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Impaired memory B-cell development and antibody maturation with a skewing toward IgE in patients with STAT3 hyper-IgE syndrome

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Abstract

Background: Signal transducer and activator of transcription 3 hyper-IgE syndrome (STAT3-HIES) is caused by heterozygous mutations in the *STAT3* gene and is associated with eczema, elevated serum IgE, and recurrent infections resembling severe atopic dermatitis, while clinically relevant specific IgE is almost absent.

Methods: To investigate the impact of STAT3 signaling on B-cell responses, we assessed lymph node and bone marrow, blood B and plasma cell subsets, somatic hypermutations in Ig genes, and in vitro proliferation and antibody production in STAT3-HIES patients and healthy controls.

Results: Lymph nodes of STAT3-HIES patients showed normal germinal center architecture and CD138$^+$ plasma cells residing in the paracortex, which expressed IgE, IgG, and IgM but not IgA. IgE$^+$ plasma cells were abundantly present in STAT3-HIES bone marrow. Proliferation of naive B cells upon stimulation with CD40L and IL-4 was similar in patients and controls, while patient cells showed reduced responses to

Abbreviations: CSR, class switch recombination; FDC, follicular dendritic cell; GC, germinal center; HIES, hyper-IgE syndrome; IGH, immunoglobulin heavy chain; IGHV, immunoglobulin heavy chain variable region gene; PBMC, peripheral blood mononuclear cell; SHM, somatic hypermutation; STAT, signal transducer and activator of transcription; T_{FH}, T follicular helper; T_H, T helper type.

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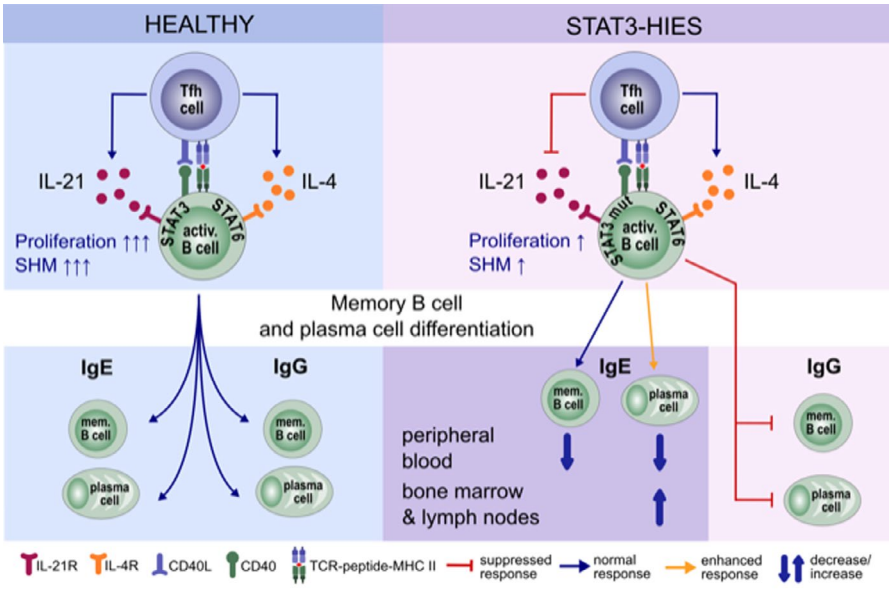
IL-21. IgE, IgG1, IgG3 and IgA1 transcripts showed reduced somatic hypermutations. Peripheral blood IgE$^+$ memory B-cell frequencies were increased in STAT3-HIES, while other memory B-cell frequencies except for IgG4$^+$ cells were decreased.

Conclusions: Despite impaired STAT3 signaling, STAT3-HIES patients can mount in vivo T-cell–dependent B-cell responses, while circulating memory B cells, except for those expressing IgG4 and IgE, were reduced. Reduced molecular maturation demonstrated the critical need of STAT3 signaling for optimal affinity maturation and B-cell differentiation, supporting the need for immunoglobulin substitution therapy and explaining the high IgE serum level in the majority with absent allergic symptoms.

KEYWORDS

B cell maturation, IgE, STAT3 hyper-IgE syndrome



GRAPHICAL ABSTRACT

STAT3-HIES patients have reduced plasmablast and memory B-cell frequencies, while showing normal IgE$^+$ B-cell frequencies. Despite normal lymph node GC architecture, STAT3-HIES B cells show reduced proliferation in response to IL-21 and reduced IGHV SHM levels. Relatively large numbers of IgE$^+$ plasma cells were detected in STAT3-HIES patients' bone marrow and lymph node tissue.

1 | INTRODUCTION

The prevalence of allergic diseases such as atopic dermatitis, allergic rhinitis, and asthma has significantly increased during the last 50 years.[1] The precise mechanisms underlying this increase of allergic diseases remain incompletely understood. As in many chronic diseases, effective prevention, curative treatment, and early diagnosis are essential and start with a detailed understanding of disease mechanism. However, the pathophysiologic complexity of allergic diseases poses a major challenge. Even the mechanisms driving the regulation of IgE production and specificity, known as a major cause of allergic symptoms, have not been completely elucidated.[2]

T-dependent B-cell responses occur mainly in germinal centers (GCs) of lymphoid tissue. The majority of T cells in these GCs are follicular T helper (T_{FH}) cells, which produce interleukin-21 (IL-21). IL-21 is a strong activator of the signal transducer and activator of transcription (STAT)3 signaling pathway, which induces immunoglobulin class switch recombination (CSR)[3] and augments IgE production and proliferation of human naive B cells in response to CD40L and IL-4.[4-6] Furthermore, IL-21 is a key cytokine for maintenance and regulation of a high-titer and high-affinity antigen-specific antibody response,[7] while there are indications that IgE production is less dependent on the formation of mature GCs than other Ig isotypes.[8] In fact, IgE production may be disfavored in a GC environment, as IL-21 has been shown to suppress antigen-induced IgE production by inhibiting germline Cε transcription of IL-4–stimulated B cells[9] and through induction of apoptosis in B cells committed to produce IgE.[10]

The importance of STAT3 in the regulation of IgE production and T-cell–dependent antibody responses is emphasized by patients with

STAT3 hyper-IgE syndrome (STAT3-HIES). STAT3-HIES is an autosomal dominant disorder caused by heterozygous mutations in the *STAT3* gene that result in a dominant negative effect on STAT3 function.[11-16] Eczema in this primary immunodeficiency resembles severe forms of atopic dermatitis. Characteristic allergic findings, however, are mainly missing, and the excessive elevated serum IgE is not directed to known allergens in the majority of patients.[11,17] STAT3-HIES patients frequently develop recurrent staphylococcal skin abscesses and lung infections and mucocutaneous candidiasis. Despite having overall normal total serum IgG levels, patients have a reduced memory B-cell compartment and lack *S aureus*-specific IgG, which likely explains why STAT3-HIES patients benefit from immunoglobulin replacement therapy.[13,14,18] Both intrinsic B-cell defects and reduced T-cell help, resulting from impaired STAT3 signaling, have been associated with a lack of high-affinity antigen-specific antibody production in STAT3-HIES.[12,17,19-23] It remains unclear whether this results only from a defect in the amount of antibody production or whether the antibodies produced have undergone crippled affinity maturation due to impaired induction of somatic hypermutations (SHMs).

