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

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Short title

B cell memory in STAT3-HIES patients

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 cells
SHM	somatic hypermutation
STAT	signal transducer and activator of transcription
T _{FH}	T follicular helper
T _H	T helper type

Abstract:

Background: STAT3 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, *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 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.

Introduction

The prevalence of allergic diseases such as atopic dermatitis, allergic rhinitis and asthma has significantly increased during the last 50 years¹. 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 elucidated².

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-4⁴⁻⁶. Furthermore, IL-21 is a key cytokine for maintenance and regulation of a high titer and high affinity antigen-specific antibody response⁷, while there are indications that IgE production is less dependent on the formation of mature GCs than other Ig isotypes⁸. 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 C ϵ transcription of IL-4-stimulated B cells⁹, and through induction of apoptosis in B cells committed to produce IgE¹⁰.

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 function¹¹⁻¹⁶. 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 as well as reduced T cell help, resulting from impaired STAT3 signaling, have been associated with a lack of high affinity specific antibody production in STAT3-HIES^{17,19-24}. It remains unclear if this results only from a defect in the amount of antibody production or if the antibodies produced have undergone crippled affinity maturation due to impaired induction of somatic hypermutations (SHM).

Recently, advanced methodologies have enabled to identify and study IgE-expressing and IgG-subclass-expressing B cells²⁵⁻²⁹. 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.

Methods

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 immunological assessment. Immune histology was performed on tissues of two additional (deceased) patients (P7, P8). All patients carried a heterozygous dominant negative mutation in the *STAT3* gene (Table 1). Eleven non-atopic 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, written informed consent was obtained from all study subjects.

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.

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 the online supplement methods section

Statistics

Non-parametric statistical procedures were applied. Two-group comparisons were assessed by Mann-Whitney test. P values of below 0.05 were considered significant. Calculations and graphics were generated with GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

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 non-immunologic findings was present in all patients (table 1). All patient had normal total serum IgG levels prior to immunoglobulin substitution therapy. Two patients had reduced serum IgA (P5, P8) and IgG subclass (P3, 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 improvement¹⁸.

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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 sensitization (positive specific serum IgE and skin prick test) and allergic clinical manifestation was only mildly present in two patients (P3 and P6). There was no correlation between the level of serum IgE and clinical findings.

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 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 presence of CD20⁺ B cells, PD-1⁺ follicular T cells and CD21⁺ follicular dendritic cells (Fig 1A). CD138⁺ plasma cells were mainly found in the paracortex and predominantly expressed IgG or IgM (Fig 1A, B). IgA⁺ plasma cells were hardly detectable (Fig 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 (Fig 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.

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 (Fig 2). The overall cell content and bone marrow structural organization of the STAT3-HIES patient was comparable to controls.

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

Next we assessed the capacity of STAT3-HIES naïve B cells to respond to T-dependent activation signals and determined whether these cells are skewed towards IgE production. CD27⁺IgM⁺IgD⁺ naïve B cells were purified from peripheral blood by flow cytometry-based cell sorting using a gating strategy aimed at excluding switched- and non-switched 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 IL21 and assessed for Ig isotype secretion, cell differentiation, and proliferation.

The proliferation rate of naïve B cells after 6 days 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 (Fig 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 (Fig 3B). IL-4 increased the levels of all secreted Ig 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 (8-fold) and IgE (11-fold) (Fig 3B).

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 (Table 1 and S1), the frequency of CD27⁺IgD⁻, CD27⁻IgD⁻, and CD27⁺IgD⁺ memory B cells of total CD19⁺ B cells were significantly lower in STAT3-HIES patients compared to healthy controls (Fig 4A, B). 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 (Fig 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 towards plasmablast over memory B cell differentiation in STAT3-HIES patients (Fig 4D).

Reflecting the overall reduction of IgD⁻ memory B cells (Fig 4B, C), the frequencies of IgA⁺, IgG1⁺, IgG2⁺ and IgG3⁺ switched B cells within total CD19⁺ cells were significantly reduced in STAT3-HIES patients (Fig 4E, F). 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 (Fig 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 (Fig 4H).

Reduced immunoglobulin heavy chain variable region gene (IGHV) 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 (Fig 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 (Fig 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.

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 ³⁰. 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 ^{14,31}.

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^{32,33}. However, given the fact that much of the IgE in STAT3-HIES patients does not appear to be allergen-specific¹⁷, 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 with impaired affinity maturation in antibody responses.

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 signalling 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 of STAT3-independent pathways such as MAPK and PI3K³⁴. STAT3-HIES patient B cells did not produce more IgE than control B cells in these conditions, 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 SHM⁷. 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 IgG1³⁵. Hence, SHM levels are usually higher in transcripts involving IGHG2 and IGHG4^{26,36} and both, IgG2 and IgG4 have been associated with chronic antigen exposure and inflammation^{29,37-39}. 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 consist 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 GC⁴⁰, while most IgE⁺ B cells in the lymph nodes were present as plasma cells outside of the GC^{40,41}. Moreover, IgE⁺ cells are characterized by a rapid differentiation to plasma cells that showed reduced affinity maturation⁴⁰. 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 signaling²⁰. BLNK in concert with CD5 and CK2 has been shown to induce phosphorylation of STAT3^{22,42}. 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 signalling 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-4⁴³, a cytokine that signals independent of STAT3 and promotes GC responses in a STAT6-dependent manner⁴⁴. Thus, the lack of IL-21R signalling 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 levels⁴⁵⁻⁴⁷. 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 allergens¹⁷. 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 C μ to C ϵ with impaired rates of affinity maturation in STAT3-HIES patients. This hypothesis is supported by animal models showing that weak antigenic stimulation favours the development of plasma cells through direct CSR to IgE while high affinity IgE antibodies require sequential CSR through an IgG intermediate^{23,48}. 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 stimulation⁴⁹.

