

Short Communication

Somatic mosaicism in B cells of a patient with autosomal dominant hyper IgE syndrome

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Hyper IgE syndrome (HIES) is characterized by recurrent skin abscesses, eczema, pneumonia, and high levels of serum IgE. Nonimmunologic manifestations of HIES include a characteristic face, pathologic dentition, scoliosis, bone alterations, hyperextensible joints, and vascular abnormalities. Somatic mosaicism is defined by the presence of two or more populations of cells with different genotypes in one individual. In this report, we describe one patient with classical HIES and another patient with a mild phenotype, both harboring the same genetic mutation. The patient with a mild phenotype did not present the characteristic face, had normal production of IL-17A by T CD4⁺ cells, but had low phosphorylation of STAT-3 in B cells. Interestingly, the mutation found in B cells was absent in other cell types analyzed, in agreement with the presence of a somatic mosaic genotype. The clinical and functional differences observed between these patients justify the use of complementary tools for a better definition of the cases. These approaches allow for a better understanding of complex phenotypes associated with somatic mosaicism, and present the possibility to analyze the role of B lymphocytes in the pathophysiology of this disease. This knowledge has an impact on not only the treatment but also the provision of appropriate genetic counseling.

Keywords: Hyper IgE syndrome · IL-17A · Somatic mosaic · STAT3 phosphorylation



Additional supporting information may be found in the online version of this article at the publisher's web-site

Introduction

Hyper IgE syndrome (HIES; OMIM #147060) is classified as one of a group of combined immunodeficiencies with associated or syndromic features [1]. HIES is characterized by recurrent skin abscesses, eczema, pneumonia, high levels of serum IgE, and reduced Th17 differentiation. Nonimmunological manifestations

of HIES include a characteristic face, pathologic dentition, scoliosis, bone alterations (fractures with minor trauma), hyperextensible joints, and vascular abnormalities [2–5]. The disease can be transmitted as an autosomal dominant trait [6], with full penetrance and variable expressivity [7]. Somatic mosaicism is defined by the occurrence of two or more cell lines with different genotypes in the same individual.

Additionally, there have been reports of HIES in somatic mosaics that show mild clinical manifestations associated with signal transducer and activator of transcription 3 (STAT3 MIM #102582) [8, 9]. In contrast to patients with somatic mosaicism,

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patients with germ line mutations in STAT3 show full disease penetrance [2, 10].

Here, we describe a patient with classical HIES, and a patient with a mild phenotype with normal production of IL-17A, but low phosphorylation of STAT3 on Tyr705 and Ser727 after IL-6 or IL-21 stimulation. The electropherograms for genotypic characterization showed no proportional peaks, suggesting the possibility of a mosaic. We then decided to sequence different tissues for the presence or absence of the mutant *STAT3* allele. We discovered the presence of a mutant allele in B cells with the absence of this allele in other tissues; this was in agreement with a somatic mosaic associated with STAT3. The clinical and functional differences observed between patients justify the use of complementary tools to analyze the possibility of somatic mosaicism in patients with a mild phenotype or with atypical features.

Results and discussion

Case reports

We present two patients carrying the same mutation (g.66083, Tyr657Cys) in *STAT3*. The patient with classical HIES was a 22-year-old female, with high concentrations of IgE range (8517–63300 IU/mL), abscesses, pneumatocoeles, a high eosinophil count range (440–2572 cell/ μ L), severe eczema, oral candidiasis, scoliosis, high palate, and characteristic face, with an 85-point score in the NIH scoring system. The patient with a mild phenotype (HIES mosaic) was a 23-year-old male, having a history of upper airway infections during childhood, with scaly pseudoalopecic plaques and abscesses on the scalp and axillae. The patient had a 47-point NIH score (see Supporting Information Table 6).

Apart from that, the HIES mosaic patient showed plaques of erythema with scales and pustules in the nostrils, anterior thorax, gluteal, and inguinal folds. The HIES mosaic patient had cold abscesses in several body parts such as the scalp, frontal region, and limbs. He was treated with antibiotics and antifungals, and on some occasions, surgical drainage was performed. The patient also had high concentrations of IgE range (552–4988 IU/mL), normal eosinophil count range (58–700 cell/ μ L) see Supporting Information Tables 4 and 5, but low compared to the classical HIES patient; in addition, *Staphylococcus aureus* was isolated several times. Nonimmunological features such as scoliosis, high palate, and hyperextensibility were also present.

Upon physical examination the HIES mosaic patient did not have the characteristic face. During the first physical examination, he presented polymorphic dermatosis disseminated to the scalp, antecubital folds on the arms, and popliteal fossae on the lower limbs, characterized by lichenification and plaques with large scales on the surface. At the latest examination, flat scars with unusual shapes on the scalp, axillae, and intergluteal cleft were also documented.

Finally, the HIES mosaic patient was evaluated by the nephrology, neurology and cardiology departments, who ruled out any other illness that may have been present. The ophthalmology

department diagnosed phlyctenular conjunctivitis; thus, a treatment of prednisolone drops was initiated and recovery was achieved.

