

## 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 (slgAD) 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 slgAD patients (12 children, 18 adults) through detailed phenotyping of peripheral B-cell, CD8<sup>+</sup> T-cell and CD4<sup>+</sup> 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<sup>+</sup>) and T-cell-independent (TI; CD27<sup>-</sup>) IgA memory B cells and increased CD21<sup>low</sup> B-cell numbers. IgM<sup>+</sup>IgD<sup>-</sup> memory B cells were decreased in children and normal in adult patients. *IGA* and *IGG* transcripts contained normal SHM levels. In slgAD 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 slgAD 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 slgAD 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

immunological focus on genomics and functional analyses to unravel the pathogenesis of slgAD.

**Keywords:** B-cell memory, cytokine concentration, IgA, selective IgA deficiency, Th1 cells, Th17 cells

## INTRODUCTION

Selective IgA deficiency (slgAD), the most common primary immunodeficiency in Western countries, is defined by very low to absent serum levels of IgA with normal IgG and IgM.<sup>1</sup> The prevalence differs between countries and ranges between 1:328 and 1:3040.<sup>2</sup> The majority of the individuals with absent serum IgA are asymptomatic and identified by coincidental findings.<sup>3</sup> Still, a number of patients are prone to suffer from recurrent infections and to develop autoimmune diseases and/or allergies.<sup>3</sup> Altogether, the clinical symptoms of immunodeficiency and immune dysregulation are much higher in slgAD 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.<sup>4,5</sup> In addition, between 18% and 56% of slgAD patients suffer from one or more allergies.<sup>4</sup> The pathogenesis of the disease is unknown.

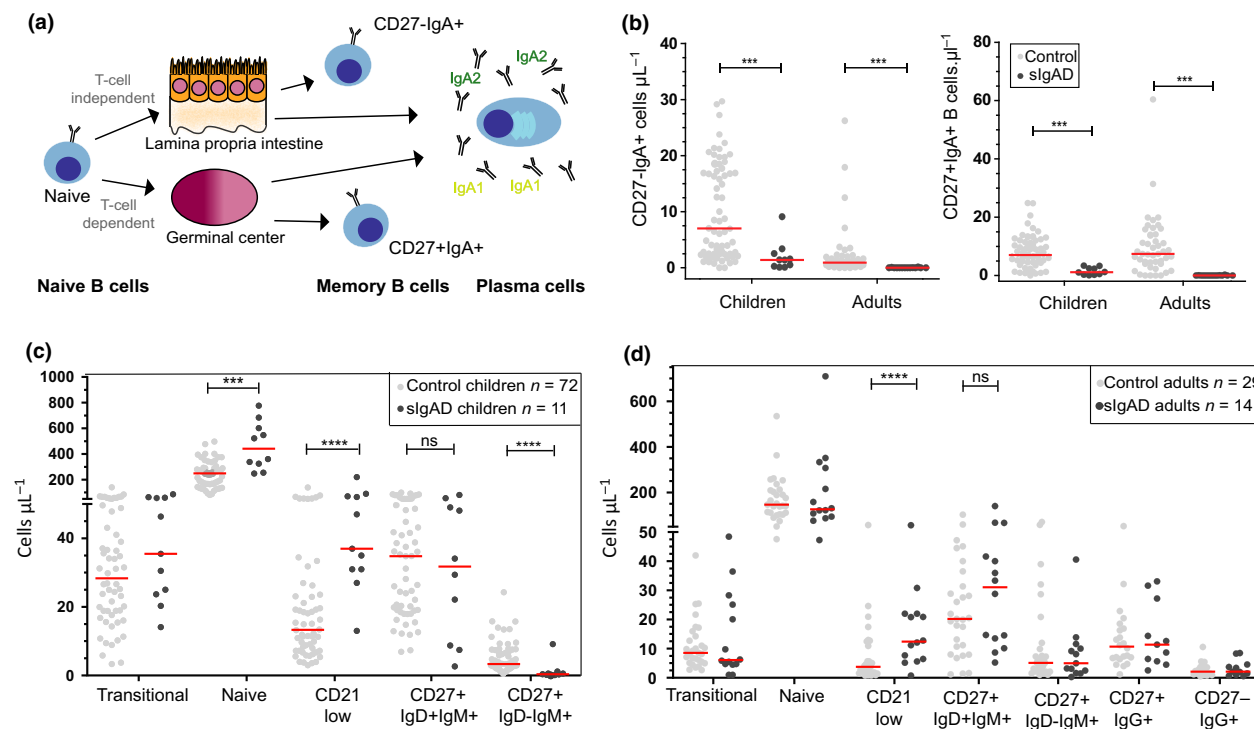
We and others have shown that IgA memory B cells can derive from T-cell-dependent (TD) and T-cell-independent (TI) responses (Figure 1a).<sup>6,7</sup> In human peripheral blood, most IgA memory B cells express CD27 and originate from CD40L-mediated T-cell help in germinal centres. In contrast, CD27-negative 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.<sup>8</sup> 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).<sup>7,9</sup> Additional cytokines, such as transforming growth factor-beta (TGF- $\beta$ ), vasoactive intestinal peptide (VIP) and IL-10, induce germline *IGA* transcripts leading to *IGA* class switching.<sup>7,10,11</sup> Previous studies described reduced numbers of Ig class-switched memory B cells and CD19<sup>+</sup>IgA<sup>+</sup> B cells in slgAD patients.<sup>12–14</sup> Recently, Blanco *et al.* grouped slgAD and other antibody deficiency patients based on the