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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 signalling. STAT3-signaling is critical for the generation of fully mature GCs that support affinity maturation, while impaired STAT3 signalling results in a weakened GC reaction, which favours direct CSR to IgE and differentiation to plasma cells that produce low affinity IgE.

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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, CM, PA, CN, OW, 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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Figure legends

FIG 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.

FIG 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.

FIG 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 stimulation with CD40L + IL-4 and CD40L + IL-4 + IL-21. * P<0.05, ** 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 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, **** P<0.0001, n=3 (ctrl) and n=4 (STAT3-HIES), two-way ANOVA.

FIG 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, ***P<0.001, Mann-Whitney test, mean with interquartile range is shown, n=11 (ctrl) and n=6 (STAT3-HIES).

FIG 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 χ^2 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 non-parametric Mann-Whitney U test; *, p<0.05; **, p<0.01; ****, p<0.0001.

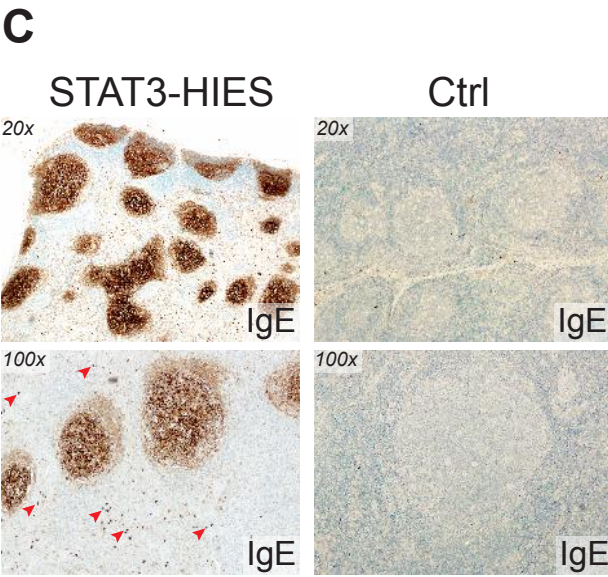
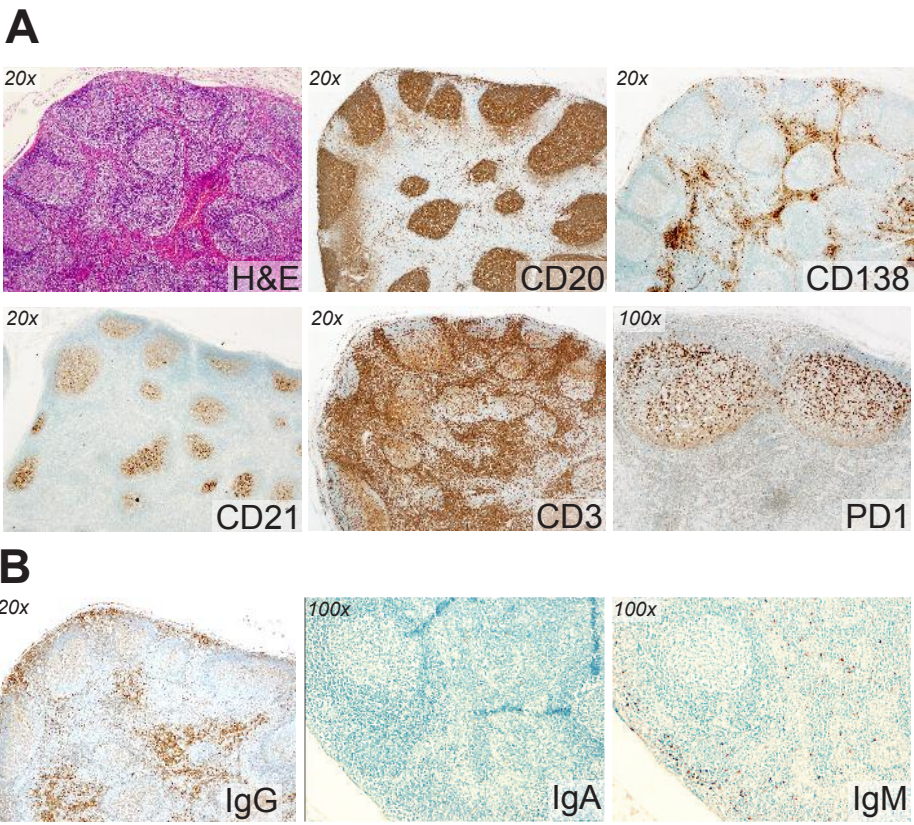
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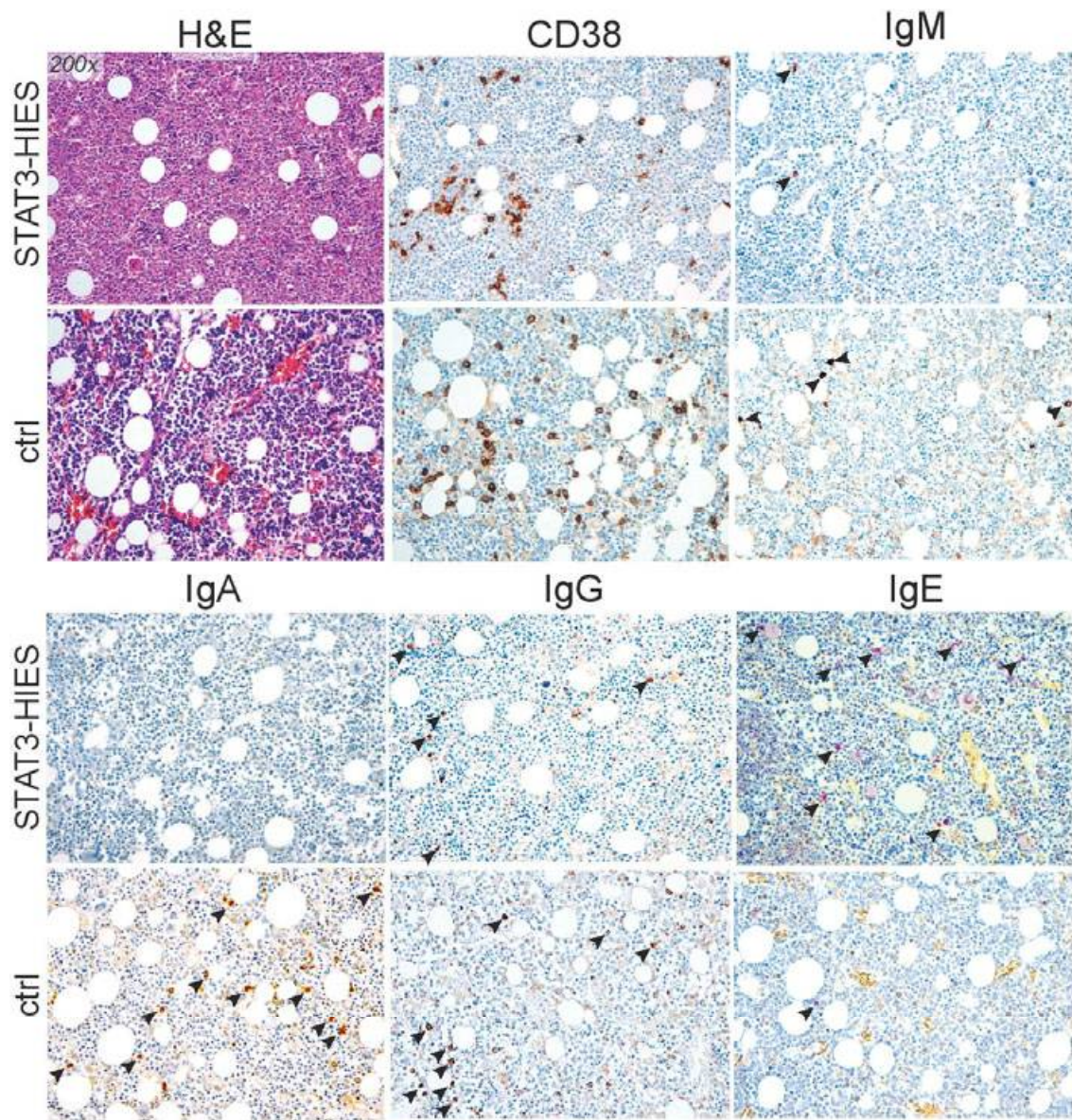
Figure S1. Purification of naive B cells from PBMC. **A**, Cells negative for CD3, CD14, CD16, FcεRI, CD235a and CD36 (DUMP) and positive for CD19 were further gated as CD27⁺IgG⁻IgA⁻. **B**, confirmation of purity of isolated naive B cells. Purified CD19⁺CD27⁺IgG⁻IgA⁻ cells were stained with IgM and IgD in order to confirm that this approach yielded highly pure IgM⁺IgD⁺ naive B cells. PBMC were stained as a control.

Figure S2. Effect of cell density on IgE production by B cells stimulated with CD40L+IL-4+IL-21. Purified B cells of healthy controls were stimulated for 12 days with CD40L+IL-4+IL-21 at different densities ranging from 100 to 25000 cells per well in a 96-well flat bottom plate. The concentration of secreted IgE was measured by ELISA.