B cells carry the mutant allele in HIES mosaic patient

A heterozygous mutation in exon 21 (g.66083, Tyr657Cys) in *STAT3* was found in peripheral blood mononuclear cells (PBMCs) of both patients. In the patient with mild phenotype, the electropherograms indicated a double peak in both forward and reverse sequences, but not in the expected proportion; these observations were replicated using two different high fidelity polymerases (Fig. 1A). Together with this evidence and a mild phenotype, a somatic mosaic was suspected.

To corroborate this hypothesis, and because of the continuous history of skin pathology, we sequenced samples of the patient's skin, hair follicles, and mouthwash to determine the presence of the mutant allele; however, we did not find any change in genomic DNA. To identify the lineage that carried the mutant allele in PBMCs, we isolated B cells, PMN, CD4⁺, and CD8⁺ T cells, and monocytes. We did not find any change in genomic DNA, except in B cells, where a double peak showing the two alleles appeared again. We used an SNP at c.1233 + 44 C > G to demonstrate how the heterozygous genotype was maintained in different tissues, and to rule out any technical error due the method employed (Fig. 1B).

The phosphorylation of STAT3 is reduced and that of STAT1 increased in B cells

To test the signaling through STAT3, PBMCs were stimulated with rhIL-6 or rhIL-21 for 15 min. The phosphorylation of STAT3 (pY705, pS727) was reduced in the HIES mosaic patient compared with that of the control. This result is in agreement with that of other patients who carried mutations in the SH2 domain (Fig. 2A). The HIES mosaic patient also presented increased phosphorylation of STAT1 on B cells after stimulation with rhIL-21 and IFN gamma for 15 min (see Supporting Information Fig. 2B).

The memory B-cell component is reduced and naïve B cells are increased in HIES mosaic patient

To analyze the memory B-cell component, PBMCs were stained with CD19, CD27, and IgD. We found that in the HIES mosaic patient, 80% were naïve B cells (CD19⁺ CD27[−] IgD⁺) and approximately 6% of class-switched memory B cells (CD19⁺ CD27⁺ IgD[−]), in agreement with Avery et al. [11] (see Supporting Information Fig. 1A and B, Table 3).

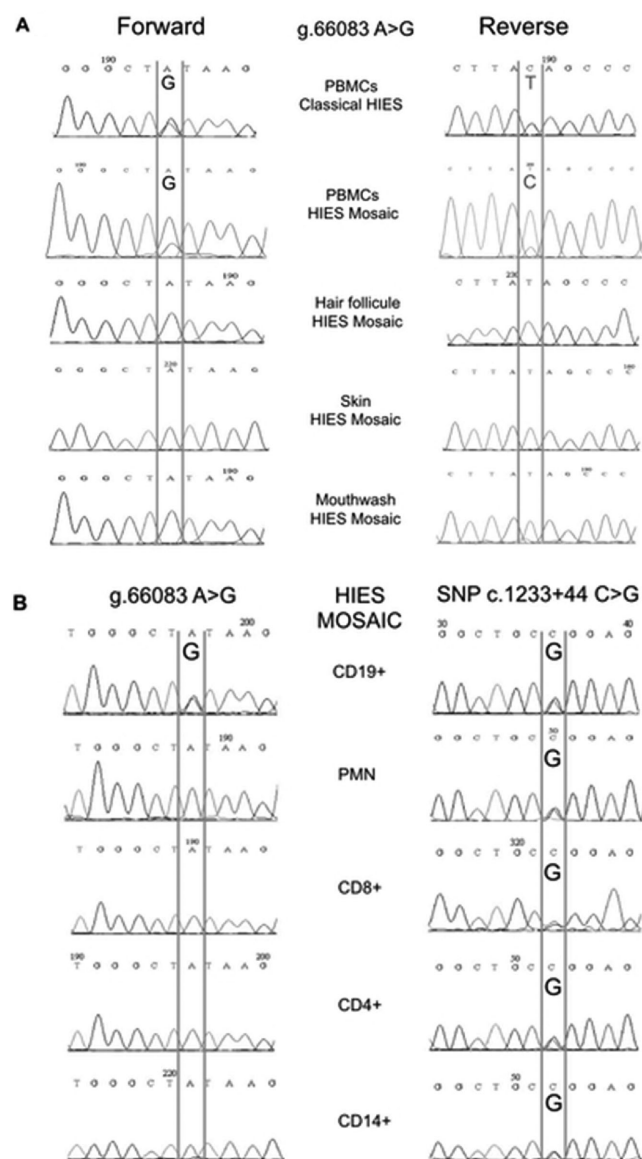


Figure 1. Electropherograms of Exon 21 of STAT3. Genomic DNA was extracted from PBMCs; next, Sanger DNA sequencing was performed two times for each tissue. (A) Forward and reverse electropherograms of a patient with classical hyper IgE syndrome and a HIES patient with somatic mosaicism (g.66083A > G) in different tissues. (B) Electropherograms of HIES mosaic patient (g.66083A > G) and SNP 1233+44 C > G as a control.

Normal production of IL-17A by Th17 cells

To analyze the production of interleukin 17A (IL-17A) and IFN- γ , PBMCs were stimulated with phorbol-12-myristate-13-acetate (PMA)/ionomycin in the presence of Brefeldin A for 6 h. The HIES mosaic patient showed normal production of IL-17A. In contrast, the patient with classical HIES consistently showed low production of IL-17A (Fig. 2B).