Recently, advanced methodologies have enabled to identify and study IgE-expressing and IgG-subclass–expressing B cells.[24-28] Here, we studied the B-cell and plasma cell compartment in peripheral blood, lymph nodes, and bone marrow of STAT3-HIES patients to better understand the mechanisms controlling IgE production and the role of STAT3 signaling in B-cell differentiation, CSR, and IgE production.

2 | METHODS

2.1 | Patients and control samples

Six STAT3-HIES patients (3 females and 3 males, age range 5-49 years; median 18.5 years) were included for functional immunologic assessment. Immune histology was performed on tissues of two additional (deceased) patients (P7 and P8). All patients carried a heterozygous dominant negative mutation in the *STAT3* gene (Table 1). Eleven nonatopic control subjects were included (Table S1). The types of analyses that were performed with each patient's material are indicated in Table 1. The study was approved by the local ethical institute (Ludwig Maximilian University reviewing board #381-13) and the Monash University, and written informed consent was obtained from all study subjects.

2.2 | Sample collection

Peripheral blood samples were obtained from STAT3-HIES patients and healthy donors. Differential blood count, immunoglobulin serum levels (IgE, IgA, IgM, total IgG, and IgG subclasses), vaccine titers, IgE immunoblot, and skin prick test were assessed according to routine measures at the Institute of Laboratory Medicine LMU. Peripheral blood mononuclear cells (PBMCs) were isolated using Biocoll separating solution (Biochrom GmbH, Berlin, Germany) and cryopreserved. Lymph nodes (P7 and P8) and bone marrow (P7) samples were obtained during autopsies of deceased patients. Lymph nodes showed signs of lymphadenopathy, which could be related to recurrent infections.

2.3 | Online supplement methods

Details regarding histology and immunohistochemistry of lymph nodes, in vitro proliferation and antibody production of naive B cells, flow cytometric analysis, and molecular analysis of IgG, IgA, and IgE transcripts are described in Online supplement methods section.

2.4 | Statistics

Nonparametric statistical procedures were applied. Two-group comparisons were assessed by Mann-Whitney test. *P* values of below .05 were considered significant. Calculations and graphics were generated with GraphPad Prism 6.0 (GraphPad Software Inc).

3 | RESULTS

3.1 | Patients show characteristic clinical STAT3-HIES presentation

The full clinical phenotype of STAT3-HIES including eczema, recurrent mucocutaneous and sinopulmonary infections, elevated serum IgE, and associated nonimmunologic findings was present in all patients (Table 1). All patients had normal total serum IgG levels prior to immunoglobulin substitution therapy. Two patients had reduced serum IgA (P5 and P8) and IgG subclass (P3 and P4) levels. Despite reduced memory B-cell counts in STAT3-HIES, vaccination responses to diphtheria and tetanus were unremarkable in all patients, while only patient P4 had reduced vaccine responses to *Pneumococci* and *Haemophilus influenzae type b*. Immunoglobulin replacement therapy was given based on the knowledge that STAT3-HIES patients mount impaired protective IgG responses and that immunoglobulin substitution therapy has been associated with reduction of recurrent infections and clinical improvement.[18]

Positive testing of specific IgE against aeroallergens (P1, P3-6) and food allergens (P3-6) did not correlate with allergen skin prick testing except for cat protein in two patients (P1 and P5). Only one patient (P6) mentioned aeroallergic symptoms such as rhinoconjunctivitis and dyspnea in clinical history, and only two patients (P3, P6) indicated food allergic symptoms such as pruritus of ear and palate or skin condition worsening. Taken together, the allergic trias of positive specific serum IgE and skin prick test in combination with allergic clinical manifestations were only mildly present in two patients (P3 and P6). There was no correlation between the level of serum IgE and clinical findings.

3.2 | Normal GC morphology in lymphoid tissue of STAT3-HIES patients

In order to determine whether the reduced memory B-cell numbers in STAT3-HIES patients resulted from compromised T-cell–dependent

TABLE 1 STAT3-HIES patient characteristics

ID	Age at last evaluation	Gender	Identified heterozygouse *STAT3* Mutation	Analysis performed	Recurrent mucocutaneous infections: Eczema	Recurrent mucocutaneous infections: Bacterial	Recurrent mucocutaneous infections: Fungal	Recurrent sinopulmonary infections: Sinusitis/ Otitis	Recurrent sinopulmonary infections: Pneumonia	Recurrent sinopulmonary infections: Pneumato-celes	Values at time of investigation: NIH score[a]	Values at time of investigation: $CD19^+$ cells (% of lymphocytes)
P1	49	Male	c.1144C>T; p.R382W	Flow cytometry	Yes	Yes	Yes	Yes	Yes	No	53	16.55
P2	37	Female	c.1144C>T; p.R382W	Flow cytometry, in vitro B cell stimulation, SHM	Yes	Yes	Yes	Yes	Yes	Yes	67	11.9
P3	5	Male	c.1144C>T; p.R382W	Flow cytometry, in vitro B cell stimulation, SHM	Yes	Yes	Yes	Yes	No	No	42	16.8
P4	13	Female	c.1909G>A; p.V637M	Flow cytometry, in vitro B cell stimulation, SHM	Yes	Yes	Yes	Yes	Yes	Yes	62	17.8
P5	17	Male	c.1145G>A; p.R382Q	Flow cytometry, in vitro B cell stimulation, SHM	Yes	Yes	Yes	Yes	Yes	Yes	56	20.4
P6	20	Female	c.1145G>A; p.R382Q	Flow cytometry	Yes	Yes	Yes	Yes	Yes	Yes	86	21.25
P7	28 (death age)	Male	c.1144C>T; p.R382W	Immunhistology	Yes	Yes	Yes	Yes	Yes	Yes	85	ND
P8	16 (death age)	Female	c.1145G>A; p.R382Q	Immunhistology	Yes	Yes	Yes	Yes	Yes	Yes	67	ND
				Positive for finding:	100% 8/8	100% 8/8	100% 8/8	100% 8/8	87.5% 7/8	75% 6/8		

Abbreviation: ND, not done; NI, not indicated; ↑ above or ↓ below normal range.
[a]Grimbacher B et al. Genetic linkage of hyper-IgE syndrome to chromosome 4. Am J Hum Genet. 1999;65:735-44.

