lack of TGF-β1 receptor expression on B cells leads to defective IgA production.41 Therefore, it will be interesting to measure TGF-β1 receptor expression, since a significant decrease might explain the high levels of this cytokine observed in some of our patients. Searching for possible genetic defects affecting the signalling cascade of costimulatory molecules beyond TNFRSF13B or costimulatory T-helper cells might provide additional insights into the pathogenesis of slgAD.

In summary, we show here that in addition to the absence of IgA in blood, symptomatic slgAD patients are defective in the formation of IgA memory B cells, despite normal B-cell activation and affinity maturation of their IGA and IGG transcripts. Instead, decreased Th1 and Th17 cell numbers and high levels of BAFF, APRIL and TGF-β1 might reflect disturbed regulation of IgA responses in vivo. We propose that the severe impairment in IgA class switch in symptomatic slgAD patients might result from shifts in Th cell subsets and cytokine dysregulation.

METHODS

Human subjects

We collected clinical data and blood samples of 30 patients (children n = 12; adults n = 18) with slgAD and 108 healthy controls (children n = 72; adults n = 29) after written
inform consent (Supplementary table 1). All patients were diagnosed based on the absence of serum IgA (< 0.07 g L⁻¹) with normal IgM and IgG. This study was performed according to the Declaration of Helsinki and the guidelines of the Medical Ethics Committees of the Erasmus MC, Karolinska University Hospital, and ethics committee at the University of Antioquia.

**Flow cytometry and cell sorting**

Absolute counts of blood CD3⁺ T cells, CD16⁺/56⁺ natural killer cells and CD19⁺ B cells were obtained with a diagnostic lyse-no-wash protocol (BD Biosciences, San Jose, CA, USA). Detailed immunophenotyping of B and T cells was performed by 11-colour flow cytometry using an LSRII Fortessa (BD Biosciences) with a standardised configuration, according to Euroflow protocols. Data were analysed using CytoSoft software (Cytognos, Salamanca, Spain). Control subjects were described previously. All antibodies used for flow cytometry are listed in Supplementary table 2. The gating strategy for cell subsets is shown in Supplementary figure 1. T-cell subsets were defined as follows: naive T cells (CD45RO⁻/CD45RA⁺), central memory (CM) T cells (CD45RO⁻/CCR7⁺), effector memory (EM) T cells (CD45RO⁺/CCR7⁻), Th1 (CD45RA⁻/CCR6⁺/CXC3⁻/CCR4⁻), Th2 (CD45RA⁻/CCR6⁻/CXC3⁻/CCR4⁺), Th17 (CD45RA⁺/CXC3⁺/CCR4⁻), regulatory T cells (Treg; CD25⁺/CD127⁻) and follicular helper T cells (Tfh; CD45RO⁺/CXC5⁻/CD8⁻/CD27⁻/IgD⁻) were high-speed cell sorted to >95% purity using a FACSAria III (BD Biosciences).

**In vitro B-cell activation and cell cultures**

Purified naive mature B cells were cultured at a density of 30,000 cells/200 µL per well in round-bottom 96-well plates with RPMI medium (Lonza, Basel, Switzerland) containing 10% foetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA), 1% penicillin/ampicillin and 0.5 µM 2-mercaptoethanol (Thermo Fisher Scientific). Cells were stimulated for 48 h with either 10 µg mL⁻¹ of anti-CD40 (Biocerus B.V., Utrecht, the Netherlands), 10 µg mL⁻¹ F(ab)² anti-IgM (Southern Biotech, Birmingham, AL, USA), 0.5 µM CpG ODN2006 (InvivoGen, San Diego, CA, USA) or 1 µg mL⁻¹ APRIL (AdipoGen Life Sciences, San Diego, CA, USA) in combination with 0.5 ng mL⁻¹ TGF-β1 (R&D Systems, Minneapolis, MN, USA) and harvested to determine the expression levels of surface activation marker by flow cytometry (Supplementary table 2).

**Molecular analysis of somatic hypermutations and Ig subclass usage**

RNA was isolated from PBMCs with the GeneElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA), followed by cDNA synthesis. IGA and IGG transcripts were amplified using IGHV3 and IGHV4 leader or FR1 primers in combination with a Cα or Cγ reverse primer. PCR products were cloned into the pGEM-T easy vector (Promega, Leiden, the Netherlands) and prepared for sequencing on an ABI PRISM 3130XL (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared with reference sequences from the IMGT database (http://imgt.org). Targeting of SHM in framework regions (FR) and complementarity determining regions (CDR) was analysed with the extended version of IGGAlyx (http://bioinf-galaxian.erasmusmc.nl/galaxy). The selection strength for replacement mutations in the FR and CDR was determined with the Bayesian estimation of Antigen-driven SElectIoN program (http://selection.med.yale.edu/baseline). IgG and IgA subclasses were determined using the germline sequence of IGH locus (NG_001019).

**TNFRSF13B mutation analysis**

Five exons of the TNFRSF13B gene encoding TACI were amplified by PCR (primers listed in Supplementary table 3) and sequenced on an ABI PRISM 3130XL (Applied Biosystems).

**Anti-IgA antibody analysis**

Plasma antibodies directed against IgA (anti-IgA) were analysed at Sanquin laboratories (Amsterdam, the Netherlands), using the commercially available EIA™ Anti-IgA enzyme immune assay (Phadia, Thermo Fisher Scientific, Uppsala, Sweden). The assay was performed on the Phadia250 analyser according to the manufacturer’s instructions without modification. Reference values used in this assay were < 3 U mL⁻¹ negative; 3−10 U mL⁻¹ borderline; >10 U mL⁻¹ positive.

**Quantification of BAFF, APRIL and TGF-β1**

An ELISA was used to measure BAFF, APRIL and TGF-β1 concentrations in plasma and serum samples of sIgAD patients (adults n = 14; children n = 12) and controls (only adults n = 24), according to the manufacturer’s instructions (BAFF and TGF-β1 R&D systems; APRIL, eBioscience, San Diego, CA, USA).

**Statistics**

Statistical analyses were performed with the Mann-Whitney U-test or chi-square test, as indicated in the figure legends. P-values of < 0.05 were considered to be statistically significant as follows: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

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The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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