Since the first report by Davis et al. [12] immunological and nonimmunological features have been added to the original description of HIES. In addition to very specific features of HIES,

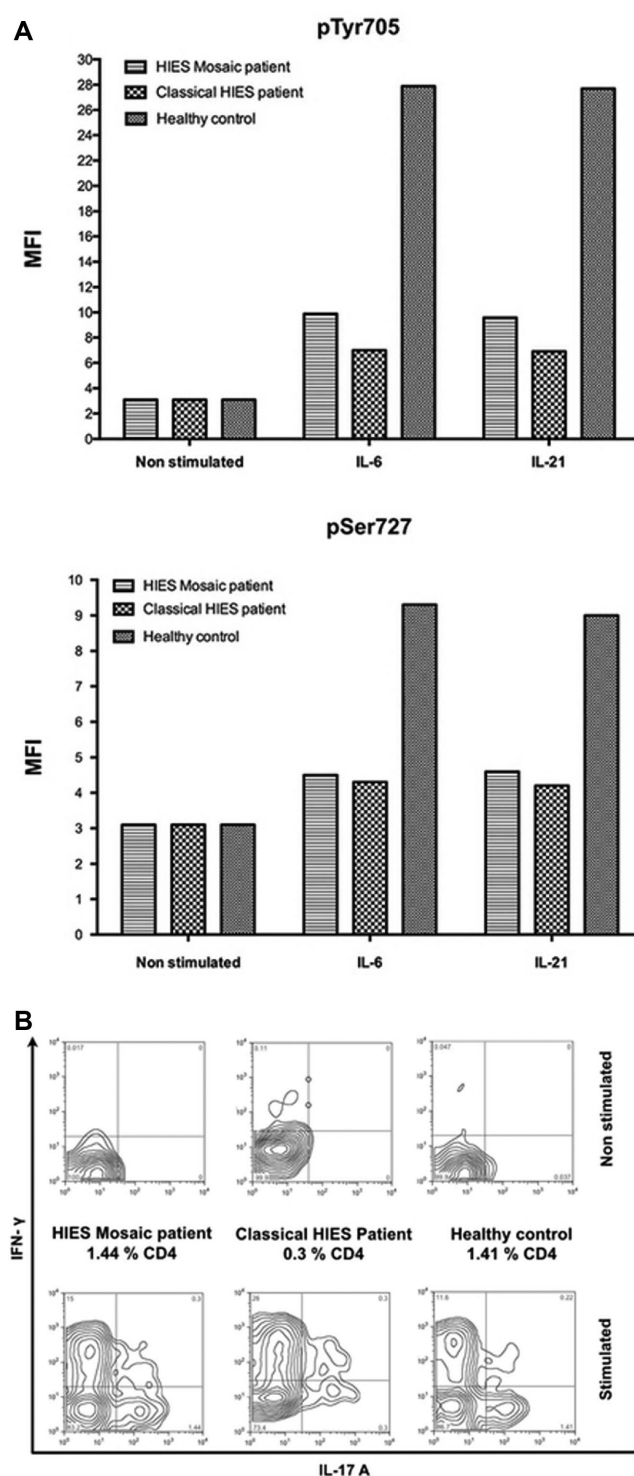


Figure 2. Phosphorylation and production of IL-17A. (A) PBMCs were stimulated with rhIL-6 or rhIL-21. Phosphorylation of STAT3 was determined by flow cytometry. Representative phosphorylation of STAT3 on Tyr705 and Ser727 in the HIES mosaic patient. Each bar represents the mean fluorescence intensity (MFI) of CD19⁺ B cells. (B) PBMCs were stimulated with PMA/ionomycin in the presence of Brefeldin A. Representative FACS analysis of production of IL-17A and IFN- γ by untreated and stimulated cells from a HIES mosaic patient, a patient with classical HIES, and a healthy control, observed as total percentage of CD4⁺ cells. Data from both figures are from two independent experiments.

other authors have proposed additional criteria, including the percentages of Th-17 cells or the phosphorylation status of STAT-3 to complement the study of HIES patients [13, 14].

Our patient with classical HIES carried a constitutive mutation that confers the full phenotype, including the characteristic face. The HIES mosaic patient did not present the characteristic face. During his lifetime, this patient has been diagnosed with neutropenia, oral candidiasis, phlyctenular conjunctivitis, and cold abscess, the latter two caused by *S. aureus*. A cold abscess has been described as a very specific symptom of the disease [13]. The susceptibility of patients to experience recurrent mucocutaneous infections is partially due to Th-17 cell differentiation. Cytokines IL-17A, IL-17F, and IL-22 exert their functions on keratinocytes, which are responsible for the production of antimicrobial peptides such as beta-defensin 2 and 3, and histatins, which directly affect the immunological response of the epithelium [4, 15–17].

Our HIES mosaic patient did not have mutations in T cells; in agreement with this observation, differentiation and production of ILs by T cells were normal. However, the patient had neutropenia and still presented with cold abscess and occasionally mucocutaneous infections (see Supporting Information Tables 1 and 2).