B-cell responses, we performed immunohistochemistry on lymph nodes of two STAT3-HIES patients to visualize GC and antibody-secreting cells in lymphoid tissue. Normal GC formation was observed in STAT3-HIES patients' lymph nodes with the presence of $CD20^+$ B cells, PD-1^+ follicular T cells, and $CD21^+$ follicular dendritic cells (Figure 1A). $CD138^+$ plasma cells were mainly found in the paracortex and predominantly expressed IgG or IgM (Figure 1A,1). IgA^+ plasma cells were hardly detectable (Figure 1B). It should be noted that the IgA and IgM stainings only detected intracellular IgA and IgM, respectively, in plasma cells and not IgM and IgA expressed as surface BCR on B cells, while anti-IgG also weakly stained surface IgG on B cells. IgE^+ cells were readily detected in STAT3-HIES lymph nodes, while they were largely undetectable in controls (Figure 1C). Those IgE^+ cells consisted partly of IgE^+ plasma cells in the paracortex. The vast majority of IgE^+ cells, however, were found in the follicles where plasma cells were absent, suggesting unspecific binding of secreted IgE to follicular dendritic cell (FDC) networks through Fcε receptors.

3.3 | Accumulation of IgE^+ plasma cells in bone marrow of STAT3-HIES

To investigate the primary location of long-lived plasma cells, we performed immunohistochemistry on bone marrow of STAT3-HIES patient P7. The numbers of total $CD38^+$ plasma cells and IgG^+ plasma cells were comparable between STAT3-HIES and control bone marrow, whereas fewer IgM^+ and IgA^+ plasma cells were detected in the STAT3-HIES patient. IgE^+ plasma cells were more frequent in STAT3-HIES patient bone marrow than in control bone marrow (Figure 2). The overall cell content and bone marrow structural organization of the STAT3-HIES patient were comparable to controls.

3.4 | STAT3-HIES B-cell activation in response to IL-21 is impaired

Next, we assessed the capacity of STAT3-HIES naive B cells to respond to T-dependent activation signals and determined whether these cells are skewed toward IgE production. $CD27^-IgM^+IgD^+$ naive B cells were purified from peripheral blood by flow cytometry–based cell sorting using a gating strategy aimed at excluding switched and nonswitched memory B cells by gating on $CD19^+CD27^-IgG^-IgA^-$ cells to avoid surface Ig crosslinking (Figure S1). Naïve B cells were co-cultured with CD40L-expressing cells with and without IL-4 and IL-21 and assessed for Ig isotype secretion, cell differentiation, and proliferation.

The proliferation rate of naïve B cells after 6 days of stimulation with CD40L + IL-4 did not differ between controls and STAT3-HIES patients, whereas B cell of STAT3-HIES patients showed significantly less proliferation in response to CD40L + IL-4 + IL-21 than those of healthy controls (Figure 3A).

Secretion of Ig isotypes in response to CD40L stimulation occurred at low levels for IgG1, IgG2, IgM, and IgA, while IgE, IgG3, and IgG4 were mostly below detection limit in patients and controls after 12 days (Figure 3B). IL-4 increased the levels of all secreted Ig

										Values prior to immunoglobulin substitution				
$CD19^+$ cells (cells/μL)	Serum IgE (IU/ml)	Serum IgA (mg/dL)	Serum IgM (mg/dL)	Serum IgG (mg/dL)	Serum IgG1 (mg/dL)	Serum IgG2 (mg/dL)	Serum IgG3 (mg/dL)	Serum IgG4 (mg/dL)	Immunglobulin substitution	Serum IgG (mg/dL)	Serum IgG1 (mg/dL)	Serum IgG2 (mg/dL)	Serum IgG3 (mg/dL)	Serum IgG4 (mg/dL)
129.09	17 259 ↑	117	115	1508	1000 ↑	358	79	200 ↑	No substitution	NI	NI	NI	NI	NI
127.33	1365 ↑	167	86.1	1271	864 ↑	242	56	10.5	Since 10/2011	1432	972 ↑	351	79	95
500.64	11 992 ↑	155	74.8	1115	801	133	16 ↓	18	Since 06/2011	270 ↓	ND	ND	ND	ND
414.74	12 027 ↑	202	168	1736 ↑	1230 ↑	386	257 ↑	15.8	Since 01/2013	976	512	90 ↓	31	12
328.44	1436 ↑	46,4 ↓	98	1602 ↑	1100 ↑	362	61	22.2	Since 06/2010	1417	886	156	84	17.4
99.45	9928 ↑	242	171	1495	ND	ND	ND	ND	Since 12/2011	1146	900 ↑	276	165 ↑	60
ND	38 268 ↑	ND	ND	ND	ND	ND	ND	ND	No substitution	NI	NI	NI	NI	NI
108	1644 ↑	46,9 ↓	148	1480	1050 ↑	2940 ↑	43	14.8	Since 01/2014	1107	ND	ND	ND	ND

isotypes and subclasses, and did not result in significant differences between patients and controls. CD40L + IL-4 + IL-21–stimulated cells strongly increased the production of all Ig isotypes in controls compared to STAT3-HIES patients. IL-21–induced increased Ig production determined as the fold change of mean secreted antibody in control over HIES samples was strong for IgG1 (184-fold), IgG3 (510-fold), and IgG4 (169-fold), intermediate for IgM (50-fold) and IgA (31-fold), and low for IgG2 (eightfold) and IgE (11-fold) (Figure 3B).

3.5 | Reduced total memory B cells with normal IgE^+ B-cell numbers in STAT3-HIES patients

Because of the high serum IgE in STAT3-HIES, we expected elevated numbers of IgE^+ B cells in STAT3-HIES patients and analyzed the peripheral blood B-cell compartment in STAT3-HIES patients by flow cytometry, and determined absolute cell counts as well as frequency of naive, memory, and plasmablast subsets within total $CD19^+$ B cells. While absolute numbers of $CD19^+$ B cells were not different between controls and STAT3-HIES patients (Tables 1 and S1), the frequency of $CD27^+IgD^-$, $CD27^-IgD^-$, and $CD27^+IgD^+$ memory B cells of total $CD19^+$ B cells was significantly lower in STAT3-HIES patients compared to healthy controls (Figure 4A,4). Similarly, plasmablast frequencies were significantly reduced in STAT3-HIES patients. These reduced frequencies of memory B cells and plasmablasts were accompanied by a higher frequency of $CD27^-IgD^+$ naive B cells. Absolute cell counts showed a similar increase in $CD27^-IgD^+$ naive B cells and a decrease in memory B-cell subsets in STAT3-HIES patients, whereas absolute numbers of plasmablasts were not significantly reduced (Figure 4C). Interestingly, the ratio of circulating plasmablasts over IgD^-CD27^+–class-switched B cells was significantly higher in STAT3-HIES patients (median 0.16) compared to controls (median 0.05), indicating a preference toward plasmablast over memory B-cell differentiation in STAT3-HIES patients (Figure 4D).