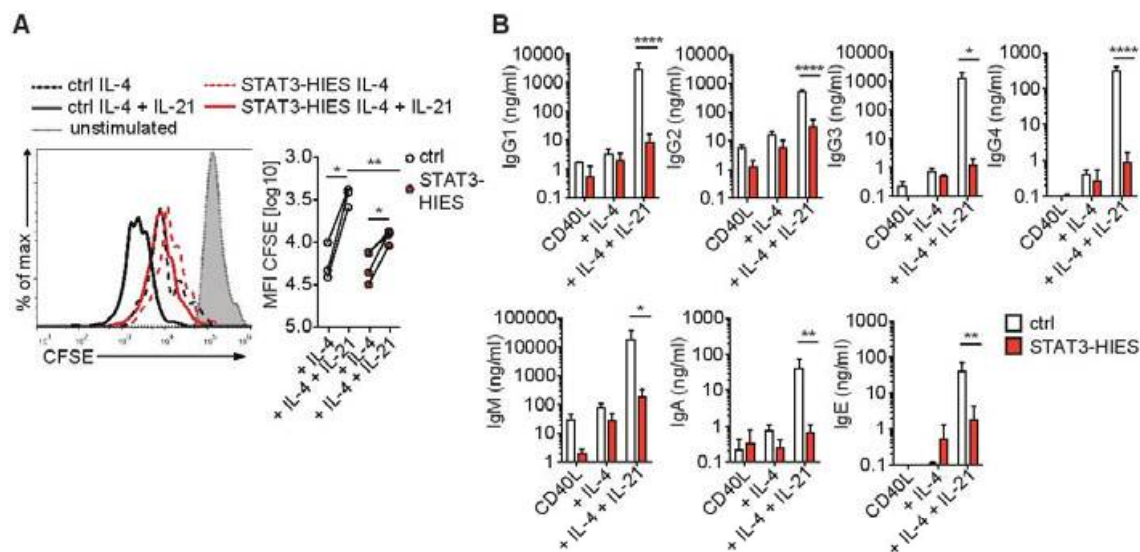
Figure S3. Frequencies of mutated nucleotides in rearranged *IGHV* genes of IgG, IgA and IgE sequences. Each *gray dot* represents a unique rearrangement, and *red lines* indicate median values, n indicates number of sequences analyzed per individual.

Figure S4. Illustration of specificity of IgE staining on peripheral B cells. Percentage of B cells that are positively stained with anti-IgE on different B cell subsets. B cells were gated as CD19⁺ and further gated as indicated above each dot plot. As expected, IgM⁺IgD⁻IgG⁻IgA⁻IgM⁺ had the highest frequency of IgE⁺ B cells while IgM⁻IgD⁻IgA⁺ and IgM⁻IgD⁻IgG⁺ class-switched B cells had no detectable IgE⁺ B cells. IgM⁺IgD⁺ naive B cells showed some IgE⁺ B cells, confirming previous reports that naive B cells can bind IgE through CD23