phenotyping of their B-cell compartment. They proposed that slgAD patients could segregate into two groups based on differences in IgA<sup>+</sup> memory B-cell numbers.<sup>15</sup> However, the authors did not discriminate between CD27<sup>+</sup> and CD27<sup>-</sup> IgA<sup>+</sup> memory B cells. The analysis of the T-cell compartment in slgAD patients has shown that CD4<sup>+</sup> T cells were reduced.<sup>12</sup> T-helper cells in slgAD have only been studied after *in vitro* stimulation and data on cell counts are lacking.<sup>14</sup>

Several studies have addressed the issue of genetic defects in the pathogenesis of slgAD. In some patients, chromosome 18p deletions or mutations in *TNFRSF13B*, the gene encoding TACI, have been found.<sup>16–18</sup> However, these genetic variants might be disease-modifying rather than disease-causing. Furthermore, distinct HLA haplotypes seem to confer risk for the development of slgAD.<sup>19,20</sup> More recently, Bronson *et al.*<sup>21</sup> performed a genome-wide association study (GWAS) meta-analysis of 1635 patients with IgAD and 4852 controls and found that the *PVT1*, *ATG13-AMBRA1*, *AHI1* and *CLEC16A* genes were significantly associated with slgAD. Therefore, cytogenetic abnormalities, HLA haplotype associations and known monogenetic disorders are likely involved in the aetiology of slgAD.<sup>22</sup>

Previously, Wang *et al.*<sup>23</sup> proposed that the lack of serum IgA results from the absence of *IGA* class switching in B cells in slgAD patients. They observed that unstimulated peripheral blood mononuclear cells (PBMC) from patients had fewer  $C\alpha$  germline transcripts and S $\mu$ -S $\alpha$  fragments than control subjects.<sup>24,25</sup> However, whether these  $C\alpha$  transcripts are different in somatic hypermutation (SHM) levels or there is preferential class switching towards either *IGA1* or *IGA2* in these patients is currently unknown. It would be noteworthy to solve this in order to gain insight into the potential genetic defects involved in molecular maturation of IgA<sup>+</sup> B cells.