These observations suggest that there are other important mechanisms involving in the pathogenesis of this disease. Similar observations have been reported in other patients with somatic mosaicism [8, 9].

The patient carried a mutant allele on exon 21 of *STAT3* (g.66083, Tyr657Cys) exclusively in B cells. This residue has been previously reported to be hot spot for mutation and is found in the SH2 domain. Without further evidence, we could only speculate that the mutation occurred after differentiation from a common lymphoid progenitor, because NK and T cells were not affected.

The phosphorylation of Tyr705 and Ser727, through stimulation with IL-6 and IL-21, was low in B cells compared to healthy controls. *STAT3* facilitates the differentiation of B and memory cells through IL-21. Our HIES mosaic patient had a substantial amount of naïve B cells with low numbers of class-switched memory B cells. In addition, there is a threshold of *STAT3* activation depending on whether naïve or memory B cells are activated. Naïve B cells require a higher threshold of stimulation compared to memory B cells [18]. Patients with HIES do not present defects in isotype switching or somatic hypermutation, but fail to generate long-lived antibody responses. According to this, our HIES mosaic patient showed poor proliferation and reduced differentiation of plasmablast cells (see Supporting Information Figs. 1B and 3, respectively). The reduction in IgA secretion and memory B cell generation, together with an enhanced proinflammatory environment driven by high levels of IgE in the skin, could contribute to infection susceptibility through an impaired response to pathogens such as *S. aureus* and *Candida* spp. [11].

We cannot discount the possibility of mutations present in other tissues that could explain some of the nonimmunological features found in the patient with a somatic mosaic genotype; however, we did not obtain authorization for further studies in this patient.

Concluding remarks

We present a patient with somatic mosaicism carrying a mutant allele on *STAT3*, functional defects in B cells, and a mild phenotype. Good clinical judgment cannot be replaced by laboratory tests; however, complementary tools are very useful to characterize patients with a mild phenotype or unusual characteristics. These approaches allow for better understanding of complex phenotypes such as somatic mosaicism, and they facilitate analysis of the role of B lymphocytes in the pathophysiology of this disease. This knowledge influences not only the patient treatment but also the appropriate genetic counseling.

Materials and methods

Patients and sample collection

Patients were diagnosed with HIES at the Hospital of High Specialties (Monterrey, Nuevo León México). Healthy individuals, age and gender matched, were included as controls. The research protocol was approved by the ethics committees of the National Institute of Pediatrics and CINVESTAV. Blood samples were collected in either EDTA-containing tubes (BD Vacutainer Franklin Lakes, NJ, USA), from which DNA was isolated and sequenced for genotype analysis, and heparinized tubes (BD Vacutainer Franklin Lakes, NJ, USA), from which PBMCs were isolated for *STAT3* sequencing studies.

DNA sequencing

Total RNA was extracted from PBMCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA was amplified by polymerase chain reaction (PCR; see Supporting Information). Genomic DNA was extracted from PBMCs of patients and healthy controls using DNAzol (Invitrogen) according to the manufacturer's instructions. Subsequently, *STAT3* was sequenced using the BigDye Terminator v3.1 Sanger sequencing kit and the ABI Prism 3100 instrument (Applied Biosystems, Foster City, CA, USA). The resulting sequences were analyzed using BioEdit Sequence Alignment Editor 7.2.5 software (Ibis Biosciences, Carlsbad, CA, USA). Genomic DNA sequences were used to corroborate mutations.

PBMC isolation

PBMCs were isolated from whole blood using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation. PMNs were isolated by Percoll (Sigma-Aldrich). The different components of PBMCs were isolated by magnetic separation using anti

CD14, CD4, and IgM microbeads according to the manufacturer's instructions.

Flow cytometry

To evaluate IL-6 and IL-21 signaling pathways, PBMCs from patients and healthy controls were incubated in 1 mL AIMV medium (Life Technologies, Carlsbad, CA, USA). Cells (1×10^6) were incubated at 37°C with 5% CO₂ for 2 h. They were then stimulated with 100 ng of recombinant IL-6 or IL-21 (Miltenyi Biotec, Auburn, CA, USA), and incubated at 37°C with 5 % CO₂ for 20 min. Next, cells were fixed with 100 µL of 16% paraformaldehyde (PFA) for 10 min. The pellets were resuspended, and permeabilized with cold methanol followed incubation at 4°C for 20 min. Cells were then washed with 3 mL of PBA. Cell staining was performed by incubation with the following antibodies: 5 µL anti-CD19 APC, 7 µL anti-STAT3 Alexa Fluor 488 (pS727), 5 µL mouse IgG1 (isotype control) (BD Pharmingen, San Jose, CA, USA), and 3 µL anti-STAT3 PE (pY705) (Santa Cruz Biotechnology, Dallas, TX, USA). Finally, cells were washed twice with 3 mL of PBA, and fixed with 300 µL of 4% PFA.