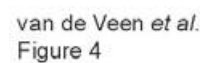


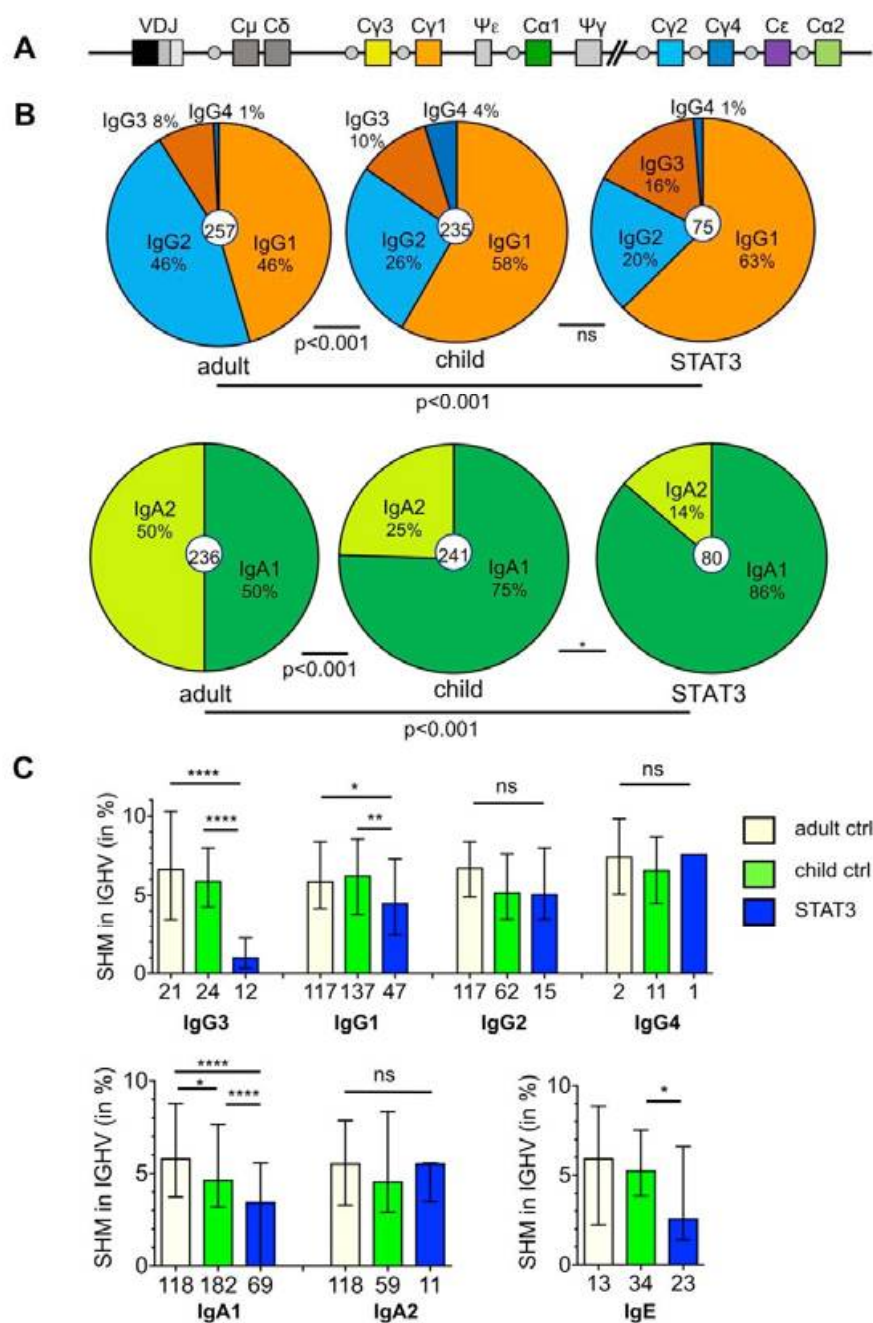


van de Veen *et al.*
Figure 2

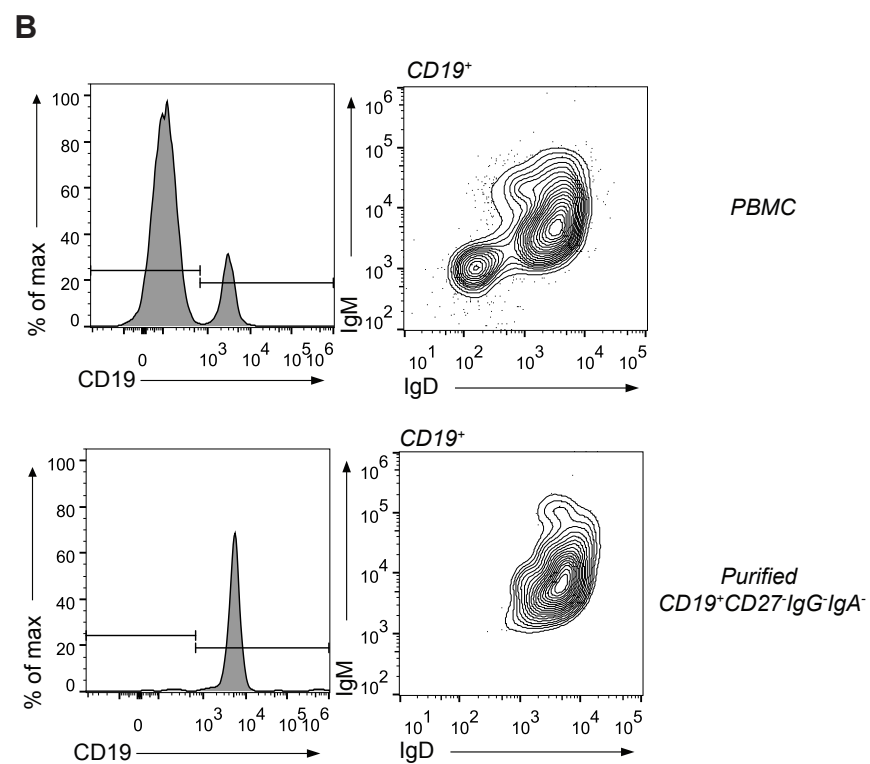
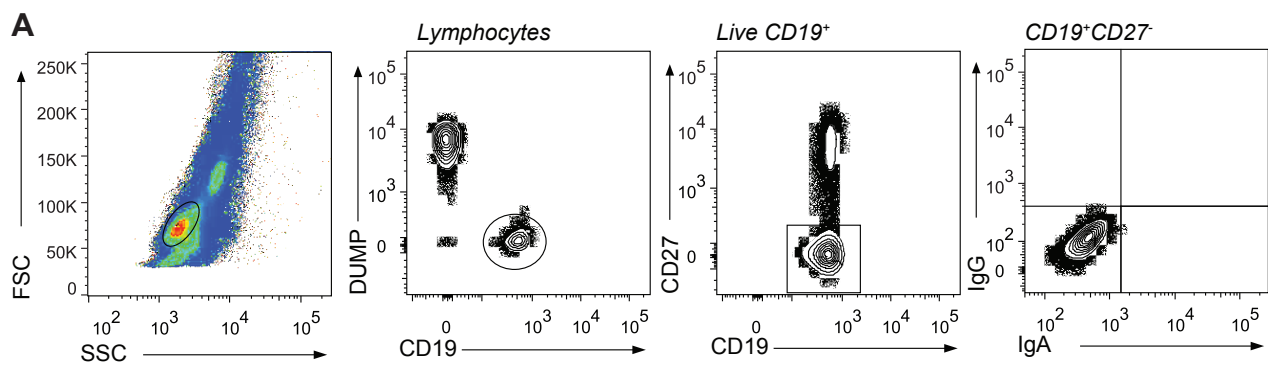


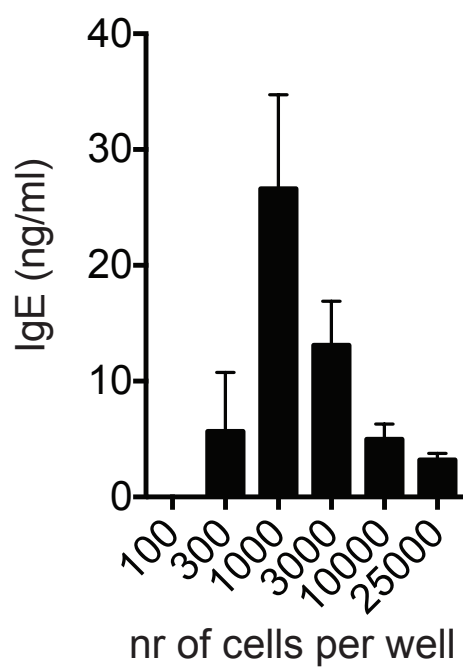
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Figure 3