To study underlying B-cell intrinsic and extrinsic defects in slgAD, we performed immunophenotyping of the B-cell, CD8 T-cell and CD4 T-cell compartments, as well as genetic



**Figure 1.** Reductions in CD27<sup>+</sup> and CD27<sup>-</sup> IgA<sup>+</sup> memory B cells in sIgAD patients. **(a)** Schematics of TI and TD IgA responses. **(b)** Quantification of CD27<sup>-</sup> and CD27<sup>+</sup> IgA<sup>+</sup> memory B cells. Adult controls *n* = 29, sIgAD patients *n* = 14; paediatric controls *n* = 67, sIgAD patients *n* = 10. **(c)** Quantification of B-cell subsets in children and **(d)** adults. The number of patients are indicated in the upper right corner for B-cell subsets, except for IgG<sup>+</sup> memory B cells (*n* = 11). **b–d**, red lines indicate median values. Statistics were calculated with the Mann–Whitney *U*-test; \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001. Technical replicates were not performed. Numbers depict biological replicates.

analysis of *IgA* transcripts in children and adults with sIgAD. 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 sIgAD patients. Interestingly, molecular maturation and B-cell activation were not impaired, but Th1 and Th17 numbers were decreased in adult sIgAD patients. In contrast, we observed increased cytokine concentrations in B-cell activating factor (BAFF), APRIL and TGF-β1. Together, these results show that sIgAD 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 sIgAD patients (12 children, 18 adults) with IgA serum concentrations < 0.07 g L<sup>-1</sup> (patient details in Supplementary table 1). To obtain more insights into the

pathogenesis of sIgAD 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<sup>-</sup> and CD27<sup>+</sup> IgA<sup>+</sup> memory B cells in sIgAD 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).<sup>26,27</sup> 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 sIgAD, we found increased numbers

of naive and CD21<sup>low</sup> B cells, but low IgM<sup>+</sup>IgD<sup>-</sup> memory B-cell numbers (Figure 1c). In contrast, in adult patients, only CD21<sup>low</sup> 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 blood B cells of slgAD patients showed similarities with B-cell abnormalities observed in CVID patients, such as reduced memory B cells and increased numbers of CD21<sup>low</sup> B cells.<sup>28</sup> Still, CVID patients are low in serum IgG, and the different subgroups (as described previously by Driessen *et al.*<sup>26</sup>) showed additional defects in naive and memory B-cell subsets that we did not observe in slgAD patients (Supplementary figure 2). Thus, slgAD patients showed signs of chronic inflammation as observed by increased CD21<sup>low</sup> B cells, and these were explicitly defective in IgA B-cell memory.

### Ig class switching and SHM in transcripts of slgAD patients

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

Somatic hypermutation (SHM) frequencies in *IGHV* regions in *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 *IGHV* of adult patients' *IGG* transcripts were similar to controls (Supplementary figure 3b and c). Thus, the reduction in *IGA2* usage in slgAD 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.

### Normal B-cell activation of adult slgAD patients' naive B cells

B cells from slgAD patients have previously been shown to produce IgA following *in vitro* stimulation with CD40L and IL-21.<sup>29</sup> However, it is currently unknown whether slgAD 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- $\beta$ ) 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 slgAD adults, B-cell activation did not appear to be defective upon stimulation with TD or TI stimuli.