Reflecting the overall reduction of IgD^- memory B cells (Figure 4B,4), the frequencies of IgA^+-, $IgG1^+$-, $IgG2^+$-, and $IgG3^+$-switched B cells within total $CD19^+$ cells were significantly reduced in STAT3-HIES patients (Figure 4E,4). In contrast, the frequencies of $IgG4^+$- and IgE^+-switched B cells among total $CD19^+$ cells did not differ between controls and STAT3-HIES patients. Absolute cell counts showed the same pattern with a significant reduction in IgA^+, $IgG1^+$, $IgG2^+$, and $IgG3^+$ B cells in STAT3-HIES patients, whereas $IgG4^+$ and IgE^+ B-cell counts were within the normal range (Figure 4G). Despite the overall reduction in Ig–class-switched memory B cells, the distribution of IgG subclasses was not altered in STAT3-HIES patients, whereas the frequency of IgA-expressing B cells was decreased, while the frequency of IgE-expressing B cells was increased (Figure 4H).

3.6 | Reduced immunoglobulin heavy chain variable region gene SHM levels in IgG1, IgG3, IgA1, and IgE transcripts of STAT3-HIES patients

In addition to phenotypic analysis of memory B-cell subsets, we performed molecular analysis of their SHMs and Ig class switch profiles.

All patients showed a large range of SHM levels in their IgG, IgA, and IgE transcripts, as did pediatric and adult controls (Figure S3). IgA and IgG subclass analysis revealed an increase in IgG3, IgG1, and IgA1 usage at the expense of IgG2 and IgA2 in STAT3-HIES patients that was significant when compared to adult healthy controls for IgG, and both healthy adult and children regarding IgA (Figure 5B). Analysis of SHM levels within IgG and IgA subclasses showed significant reductions in IgG3, IgG1, and IgA1 transcripts of STAT3-HIES patients as compared to controls (Figure 5C). Patients' SHM levels in IgE transcripts were significantly lower than in those of healthy children. Thus, STAT3-HIES patients appeared to have a skewed IgG and IgA repertoire with predominance for IgM-proximal IgG3, IgG1, and IgA1 subclasses with low levels of SHM.

4 | DISCUSSION

To gain a better understanding of the mechanisms that regulate IgE production in humans, we assessed the functional immunoglobulin defect and hyper-IgE phenotype in STAT3-HIES in lymphoid tissue, bone marrow, and peripheral blood of STAT3-HIES patients. We showed for the first time analysis of IgE^+, IgG^+, IgM^+, and IgA^+ plasma cells in lymph node and bone marrow tissue of STAT3-HIES patients with increased IgE^+ plasma cells abundant in both lymph node and bone marrow of STAT3-HIES patients.

GCs within lymphoid tissue are the sites where T-dependent B-cell responses take place including clonal expansion, Ig class switch recombination, affinity maturation, and differentiation to memory B cells and plasma cells.[29] The GCs of STAT3-HIES patients did not show obvious architectural abnormalities regarding B-cell follicles and T-cell areas. Thus, human GC formation is not critically dependent on STAT3 signaling as suggested in a previous report on STAT3-HIES patients.[7] Similarly, mice with a conditional deletion of *Stat3* in the B-cell lineage ($Stat3^{fl/fl}CD19^{Cre/+}$ mice) demonstrated normal GC formation but had a defect in T-dependent IgG responses upon immunization despite normal serum IgG levels.[14,30]

We observed a dense IgE staining within the B-cell follicles, which largely overlapped with the CD21 staining on FDCs. This dense IgE staining indicates that FDCs capture secreted IgE through their FcεRI. It remains to be determined whether this FDC-bound IgE in STAT3-HIES patients has a functional role, for example, in the regulation of T-cell responses. While several studies have demonstrated that APC-bound IgE can enhance T-cell responses to allergens, this was previously shown in the context of allergen-specific IgE.[31,32] However, given the fact that much of the IgE in STAT3-HIES patients does not appear to be allergen-specific,[17] the functional role of FDC-bound IgE in these patients may be limited.

The STAT3-HIES patients in this study had reduced circulating frequencies of plasmablasts and memory B cells expressing all Ig heavy chain isotypes, except IgE and IgG4. Moreover, their B cells showed reduced SHM levels in *IGHV* of IgG3, IgG1, IgA1, and IgE transcripts, reflecting suboptimal GC responses. Thus, normal GC architecture does not guarantee fully functional GC reactions and may still be accompanied by impaired affinity maturation in antibody responses.