Intracellular staining for IL-17A

PBMCs (1×10^6) from patients and healthy controls were resuspended in 1 mL of RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Biowest LLC, Kansas City, MO, USA) and penicillin/streptomycin (50 U/mL/50 µg/mL) (In Vitro S.A., Mexico DF, Mexico), and cultured at 37°C with 5% CO₂ for 6 h. PBMCs were then stimulated with 10 ng/mL PMA and 1 mg/mL ionomycin (Sigma-Aldrich) in the presence of 1 mg/mL Brefeldin A (Sigma-Aldrich) at 37°C with 5% CO₂ for 6 h. Subsequently, PBMCs were incubated with 4 µL of anti-CD3 Pacific Blue and 4 µL of anti-CD4 APC-Cy7 antibodies in the dark for 30 min at room temperature. The cells were then fixed with 300 µL of 4% PFA. For intracellular staining, PBMCs were incubated with Permeabilizing Solution 2 (BD Biosciences) for 15 min, and cells were washed twice with 3 mL of PBA and stained with 5 µL of anti-IL-17 Alexa Fluor 647 and 5 µL of anti-IFNγ PE antibodies (BD Pharmingen and BD Biosciences, respectively). Finally, the cells were washed twice with 3 mL PBA, and fixed with 300 µL of 4% PFA. Flow cytometry was performed using a CyAn ADP cytometer (Beckman Coulter, Indianapolis, IN, USA). The files were analyzed using FlowJo 8.8 software (Tree Star, Asland, OR, USA).

Acknowledgments: The authors acknowledge support from Consejo Nacional de Ciencia y Tecnología CONACyT (Salud 2013-01-20211). J.C.A.M. received a PhD scholarship from CONACyT (233351); he received additional support from CONACyT for a

short scientific visit to NIH. The authors thank Guadalupe Aguilar and Hector Romero-Ramirez for their technical support.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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- Abbreviations:** AD: autosomal dominant · HIES: hyper IgE syndrome · PFA: paraformaldehyde
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- Received: 28/12/2015
 Revised: 16/7/2016
 Accepted: 1/8/2016
 Accepted article online: 4/8/2016

Somatic mosaicism in B cells of a patient with autosomal dominant hyper IgE syndrome

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Review Timeline:	Submission date:	28-Dec-2015
	First Editorial decision:	29-Jan-2016
	First revision received:	09-May-2016
	Second Editorial decision:	02-Jun-2016
	Second revision received:	22-Jun-2016
	Third editorial decision	04-Jul-2016
	Third revision received:	16-Jul-2016
	Accepted:	01-Aug-2016

Handling Executive Committee member: Prof. Hans-Martin Jack

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

29-Jan-2016

Dear Dr. Santos-Argumedo,

Manuscript ID eji.201546275 entitled "Somatic mosaicism in B cells of a patient with autosomal dominant hyper IgE syndrome" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will

not make the requested changes.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Prof. Hans-Martin Jack

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Reviewer: 1

Comments to the Author

The paper by Alcantara-Montiel et al reports a patient with somatic mosaicism for a AD-HIES causing mutation in STAT3 that seems to be restricted to the B cell compartment. While this is potentially interesting finding that could provide new insight into the relevant role of STAT3 in different cell types I have some concerns and feel that additional work is required to clarify.

Similar to what was seen in the two previously described mosaic individuals (Hsu et al 2013) this individual suffered from recurrent abscesses associated with *S. aureus*, candidiasis and respiratory tract infections, and displayed high IgE concentrations. They also displayed non-immunological features such as scoliosis, high palate and hyperextensibility. However, in contrast to what is reported here in these previously described individuals the STAT3 mutation could also be detected in non-B cells e.g. T cells, NK cells, eosinophils. While the sanger sequencing shown in Figure 1 seems to clearly show that the mutation is only in the B cells, given the phenotype of the patient it is hard to imagine how this would be so. First, as the authors themselves point out the non-immunological phenotype suggests there must be mutations elsewhere. Second, it is difficult to explain how the candidiasis and staph infections could arise from a deficiency of STAT3 in B cells alone. Moreover, it is hard to imagine a scenario whereby the STAT3 mutation would arise only in B cells given a common lymphoid progenitor gives rise to both T and B cells.

Given this unusual genotype of the mosaic patient a much more detailed analysis is warranted.

- Given the skin phenotype of the patient it might be expected that there were patches of skin that also carried the mutation – for the sequencing shown in fig 1 were the skin taken from patches of affected or unaffected skin?
- What are the numbers of CD4+, CD8+ and CD19+ cells in PBMC? What are the percentages of naïve and memory cells in these populations? This is particularly relevant as it has previously been shown that memory T cells in these mosaic individuals are enriched for cells with a non-mutated phenotype (Siegel et al 2011), thus a high number of memory T cells could obscure the ability to detect mutant cells that may be present within the naïve T cell population. Could naïve T cells be sorted and also sequenced? Further, it is of interest to determine how the STAT3 mutation in the B cells affects memory cell development in a mosaic environment.
- What are serum levels besides of Igs besides IgE? Were the specific antibody titres (e.g. against tetanus, diphtheria etc) measured?
- It would be helpful to include things such as eosinophil counts and IgE levels for the both the classical patient and the mosaic patients.
- Did the mosaic patient have retention of primary teeth?