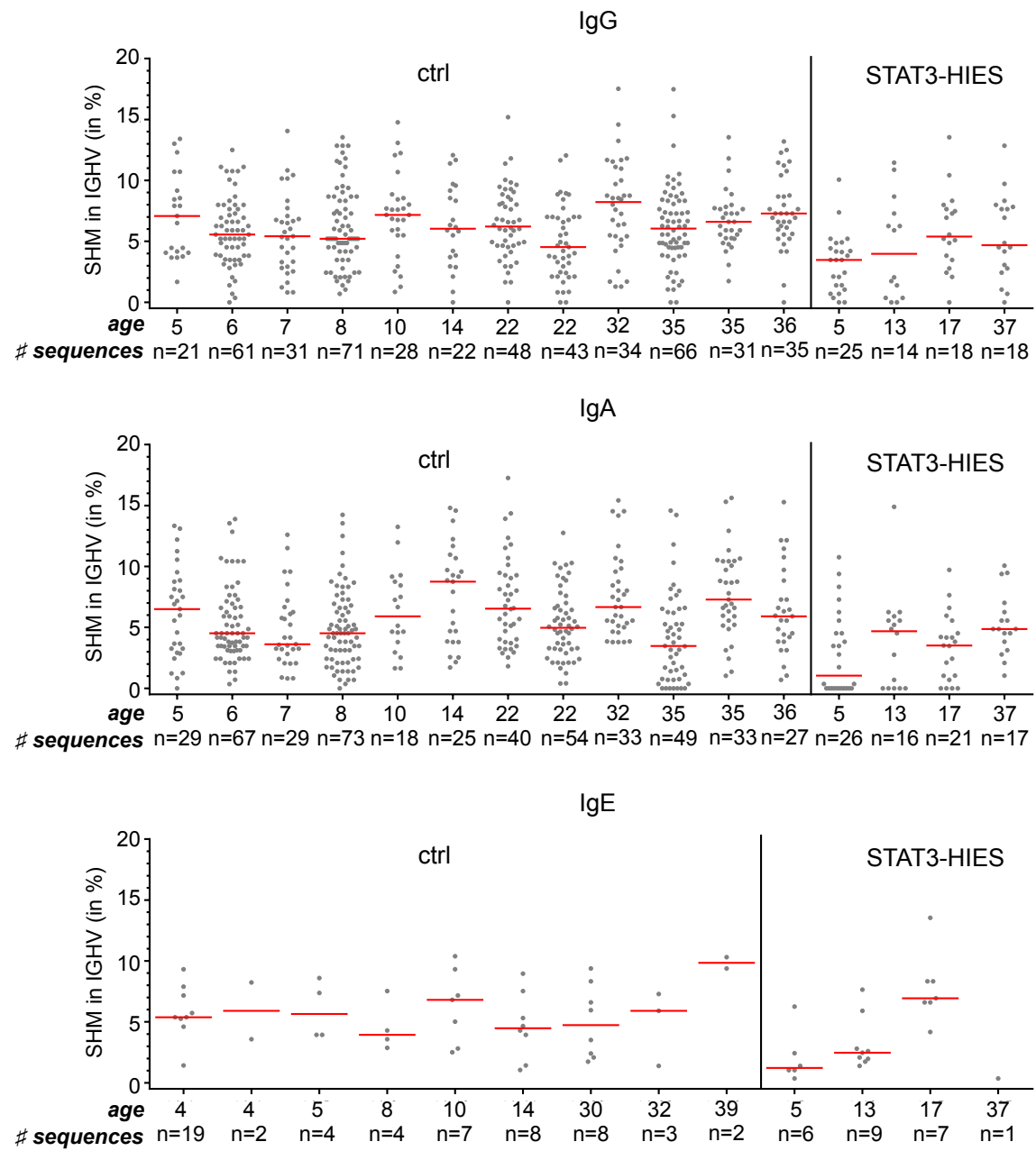




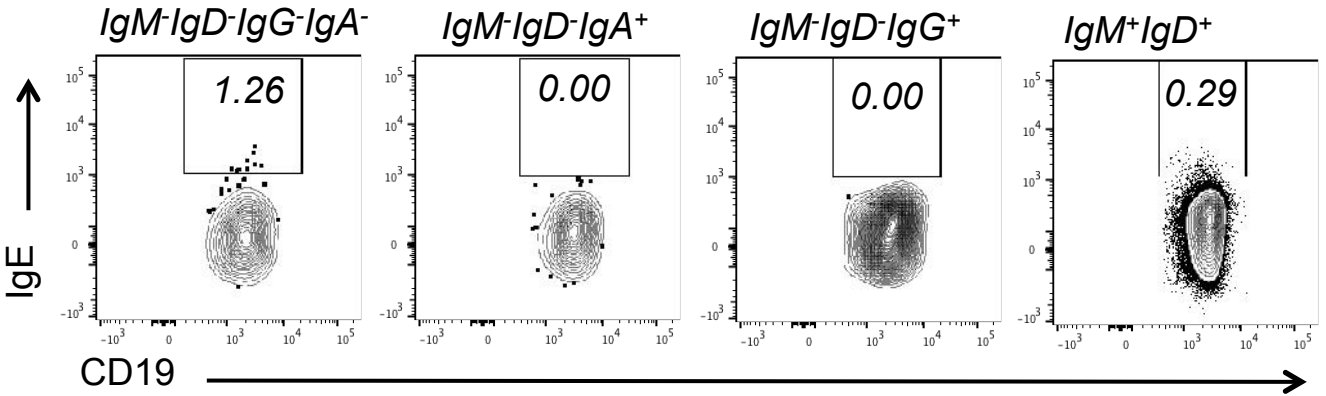
van de Veen *et al.* Figure 5







van de Veen *et al.*
Figure S3



van de Veen *et al.* Fig S4

Table S1. Control subject characteristics

ID	Age	Gender	CD19 ⁺ cells (% of lymphocytes)	CD19 ⁺ cells (cells/ μ l)	Serum IgE (IU/ml)
C01	32	female	12.7	287.0	71.8
C02	41	female	8.5	198.4	22.6
C03	49	female	7.5	188.8	85.2
C04	27	male	11.1	328.6	0.6
C05	48	female	5.3	165.0	0.7
C06	29	female	5.5	81.2	31.0
C07	29	female	10.1	192.6	66.3
C08	34	female	14.3	321.3	82.0
C09	28	female	6.2	121.4	111.0
C10	27	male	5.3	163.6	16.0
C11	33	male	7.5	106.7	15.4

Table S2. Antibodies used for immunohistochemistry

Marker	Clone	Company	Detection system
CD138	MI15	DAKO, Glostrup, Denmark	DAB
CD20	L26	DAKO, Glostrup, Denmark	DAB
CD21	2G9	Thermo scientific, Waltham, MA	DAB
CD3	SP7	Thermo scientific, Waltham, MA	DAB
CD38	MI15	Leica Biosystems	DAB
PD1	NAT105	Abcam, Cambridge, UK	DAB
IgM	rabbit polyclonal	DAKO, Glostrup, Denmark	DAB
IgA	rabbit polyclonal	DAKO, Glostrup, Denmark	DAB
IgG	rabbit polyclonal	DAKO, Glostrup, Denmark	DAB
IgG4	HP6025	Binding Site Immunologicals Group, Birmingham, UK	DAB
IgE	XTE4	Santa Cruz	AP
IgE	rabbit polyclonal	DAKO, Glostrup, Denmark	DAB

Table S3. Antibodies used for immunophenotyping and flow cytometric cell sorting

Marker	Clone	Fluorochrome	Company
CD19	H1B19	APC-Cy7	BioLegend, San Diego, CA
CD19	J3-119	PC7	Beckman Coulter, Brea, CA
CD27	M-T271	BV421	BioLegend, San Diego, CA
CD27	L128	BV510	BD Biosciences, San Jose, CA
CD27	O323	PE	BioLegend, San Diego, CA
IgM	MHM-88	PerCP-Cy5.5	BioLegend, San Diego, CA
IgM	MHM-88	BV510	BioLegend, San Diego, CA
IgD	IA6-2	FITC	BD Biosciences, San Jose, CA