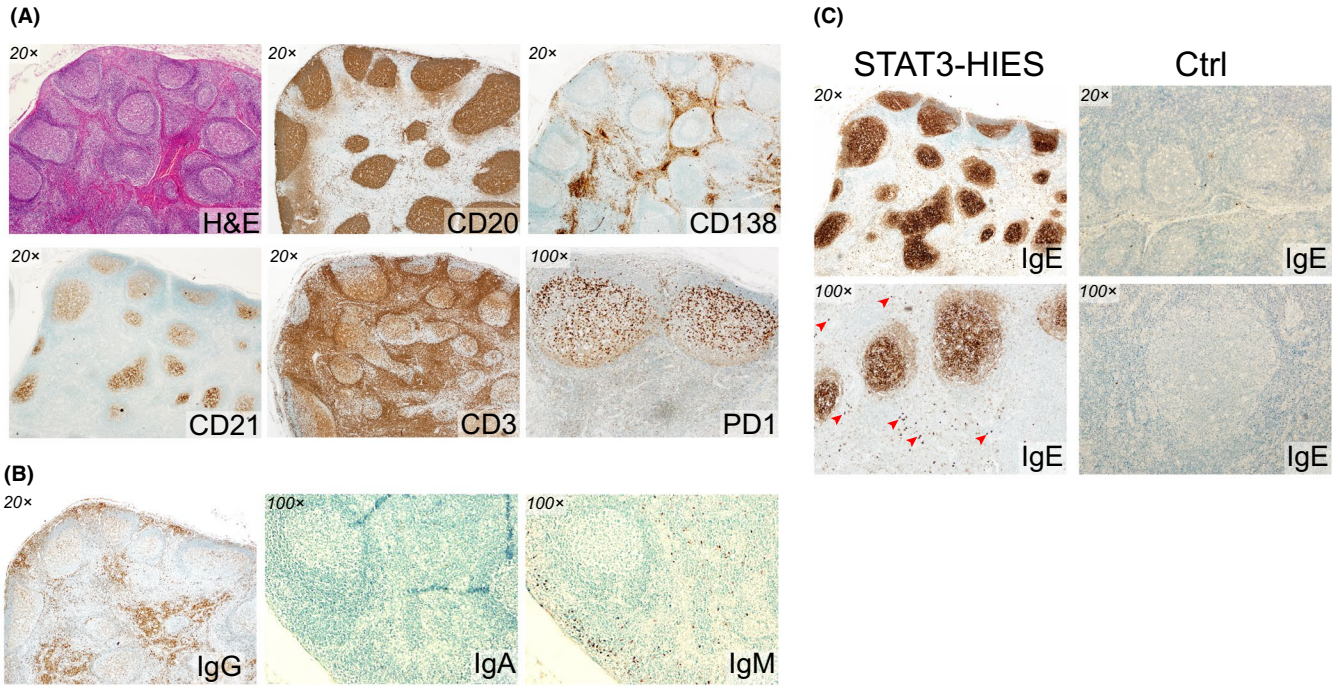


FIGURE 1 A, Sections of STAT3-HIES lymph nodes show a normal architecture with secondary lymph follicles and well-developed GC with normal cellular constitution. A, STAT3-HIES patient B-cell staining of follicles (CD20), T-cell areas (CD3), TFH cells (PD1), FDC networks (CD21), and plasma cells (CD138). B, STAT3-HIES patient staining of IgG, IgA, and IgM. C, Staining of IgE in lymph node sections of a STAT3-HIES patient and healthy control (ctrl); *H&E*, hematoxylin and eosin, antibody staining brown. Arrows highlight IgE^+ plasma cells

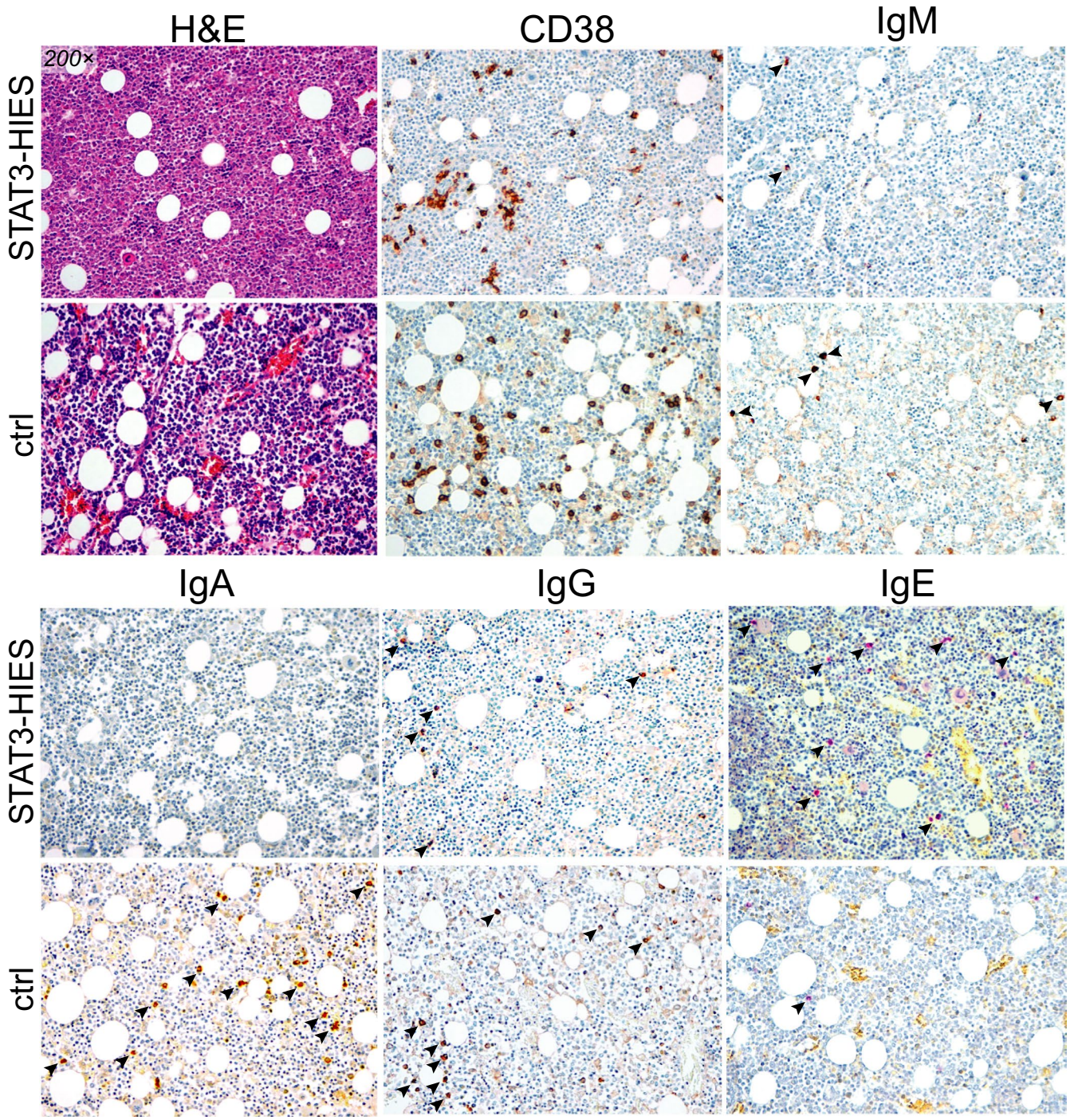


FIGURE 2 Plasma cell distribution in bone marrow sections of STAT3-HIES patient and control (ctrl) bone marrow samples stained with H&E, CD38, IgM, IgG, IgA (brown), and IgE (red). Arrows highlight IgM^+, IgA^+, IgG^+, and IgE^+ plasma cells