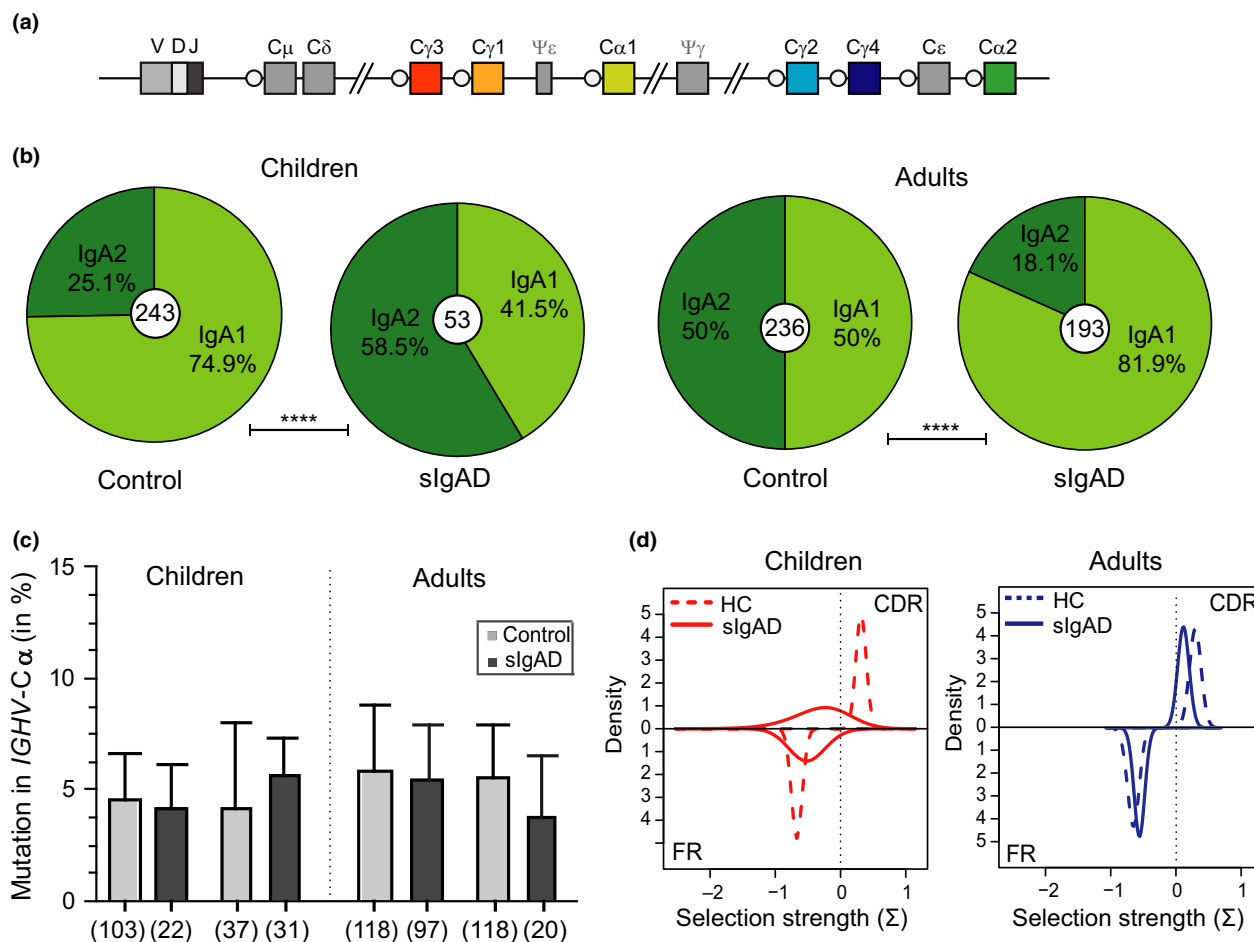
### Reduced Th1 and Th17 cell numbers and increased serum TGF- $\beta$ 1, APRIL and BAFF levels

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

Soluble TGF- $\beta$ 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 slgAD patients does not seem to be related to decreased blood concentrations of these cytokines.

## DISCUSSION

In the present study, we searched for underlying B-cell intrinsic and extrinsic defects in children and adults with slgAD by characterising the peripheral blood B-cell and T-cell compartments, *IGA*