- Further controls for the STAT3 phosphorylation should also be included – what was the phosphorylation of STAT3 like in non-B cells? Was STAT1 phosphorylation observed in B cells from both patients?
- Use of additional normal controls for cellular assays would also strengthen the data.
- Some of the English could be reworked slightly to improve clarity

Reviewer: 2

Comments to the Author

The authors present two patients with STAT3 mutations- one with classic AD-HIES with heterozygous STAT3 mutation. The other has a STAT3 mutation that appears to be isolated to B cells, but has many of the features. The authors present convincing data that this is somatic, and as expected with B cells and not T cells affected, the Th17 cell differentiation is preserved. What is harder to understand is why the patient has so many of the clinical features- including non-immunologic features but also those that would be expected with T cell involvement like candidiasis. The authors admit this and state that not all tissue types were investigated, which is understandable, but still hard to understand this, especially as if T cells are normal, the mutation occurred in a lineage that likely wouldn't involve some of the somatic features. Would add more speculation if possible.

- I think it would be interesting to include the memory/naive T/B cell populations if possible to see if the memory/naive components are affected by the STAT3 mutated B cells. In addition would add whether specific antibodies are present.

-Adding whether the somatic patient had allergies would be interesting- in general, the lack of anaphylaxis in AD-HIES is thought to be due, in part, to mast cell dysfunction. Even if testing the mast cell degranulation with morphine is possible, that would be interesting. But understand this may not be easy to do!

-For the case presentation, was a little confusing as to which patient had which features- would consider a table of the features included in the scoring system and which features present/absent. Also, the skin rash for the mosaic patient sounded atypical, was that biopsied? Maybe include a picture?

In general I found this patient to be interesting, but hard to sort out exactly what it means!

First Revision – authors' response

09-May-2016

Reviewer: 1

Comments to the Author

Similar to what was seen in the two previously described mosaic individuals (Hsu et al 2013) this individual suffered from recurrent abscesses associated with *S. aureus*, candidiasis and respiratory tract infections, and displayed high IgE concentrations. They also displayed non-immunological features such as scoliosis, high palate and hyperextensibility. However, in contrast to what is reported here in these previously described individuals the STAT3 mutation could also be detected in non-B cells e.g. T cells, NK cells, eosinophils. While the sanger sequencing shown in Figure 1 seems to clearly show that the mutation is only in the B cells, given the phenotype of the patient it is hard to imagine how this would be so.

First, as the authors themselves point out the non-immunological phenotype suggests there must be mutations elsewhere. Second, it is difficult to explain how the candidiasis and staph infections could arise from a deficiency of STAT3 in B cells alone. Moreover, it is hard to imagine a scenario whereby the STAT3 mutation would arise only in B cells given a common lymphoid progenitor gives rise to both T and B cells. Given this unusual genotype of the mosaic patient a much more detailed analysis is warranted.

Response

Thank you for your observations; we did not investigate many tissues. First, we were authorized by the ethics committee to analyze only the tissues stated; second, the patient was very reluctant to provide more biological specimens. Without further evidence, we can only speculate that the mutation potentially occurs after differentiation of the common lymphoid progenitor, because T and NK cells were not affected. We provide further evidence of defects, through mutation in STAT3, in the B cell compartment (see supporting information figures S1, S2A, S2B, S3). We strongly believe that other cell lineages might also have the mutation that contributes to these clinical features. At the time of this second analysis, the patient presented neutropenia, which contributes to the pathogenesis by *S. aureus* and *Candida* spp. The patient abandoned prophylactic antibiotic treatment and the cold abscesses in the skin reappeared.

Comment

- Given the skin phenotype of the patient it might be expected that there were patches of skin that also carried the mutation – for the sequencing shown in fig 1 were the skin taken from patches of affected or unaffected skin?

Response

We believe that some patches of skin also carried the mutation. For this reason, we took a biopsy of the skin in a zone previously affected by a cold abscess to identify the mutation; however, the scar could have arisen from healthy skin. Alternatively, the cells carrying the mutation could have been poorly represented in the biopsy, and for this reason, we failed to identify the mutation there. The main reason that we analyzed only one spot from the skin was that the patient did not approve of a second biopsy for this purpose. We are adding two pictures from the patient showing the scars of the cold abscess.

Comment

- What are the numbers of CD4+, CD8+ and CD19+ cells in PBMC? What are the percentages of naïve and memory cells in these populations? This is particularly relevant as it has previously been shown that memory T cells in these mosaic individuals are enriched for cells with a non-mutated phenotype (Siegel et al 2011), thus a high number of memory T cells could obscure the ability to detect mutant cells that may be present within the naïve T cell population. Could naïve T cells be sorted and also sequenced? Further, it is of interest to determine how the STAT3 mutation in the B cells affects memory cell development in a mosaic environment.

Response

To answer these questions, we analyzed memory T and B cells in two blood samples from the patient and compared them to our control group (matched by gender and age). We identified differences in the CD4+ CD45RO+ memory T cell population. In the B cell compartment, we found that the patient had 83 % un-switched or naïve IgD+ B cells, in agreement with the report of Avery, Deenick et al. 2010). In that study, an increased number of naïve B cells with low numbers of memory CD27+ switched B cells were found. These findings substantiated the importance of STAT3 in B cell development, specifically in the differentiation to memory B and plasma cells. Because we had collected only a limited quantity of blood, we could not sort naïve T cells; we also did not observe any increase in the numbers or proportion of naïve or memory T cells to justify sequencing the naïve compartment (see supporting information figure S1A, B).