IgD	IA6-2	PE-CF594	BD Biosciences, San Jose, CA
IgG	Goat polyclonal	Dylight-405	Jackson ImmunoResearch, West Grove, PA
IgG1	HP6188	PE*	Sanquin, Amsterdam, Netherlands
IgG2	HP6014	PE-Cy5.5*	Sanquin, Amsterdam, Netherlands
IgG3	HP6095	PC7*	Sanquin, Amsterdam, Netherlands
IgG4	RJ4	Dylight-405*	Abingdon Health, York, UK
IgE	Ige21	APC	Ebioscience, San Diego, CA
IgA	Goat polyclonal	Alexa Fluor 488	Jackson ImmunoResearch, West Grove, PA
CD38	HIT2	APC-Cy7	BioLegend, San Diego, CA
CD38	HB7	PC7	BioLegend, San Diego, CA
CD138	DL-101	APC	BioLegend, San Diego, CA
CD3	OKT3	Biotin	BioLegend, San Diego, CA
CD14	M5E2	Biotin	BioLegend, San Diego, CA
CD16	3G8	Biotin	BioLegend, San Diego, CA
FCεRI	AER-37 (CRA-1)	Biotin	BioLegend, San Diego, CA
CD235a	HIR2 (GA-R2)	Biotin	BioLegend, San Diego, CA
CD36	5-271	Biotin	BioLegend, San Diego, CA

* Antibodies were in-house labeled with fluorochromes using lightning link labeling kits (Innova Biosciences, Cambridge, UK).

Supplementary methods

Histology and immunohistochemistry of lymph nodes

The lymph node specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin according to standard procedures. For immunohistochemistry 5 μ m standard tissue sections of formalin-fixed paraffin embedded samples were stained employing a Ventana Benchmark XT autostainer (Ventana Medical Systems, Oro Valley, AZ). Immunohistochemistry was performed according to standard procedures using antibodies listed in Table S2. To exclude unspecific reactions of antibodies and/or reagents, isotype and system controls were performed. Stainings were developed with 3,3'-Diaminobenzidine (DAB), or permanent alkaline phosphatase (AP) polymer red.