To assess whether an intrinsic IgE-skewed antibody response may account for the elevated levels of circulating IgE antibodies in STAT3-HIES patients, we isolated naive B cells and studied their in vitro proliferation rate and antibody secretion in a cell culture system that mimics T-cell–dependent B-cell activation. We observed no significant difference between STAT3-HIES- and control-derived naive B cells in terms of proliferative responses and antibody production to stimulation with CD40L + IL-4. However, addition of IL-21 to this condition resulted in much stronger enhancement of these responses in control-derived B-cell cultures than in STAT3-HIES cultures, confirming the dependence of IL-21 signaling on STAT3.[4,7] Of note, the IL-21 did induce a modest increase in proliferation and immunoglobulin production in STAT3-HIES patients as well. This may be related to the fact that STAT3-HIES is an autosomal dominant disorder and these patients have one mutated and one normal copy of the STAT3 gene. As a result, these patients have impaired but not entirely ablated STAT3 signaling. Moreover, residual IL-21–mediated B-cell activation may be due to activation

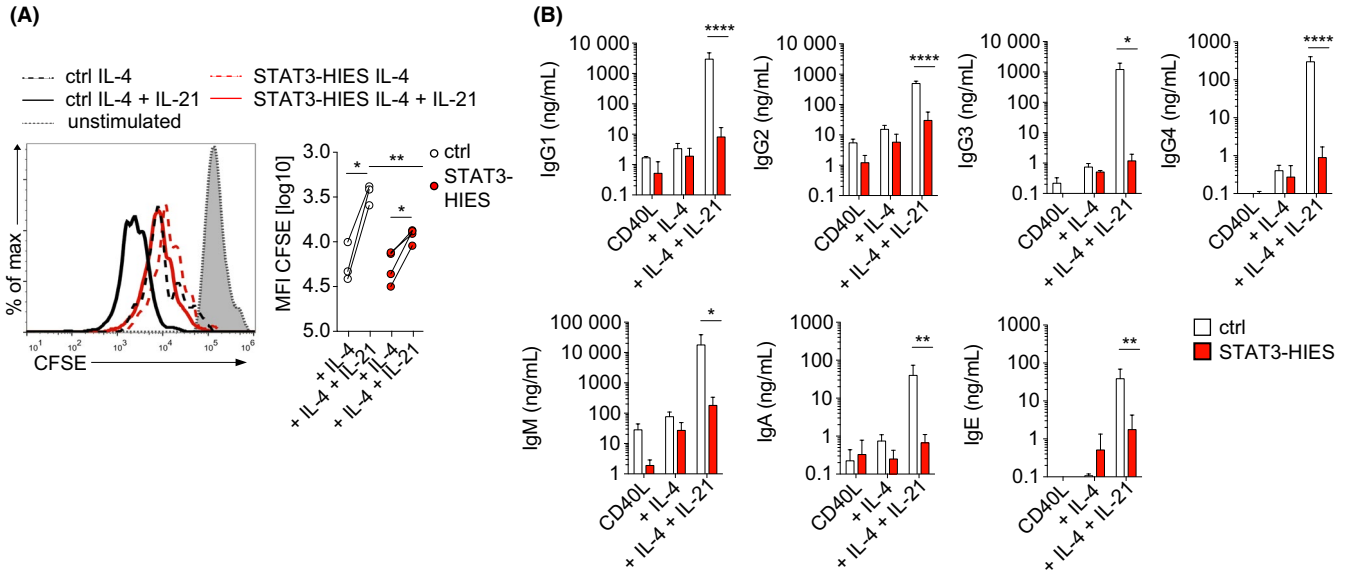


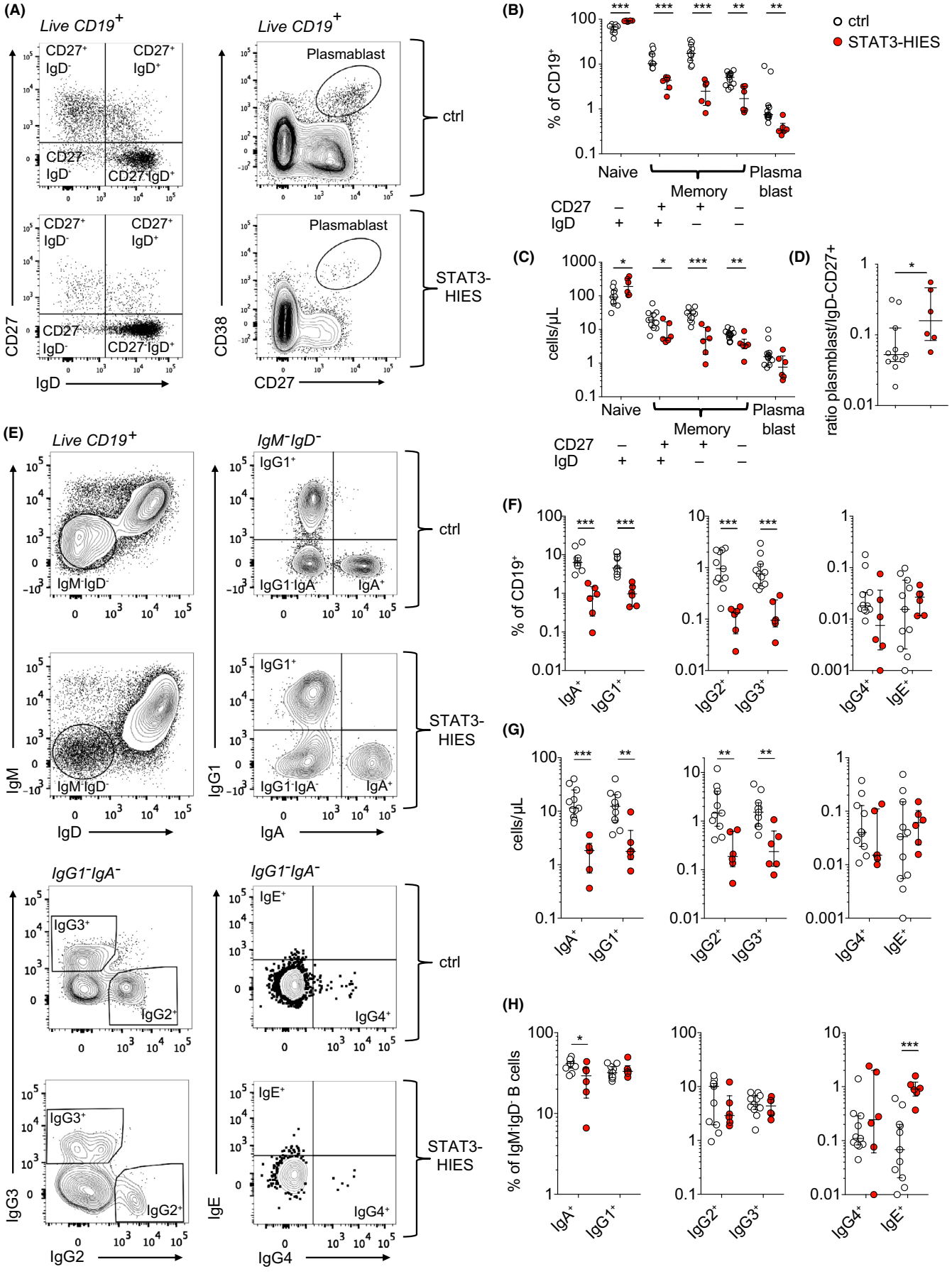
FIGURE 3 STAT3-HIES B-cell activation in response to IL-21 is impaired. In vitro response of naive IgM^+IgD^+ B cells to CD40L, IL-4, and IL-21. A, Proliferative response of naive B cells after six days of stimulation with CD40L + IL-4 and CD40L + IL-4 + IL-21. *$P < 0.05$ and **$P < 0.01$, effects of different stimuli within the group of patients and the group of controls were analyzed with paired *t* test, and effects of the same stimulus between the two groups with unpaired *t* test. B, Immunoglobulin secretion from naive B cells after 12 days of stimulation with CD40L, CD40L + IL-4, and CD40L + IL-4 + IL-21. Bar graph shows mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, n = 3 (ctrl) and n = 4 (STAT3-HIES), two-way ANOVA