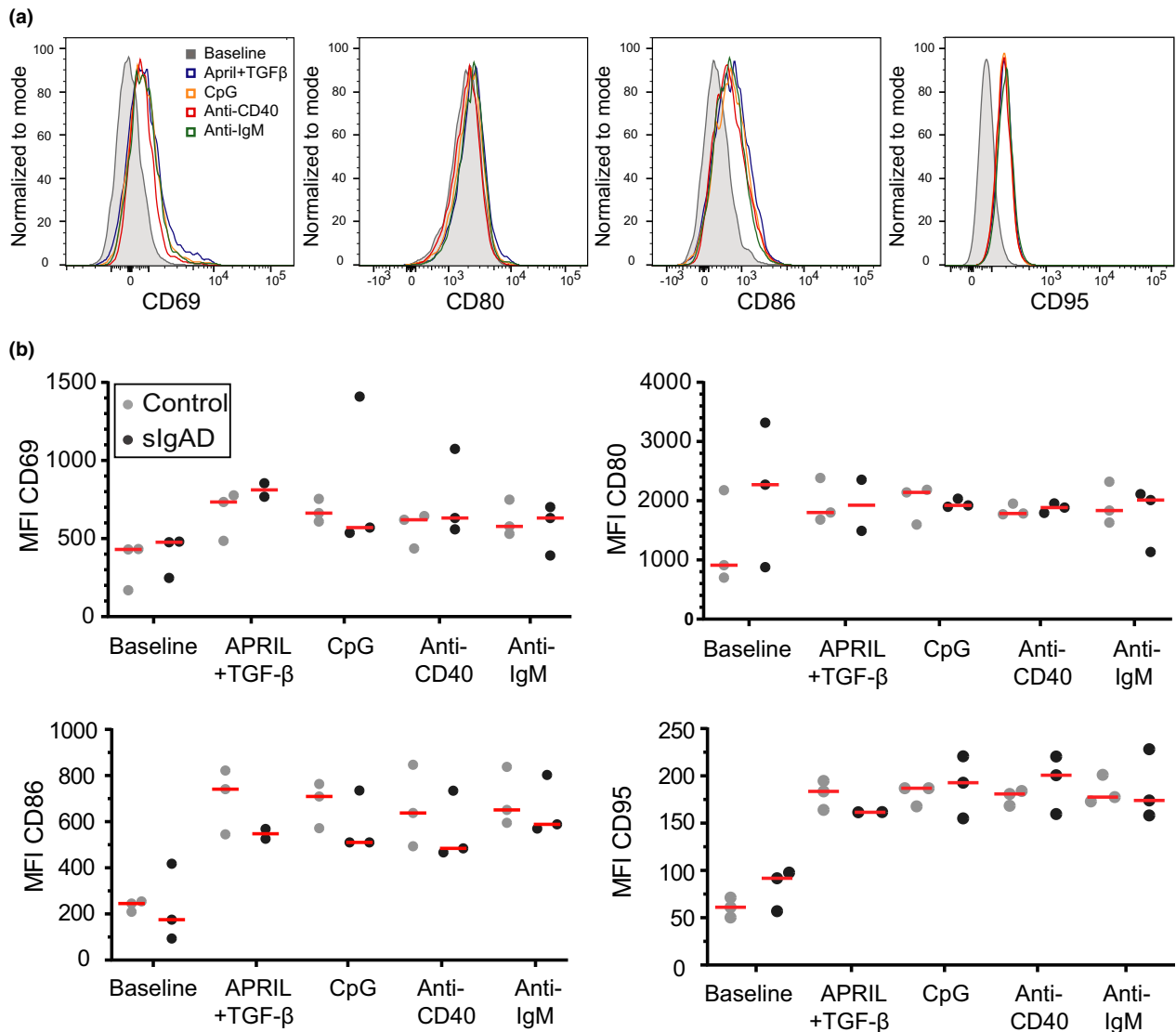


**Figure 2.** Molecular maturation of *IgA* transcripts in sIgAD patients. **(a)** Schematic overview of the human *IGH* locus depicting the positioning of *IgA* and *IgG* encoding regions. **(b)** Distribution of *IgA* subclasses in children and adults; analysed sequences are indicated with small circles. **(c)** Mutations in *IGHV-Cα* transcripts (median with interquartile range). Number of sequences analysed shown in parentheses. **(d)** Antigenic selection of *IGHV-Cα* transcripts of controls ( $n = 477$ ) and sIgAD patients ( $n = 168$ ). **(a–d)** Healthy controls: children  $n = 6$ , adults  $n = 6$ . sIgAD patients: children  $n = 6$ , adults  $n = 9$ . Statistics were calculated with the Mann–Whitney *U*-test; \*\*\*\* $P < 0.0001$ . Technical replicates were not performed. Numbers depict biological replicates.