The numbers and percentages of different populations can be seen in supplementary tables 1-3. The technical details, the antibodies used and the FACS graphs are included separately in supplementary methods.

Comment

- What are serum levels besides of Igs besides IgE? Were the specific antibody titers (e.g. against tetanus, diphtheria etc) measured?

Response

In the hospital, where this patient was treated, there are no records of antibody titers. We measured IgG titers against tetanus and diphtheria toxoids. The patient had very low titers against tetanus < 0.1 UI/mL versus 1 UI/mL for the control (OD 0.1 versus control OD 0.75). We could not detect antibody titers against diphtheria in the patient (OD 0.11 versus healthy control OD 0.80). Tetanus and diphtheria vaccination is mandatory in Mexico, but without records, we cannot guarantee that the result is directly linked to the defect.

Comment

- It would be helpful to include things such as eosinophil counts and IgE levels for the both the classical patient and the mosaic patients.

Response

We sent the manuscript as a short report; therefore, we did not have space to add this data. In supporting information supplementary tables 4 and 5, we show the latest eosinophil counts and IgE levels in classical HIES patients, and the HIES mosaic patient, respectively.

Comment

- Did the mosaic patient have retention of primary teeth?

Response

No, the mosaic patient did not have primary teeth retention, compared with the classical HIES patient that had three teeth retained. We added a table with clinical features of the classical and mosaic patients see supporting information table 6.

Comment

- Further controls for the STAT3 phosphorylation should also be included – what was the phosphorylation of STAT3 like in non-B cells?.

Was STAT1 phosphorylation observed in B cells from both patients?

- Use of additional normal controls for cellular assays would also strengthen the data.

Response

In two independent blood samples, we analyzed the phosphorylation of STAT1 and STAT3. To address the first point, the phosphorylation of STAT3 was higher in CD3⁺ cells compared to that of B cells from the mosaic patient (see supporting information supplementary figure S2A, S2B).

The phosphorylation of STAT3 and STAT1 appeared to be opposite to that of B cells. To analyze STAT1 phosphorylation, the cells were stimulated for 15 min and 12 h with IFN-gamma or IL-21, as previously demonstrated by Chi-keung Wan et al. 2015. The patient had low phosphorylation of STAT3 (Y705) but the phosphorylation STAT1 appeared to be higher in B cells than in the control cells, for both stimuli. The results are included in this letter (see supporting information supplementary figure S2B).

Comment

- Some of the English could be reworked slightly to improve clarity.

Response

English was corrected and revised by a professional company.

Reviewer: 2

Comments to the Author

The authors present two patients with STAT3 mutations- one with classic AD-HIES with heterozygous STAT3 mutation. The other has a STAT3 mutation that appears to be isolated to B cells, but has many of the features. The authors present convincing data that this is somatic, and as expected with B cells and not T cells affected, the Th17 cell differentiation is preserved. What is harder to understand is why the patient has so many of the clinical features- including non-immunologic features but also those that would be expected with T cell involvement like candidiasis. The authors admit this and state that not all tissue types were investigated, which is understandable, but still hard to understand this, especially as if T cells are normal, the mutation occurred in a lineage that likely wouldn't involve some of the somatic features. Would add more speculation if possible.

Response

Thank you for your observations; as we previously remarked, we did not investigate many tissues. First, we had authorization by the ethics committee to analyze only the tissues stated. Second, the patient was very reluctant to provide more biological material. Without further evidence, we can only speculate that the mutation potentially occurred after differentiation from a common lymphoid progenitor, because T and NK cells were not affected. We provide further evidence of defects, through a mutation in STAT3, in the B cell compartment (see supporting information supplementary figures S1, S2A, S2B, S3). We strongly believe that other cell lineages might also have the mutation that contributes to these clinical features. At the time of this second analysis,

the patient presented neutropenia that contributes to pathogenesis by *S. aureus* and *Candida*. The patient abandoned the prophylactic antibiotic treatment and the cold abscesses in the skin reappeared.

Comment

-I think it would be interesting to include the memory/naive T/B cell populations if possible to see if the memory/naive components are affected by the STAT3 mutated B cells. In addition would add whether specific antibodies are present.

Response

We are now including all of this information. I kindly ask you to refer the response to reviewer # 1. We have accordingly revised see supporting information supplementary Tables 2 and 3 and Supplementary Figure S1A and B.

Comment

-Adding whether the somatic patient had allergies would be interesting- in general, the lack of anaphylaxis in AD-HIES is thought to be due, in part, to mast cell dysfunction. Even if testing the mast cell degranulation with morphine is possible, that would be interesting. But understand this may not be easy to do!

Response

We appreciate your suggestion; of course, it would be very interesting to analyze mast cell degranulation. However, unfortunately, this was not easy to perform. As we mentioned above, the patient was very reluctant to provide new samples. He finally agreed, but only for a limited set of assays; thus, we concentrated on the most pertinent questions asked by both reviewers. To the best of our knowledge, the patient has no documented allergies. Based on the study of Valeri Hox et al. (2015), we are aware that the reduction of STAT3 inhibits vascular permeability and anaphylaxis, masking the classical signs of anaphylaxis. We believe that the mutation could be present in other tissues, possibly contributing to the absence of documented allergy; however, currently we are only certain of a mutation in B cells.