of STAT3-independent pathways such as MAPK and PI3K.[33] STAT3-HIES patient B cells did not produce more IgE than control B cells in these conditions, and it is interesting to note that the difference in antibody production between STAT3-HIES and controls in response to CD40L + IL-4 + IL-21 was smaller for IgE than for most other isotypes. This indicates that CSR to IgE is less severely impaired than CSR to most other isotypes.

We performed a detailed SHM analysis of the IGHV genes for each heavy chain isotype. The observed impaired proliferative response to IL-21 may be related to the reduced numbers of SHM in IgG3, IgG1, IgA1, and IgE transcripts that we observed in circulating B cells of STAT3-HIES patients. Patients' SHM levels of IgG2, IgG4, and IgA2 transcripts, however, were comparable to healthy controls, suggesting that the SHM defect in STAT3-HIES is not absolute. Previous reports showed that the rate of SHM in memory B cells of STAT3-HIES patients was within the normal range, suggesting that the B-cell defect lies primarily in the development of memory B cells rather than the inability of SHM.[7] IGH regions of IgG2, IgG4, and IgA2 are distally located in the IGH locus and often utilized following sequential Ig class switching via IgG3 or IgG1.[34] Hence, SHM levels are usually higher in transcripts involving IGHG2 and IGHG4[25,35] and both IgG2 and IgG4 have been associated with chronic antigen exposure and inflammation.[28,36-38] Thus, the normal SHM levels in IGHG2 and IGHG4 in STAT3-HIES patients may result from repeated responses to pathogens during which some extent of memory B-cell development takes place and additional mutations are accumulating resulting from persistent immunologic pressure due to chronic infections.

Even though we detected reduced circulating plasmablasts and memory B cells in STAT3-HIES patients, confirming previous reports,[7,13,14] the ratio between circulating plasmablasts over switched memory cells was significantly higher in STAT3-HIES patients compared to controls. This indicates that even though the B-cell population in these patients primarily consists of naive cells, the cells that do differentiate are more prone to become plasmablasts in STAT3-HIES patients than in controls. It has been demonstrated in mouse models of helminth infection as well as T-cell–dependent hapten-protein and ovalbumin sensitization that small fractions of IgE^+ B cells were transiently present in the GC,[39] while most IgE^+ B cells in the lymph nodes were present as plasma cells outside of the GC.[39,40] Moreover, IgE^+ cells are characterized by a rapid differentiation to plasma cells that showed reduced affinity maturation.[39] The expression of membrane IgE was found to autonomously trigger rapid plasma cell differentiation through the CD19-PI3K-Akt-IRF4 pathway, and apoptosis through BLNK-Jnk/p38 signaling.[20] BLNK in

FIGURE 4 STAT3-HIES patients have an impaired memory B-cell compartment but retain normal IgE^+ B-cell numbers. Flow cytometry analysis of B-cell subsets in primary peripheral blood mononuclear cells. A, Gating strategy for naive and memory B cells and plasmablasts. Frequencies (B) and absolute cell counts (C) of B-cell subsets in control and STAT3-HIES patients. D, Ratio plasmablasts/$CD27^+IgD^-$-switched memory B cells. E, Gating strategy for class-switched B-cell subsets. B cells expressing IgA, IgG1-4, and IgE represented as percentage of total $CD19^+$ cells (F), absolute cell counts (G), and frequencies among IgM^-IgD^-–class-switched B cells (H). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, Mann-Whitney test, mean with interquartile range is shown, n = 11 (ctrl) and n = 6 (STAT3-HIES)


(A)
Live CD19+
CD27+ IgD-
CD27+ IgD+
CD27- IgD-
CD27- IgD+
CD27
IgD
Plasmablast
CD38
CD27
ctrl
STAT3-HIES
(B)
ctrl
STAT3-HIES
% of CD19+
Naive
Memory
Plasma blast
CD27
IgD
(C)
cells/µL
(D)
ratio plasmablast/IgD-CD27+
(E)
Live CD19+
IgM-IgD-
IgG1+
IgG1-IgA-
IgA+
IgM
IgD
IgG1
IgA
(F)
% of CD19+
IgA+
IgG1+
IgG2+
IgG3+
IgG4+
IgE+
(G)
cells/µL
(H)
% of IgM-IgD- B cells