***In vitro* proliferation and antibody production of naive B cells**

For purification of naive B cells, PBMCs were stained with biotinylated antibodies against CD3, CD14, CD16, FC ϵ RI, CD235a and CD36 (BioLegend, San Diego, CA, USA) in staining Buffer (PBS + 2mM EDTA + 0.5% BSA). After washing, cells were stained with anti-CD19-APC/Cy7, anti-CD27-PE, streptavidin-BV605 (BioLegend, San Diego, CA, USA), anti-IgG-dylight405 and anti-IgA-AF488 (Jackson ImmunoResearch, West Grove, PA, USA). 7-AAD (BD Biosciences) was added for dead cell exclusion. Naive B cells were gated as DUMP⁻7-AAD⁻CD19⁺CD27⁺IgG⁻IgA⁻ and sorted on a FACS ARIA III (DUMP channel was used to exclude cells expressing CD3, CD14, CD16, FC ϵ RI, CD235a, CD36). To confirm that this sorting strategy yielded pure naive B cells, a small fraction of purified B cells were stained with anti-IgM-BV510 (Biolegend), anti-IgD-FITC (BD Biosciences) and analyzed on a flow cytometer (Gallios, Beckman Coulter, Brea, CA) (Figure S1). The remainder of the purified naive B cells was labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes Europe BV, Leiden, Netherlands). 1000 of CFSE labeled naïve B cells (which was found to be the optimal density for IgE induction in response to CD40L+IL-4+IL-21 stimulation as shown in Figure S2) were co-cultured with 1x10⁴ fibroblasts expressing CD40-Ligand (CD40L-Lcells), IL-4 (50 ng/ml) (Gift from Dr. C. Heusser,

Novartis, Basel, CH) with or without IL-21 (50 ng/ml) (Miltenyi Biotec, Bergisch Gladbach, Germany) per well in a 96-well flat bottom plate. Cells were harvested and analyzed on day 6 for proliferation analysis. B cells were stained with anti-CD19-APC/Cy7, and 7-AAD. Supernatants were collected 12 days after stimulation to measure secreted IgE (ELISA), IgA, IgM, IgG1, IgG2, IgG3 and IgG4 (Bio-Plex Pro™ Isotyping Panel on a Bioplex-200 system, Biorad, Hercules, CA). Results were compared to eleven healthy individuals (8 females and 3 males, age range 27-49 years; median 32 years) without signs of STAT3-HIES, a negative history for allergic symptoms, and serum IgE within the normal range below 120 IU/ml (range 0.6 to 111 IU/ml) (Table S1).

Flow cytometric analysis of B-lymphocytes in PBMC

Immunophenotyping was performed on thawed PBMCs from STAT3-HIES patients and healthy individuals. The antibodies used for staining are listed in Table S3. 7-AAD (BD Biosciences, San Jose, CA) and Zombie yellow (BioLegend, San Diego, CA) were used for dead cell exclusion. Samples were measured by multi-color flow cytometry (FACS ARIA III, BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using FlowJo v10.0.7 (Tree Star, Ashland, OR). Since the number of IgE⁺ B cells was very low we performed gating on several B cell subsets to illustrate the specificity of the staining (Fig S4). IgM⁻IgD⁻IgG⁻IgA⁻IgM⁺ had the highest frequency of IgE⁺ B cells while IgM⁻IgD⁻IgA⁺ and IgM⁻IgD⁻IgG⁺ class-switched B cells had no detectable IgE⁺ B cells. IgM⁺IgD⁺ naive B cells showed some IgE⁺ B cells, confirming previous reports that naive B cells can bind IgE through CD23 (1).

Molecular analysis of IgG, IgA and IgE transcripts

Complete immunoglobulin heavy chain (IGH) V-D-J gene rearrangements were amplified as described before (2) with L-VH forward primers (3) or IGHV-FR1 forward primers (4) in a 1-step PCR with an IGHG or an IGHA consensus reverse primer (3, 5, 6). *IGHE* transcripts were amplified in a semi-nested multiplex PCR approach using the 4 L-VH-family forward primers in combination with an outer (first PCR) and an inner (second PCR) *IGHE*-specific reverse primer (1). PCR products were cloned into the pGEM-T easy

vector (Promega, Fitchburg, WI) and single clones were prepared for sequencing on an ABI Prism 3130XL (Applied Biosystems, Foster City, CA). Obtained sequences were analyzed using the IMGT database (http://www.imgt.org/IMGT_vquest/vquest) to assign the IGHV, IGHD and IGHJ genes and alleles, and to identify somatic hypermutations (SHM) (7, 8). Of each unique clone, the position and frequency of mutations were determined within the IGHV gene (CDR1-FR3). SHM was determined as variations on the best matched V-gene and represented as the percentage of mutations of the total sequenced V-gene nucleotides. The IgA and IgG receptor subclasses were determined using the IGH reference sequence (NG_001019). Data from IgA and IgG transcripts of controls have been published before (2, 9). Additional IgE and IgA transcripts from controls were determined as part of a healthy control study (HREC 2016-0289 Monash University).

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