transcripts and cytokine concentrations. We observed a reduction in TI-derived CD27<sup>+</sup>IgA<sup>+</sup> and TD-derived CD27<sup>+</sup>IgA<sup>+</sup> 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 *IGHV-Cα* 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.<sup>15</sup> showed that the detection of IgA<sup>+</sup> memory is not related to the presence of symptoms in sIgAD patients. The majority of adult patients in our cohort had undetectable peripheral IgA<sup>+</sup> memory B cells, which is higher than in the study of Blanco et al., in which IgA<sup>+</sup> memory B cells were undetectable in 50% of the patients. The authors defined two IgAD groups: individuals in group 1 had a normal to a mild decrease in number of IgA<sup>+</sup> memory B cells. In contrast, in group 2, individuals had



**Figure 3.** Normal B-cell activation in adult slgAD 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 markers measured 48 h after *in vitro* stimulation of naive B cells from slgAD 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.

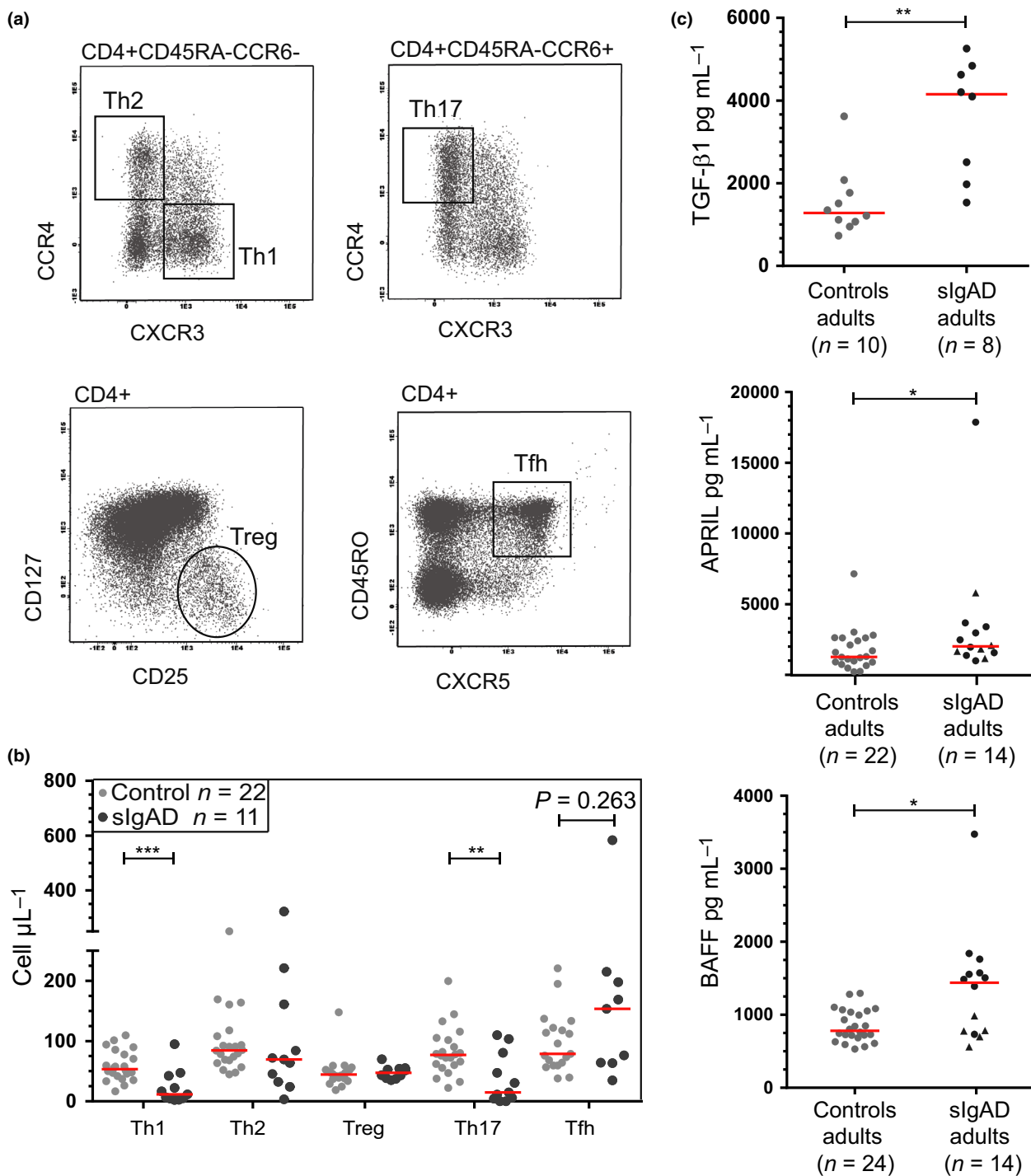
severe deficiency or undetectable IgA<sup>+</sup> memory B cells. The percentages of symptomatic patients did not differ between the two groups. However, slgAD patients from group 2 experienced more frequently respiratory tract infections and autoimmunity.<sup>15</sup> Therefore, adult patients in our study may be more comparable to the slgAD patients of group 2 than group 1.