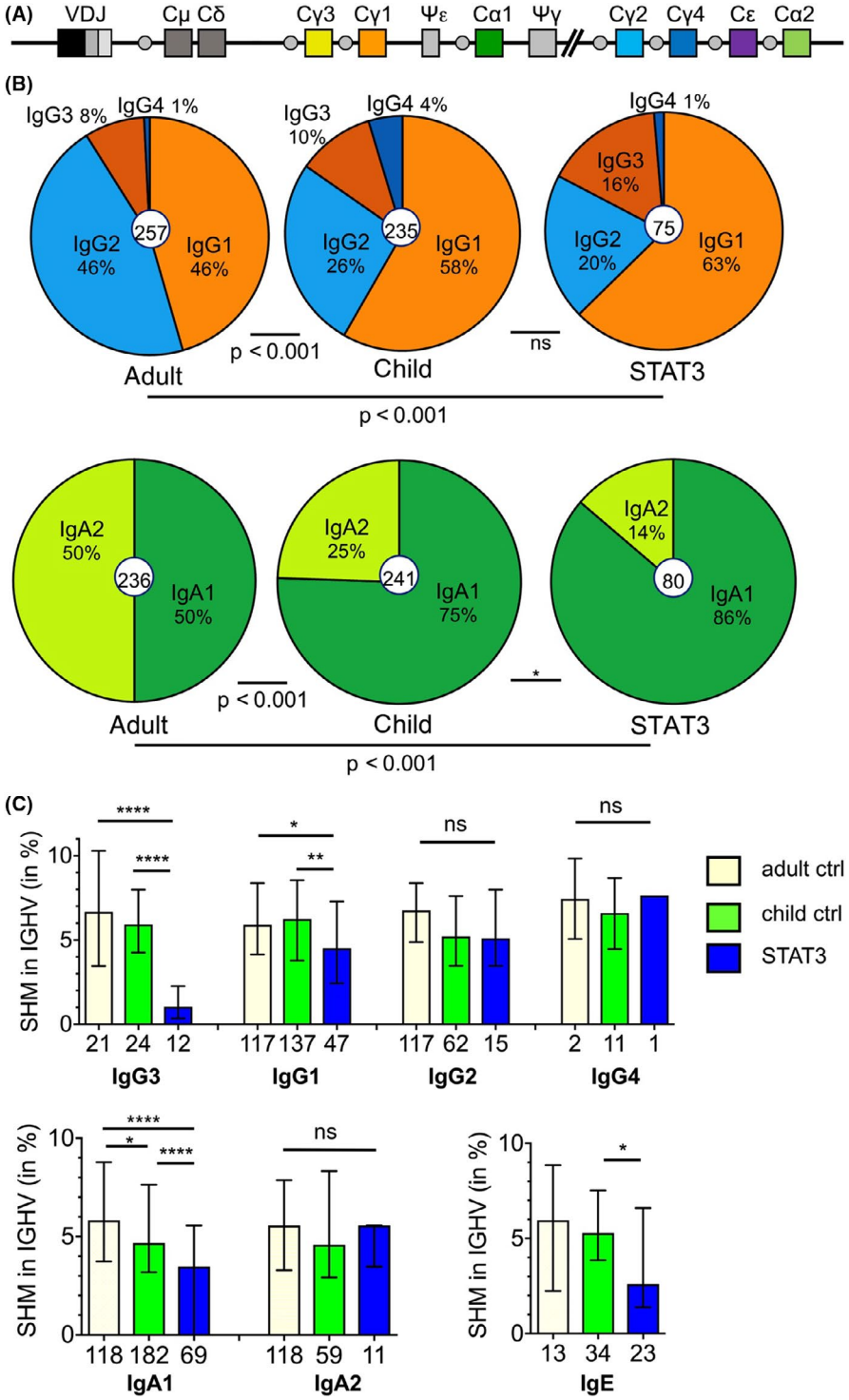


FIGURE 5 SHM levels and subclass usage in IgG, IgA, and IgE transcripts of STAT3-HIES patients. A, Schematic representation of the human *IGH* constant gene regions. B, IGHG and IGHA subclass in transcripts from healthy adults (n = 6), children (n = 6), and STAT3-HIES patients (n = 4). Statistical analysis of CSR with the chi-square test; **P* < 0.05; *****P* < 0.0001. C, SHM levels in IgG, IgA, and IgE transcripts of the same individuals as in B. Pooled clones (n indicated below each bar) of all donors are shown as median bars with interquartile ranges. Statistical analysis of SHM levels between groups was done with the nonparametric Mann-Whitney *U* test; **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001

concert with CD5 and CK2 has been shown to induce phosphorylation of STAT3.[22,41] This may lead to exaggerated IgE$^+$ plasma cell development in STAT3-HIES patients, since CD19-PI3K-Akt-IRF4–dependent plasma cell differentiation is not dependent on STAT3 signaling, while the BLNK-Jnk/p38–mediated apoptosis induction may involve STAT3 phosphorylation. IgE$^+$ plasma cells were readily detected in STAT3-HIES patients' bone marrow and lymph node tissue. These cells may be responsible for the increased levels of circulating IgE in STAT3-HIES patients.

We showed here for the first time a detailed immunophenotyping of STAT3-HIES patient blood B cells that included all immunoglobulin heavy chain isotypes. This revealed that the absolute numbers of circulating class-switched memory B cells expressing IgA, IgG1, IgG2, and IgG3 but not IgG4 and IgE were strongly reduced in STAT3-HIES patients. CSR to IgG4 and IgE is highly dependent on IL-4,[42] a cytokine that signals independent of STAT3 and promotes GC responses in a STAT6-dependent manner.[43] Thus, the lack of IL-21R signaling in STAT3-HIES B cells might tip the

balance to more abundant IgG4 and IgE class switching, which has also been observed in IL-21–deficient patients and mice showing reduced B memory cells and highly elevated serum IgE levels.[44-46] However, the effect of IL-21 on IgE regulation may be markedly different in humans than in mice. While STAT3-HIES patients develop high concentrations of serum IgE, only a minimal fraction of this IgE is specific for known aeroallergens and food allergens.[17] This, combined with our observation of a low frequency of SHM in the IgE-associated VDJ regions, suggests that CSR in IgE primarily occurs through direct switching from Cμ to Cε with impaired rates of affinity maturation in STAT3-HIES patients. This hypothesis is supported by animal models, showing that weak antigenic stimulation favors the development of plasma cells through direct CSR to IgE, while high-affinity IgE antibodies require sequential CSR through an IgG intermediate.[23,47] The relative immature state of the circulating B-cell population in STAT3-HIES patients may also play a role in their propensity to switch to IgE. It was shown in mice that immature B cells are prone to undergo direct CSR to IgE in response to anti-CD40 and IL-4 stimulation.[48]

In conclusion, to gain a better understanding of the regulation of IgE production and the impact of STAT3 on B-cell responses, we have studied the cellular and molecular maturation of B cells in STAT3-HIES patients with impaired STAT3 signaling. STAT3 signaling is critical for the generation of fully mature GCs that support affinity maturation, while impaired STAT3 signaling results in a weakened GC reaction, which favors direct CSR to IgE and differentiation to plasma cells that produce low-affinity IgE.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

WV, CEK, MCZ, MA, and EDR designed the study and wrote the manuscript; EDR, BDS, CK, BH, MCZ, and CEK recruited patients and collected patient material; WV, CEK, CIM, PMA, CJMN, OFW, BH, and JN performed and analyzed experiments; all authors supported and made contributions to data interpretation, and revised and approved the final version of the manuscript.

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