The generation of IgA2<sup>+</sup> memory B cells is mainly TI at mucosal sites such as the lamina propria in the intestine.<sup>6</sup> Our results showing a deficiency of TI- and TD-derived IgA<sup>+</sup> memory B

cells in our patients are in line with the presence of both IgA1<sup>+</sup> and IgA2<sup>+</sup> memory B cells in slgAD patients.<sup>15</sup>

Although by definition, patients with slgAD 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 slgAD, progressed over time.<sup>31-34</sup> Furthermore, genetic variants in *TNFRSF13B* are more prevalent in both disorders and can coexist within the same family.<sup>16</sup> However, our two patients in which we





**Figure 4.** Reduced Th1 and Th17 cell numbers and increased cytokine concentrations. **(a)** Gating strategy of CD4<sup>+</sup> T cells to define Th1 (CD45RA<sup>-</sup>CCR6<sup>-</sup>CXCR3<sup>+</sup>CCR4<sup>-</sup>), Th2 (CD45RA<sup>-</sup>CCR6<sup>-</sup>CXCR3<sup>-</sup>CCR4<sup>+</sup>), Th17 (CD45RA<sup>-</sup>CCR6<sup>+</sup>CXCR3<sup>-</sup>CCR4<sup>+</sup>), regulatory T cells (Treg; CD25<sup>+</sup>CD127<sup>-</sup>) and follicular helper T cells (Tfh; CD45RO<sup>+</sup>CXCR5<sup>+</sup>). **(b)** Quantification of T-helper cells in adult slgAD patients and controls. **(c)** Cytokine concentrations in blood samples (dots represent plasma samples; triangles represent serum samples) of adult slgAD 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<sup>+</sup> or IgG<sup>+</sup> 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.<sup>35,36</sup> 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.<sup>29</sup> 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 CD21<sup>low</sup> B cells.<sup>36-38</sup> Interestingly, we found decreased Th17 and Th1 cell numbers in adult slgAD patients. Recently, Lemarquis *et al.*<sup>14</sup> 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<sup>+</sup> T cells. Patients with congenital agammaglobulinemia, who lack mature B cells, exhibit decreased frequencies of Th17 cells.<sup>37</sup> 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.<sup>37</sup> We observed elevated concentrations of BAFF and APRIL in our adult SlgAD patients. This has also been described for children with slgAD.<sup>39</sup> 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<sup>+</sup> memory B cells, as well as comparisons of T helper cell numbers between slgAD patient groups 1 and 2, as recently defined.<sup>15</sup> 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- $\beta$ 1. More than two decades ago, Muller *et al.*,<sup>40</sup> using a cell bioassay, noted a moderate reduction in serum concentrations of TGF- $\beta$ 1 in slgAD patients, and these correlated negatively with the numbers of CD19<sup>+</sup> B cells.<sup>38</sup> In contrast, we found increased TGF- $\beta$ 1 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- $\beta$  receptor expression on B cells leads to defective IgA production.<sup>41</sup> Therefore, it will be interesting to measure TGF- $\beta$  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- $\beta$ 1 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



informed consent (Supplementary table 1). All patients were diagnosed based on the absence of serum IgA ( $< 0.07 \text{ g L}^{-1}$ ) 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  $\text{CD3}^+$  T cells,  $\text{CD16}^+/56^+$  natural killer cells and  $\text{CD19}^+$  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.<sup>42</sup> Data were analysed using FACS DIVA software v8 (BD Biosciences) and Infinicyt software (Cytognos, Salamanca, Spain). Control subjects were described previously.<sup>26,27</sup> All antibodies used for flow cytometry are listed in Supplementary table 2. The gating strategy of B-cell subsets is shown in Supplementary figure 1. T-cell subsets were defined as follows: naive T cells ( $\text{CD45RO}^- \text{CCR7}^+$ ), central memory (CM) T cells ( $\text{CD45RO}^+ \text{CCR7}^+$ ), effector memory (EM) T cells ( $\text{CD45RO}^{+/+} \text{CCR7}^-$ ), Th1 ( $\text{CD45RA}^- \text{CCR6}^- \text{CXCR3}^+ \text{CCR4}^-$ ), Th2 ( $\text{CD45RA}^- \text{CCR6}^- \text{CXCR3}^- \text{CCR4}^+$ ), Th17 ( $\text{CD45RA}^- \text{CCR6}^+ \text{CXCR3}^- \text{CCR4}^+$ ), regulatory T cells (Treg;  $\text{CD25}^+ \text{CD127}^-$ ) and follicular helper T cells (Tfh;  $\text{CD45RO}^+ \text{CXCR5}^+$ ).<sup>27,43</sup> Naive mature B cells ( $\text{CD3}^- \text{CD20}^+ \text{CD38}^{\text{low}} \text{CD27}^- \text{IgD}^+$ ) 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  $\mu\text{L}$  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  $\mu\text{M}$  2-mercaptoethanol (Thermo Fisher Scientific). Cells were stimulated for 48 h with either 10  $\mu\text{g mL}^{-1}$  of anti-CD40 (Bioceros B.V., Utrecht, the Netherlands), 10  $\mu\text{g mL}^{-1}$  F(ab')<sub>2</sub> anti-IgM (Southern Biotech, Birmingham, AL, USA), 0.5  $\mu\text{M}$  CpG ODN2006 (Invivogen, San Diego, CA, USA) or 1  $\mu\text{g mL}^{-1}$  APRIL (AdipoGen Life Sciences, San Diego, CA, USA) in combination with 0.5 ng  $\text{mL}^{-1}$  TGF- $\beta$  (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  $\text{C}\alpha$  or  $\text{C}\gamma$  reverse primer.<sup>8,44</sup> 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 IGGalaxy (<http://bioinf-galaxian.erasmusmc.nl/galaxy>).<sup>45</sup> 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/>)<sup>46,47</sup> 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 EliA™ 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  $< 3 \text{ U mL}^{-1}$  negative; 3–10  $\text{U mL}^{-1}$  borderline;  $> 10 \text{ U mL}^{-1}$  positive.

## Quantification of BAFF, APRIL and TGF- $\beta$ 1

An ELISA was used to measure BAFF, APRIL and TGF- $\beta$ 1 concentrations in plasma and serum samples of *slgAD* patients (adults  $n = 14$ ; children  $n = 12$ ) and controls (only adults  $n = 24$ ), according to the manufacturer's instructions (BAFF and TGF- $\beta$ 1 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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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## Supporting Information

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