Comment

-For the case presentation, was a little confusing as to which patient had which features- would consider a table of the features included in the scoring system and which features present/absent.

Response

We are adding a table with clinical features of the classical HIES and the HIES mosaic patient (see supporting information supplementary table 6).

Comment

Also, the skin rash for the mosaic patient sounded atypical, was that biopsied? Maybe include a picture?

Response

To the best of our knowledge, the patient has not been biopsied. We added two pictures of the patient's face to appreciate the skin and scars from the abscess.

The score was evaluated several years ago at the beginning of this study. We recently reevaluated the score of the mosaic patient recording a score of 39 points, whereas it was evaluated as 47 points, 4 years ago. The re-evaluation includes the lack of a clear difference in eosinophilia, the absence of a characteristic face, the nonexistence of increased nasal width 1-2

SD, and an age correction. Finally, we incorporated the new evidence in the manuscript, and referred to it in the supplementary information. We hope that the new evidence and the functional alterations documented in B cells (see supplementary figures S1, S2, S3, supplementary tables 1-6) will help to convince you of our findings for this case report, and that you might kindly recommend our work to be published in this prestigious journal.

Legends of supplementary figures

Supplementary Figure 1. Memory compartments.

The gates for memory T and B cells are from the leucocyte gate; subsequently, CD3+ or CD19+ gates were selected.

S1A) Representative dot plot of CD4+ T cells from HIES mosaic patient: (CD45RO+ memory) 56 %, (CD45RA+ Naive) 34 % in HIES mosaic patient.

S1B) Representative dot plot of B cells from HIES mosaic patient, left panel (Memory CD27+IgD-) 6.4 %; naïve B cells (CD27- IgD+) 83 %. Center panel, CD21low, 1.4 %. Right panel, transitional cells (CD24+ CD38+), 4.7 %, and plasmablast (CD38+), 0.28 %.

Supplementary Figure 2. Phosphorylation of STAT3 and STAT1.

The representative histograms from the leucocyte gate; we then selected CD19+ or CD3+ gates to show histograms from STAT3 or STAT1. Both phosphorylation assays were performed at 15 min with IL-21 and IFN- γ . In the clear gray histogram we show unstimulated cells, the dark gray histogram represents the HIES mosaic patient, and the black histogram represents healthy controls for each condition.

S2A) Representative histograms of phosphorylation of STAT3 (Y705) after stimulation of CD19+ and CD3+ cells with IL-21.

S2B) Representative histograms of phosphorylation of STAT1 (Y701) after stimulation of CD19+ and CD3+ cells with IL-21.

Supplementary Figure 3A. B cell proliferation

Representative histograms of B cell proliferation. Each gate was obtained from the leucocyte gate; we then selected CD19+ cells, and the histograms from carboxyfluorescein succinimidyl ester (CFSE) were made. The histograms in the upper rows correspond to the mosaic patient. The lower row histograms correspond to the healthy control. Each column represents one condition of stimuli. The cells were stained with CFSE and cultured for 5 days in RPMI medium with or without stimuli; next the cells were harvested, stained with antibodies, and CFSE was analyzed.

Second Editorial decision:

02-Jun-2016

Dear Dr. Santos-Argumedo,

Thank you for submitting your revised manuscript ID ej.201546275.R1 entitled "Somatic

mosaicism in B cells of a patient with autosomal dominant hyper IgE syndrome" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

Unfortunately, the referees were not satisfied with the revisions made and further major revision is requested. You will see that both referees feel that there are still inconsistencies in the data presentation, which make proper interpretation difficult. Furthermore, referee 2 feels that some better explanation, at the least, is necessary. The Executive Editor tends to agree and we therefore ask you to fully address the concerns of both referees in this final round of revisions.

The journal does not encourage multiple rounds of revision and you should fully address the concerns of the referee in this final round of revision. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Prof. Hans-Martin Jack

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

Reviewer: 1

Comments to the Author

The authors have now addressed my concerns and the paper is now acceptable for publication pending a few minor corrections.

Minor points

- The change in line 44 of the introduction to “as a” has introduced an error. I think this should read “as a combined immunodeficiency with associated syndromic features” or “as one of a group of combined immunodeficiencies with associated syndromic features” as AD-HIES is not a group of immunodeficiencies on its own.
- The text states the mosaic patient had “low eosinophil count” when in fact it was within normal ranges but low compared to the classical AD-HIES – please clarify this in the text.
- The text states “the HIES mosaic patient did not have the characteristic face” but the table says it was present.
- “The phosphorylation of STAT3 and STAT1 was reduced is increased, respectively, in B cells” – I think this would be clearer stated “The phosphorylation of STAT3 was reduced and that of STAT1 increased, in B cells” or something similar.
- Line 174 states that the mosaic cells had “poor proliferation and reduced differentiation of plasma cells (see supporting information)” but the plasma cell data is not shown in this supporting figure.
- Supplementary figure 1 – it is unclear what the two samples are, based on the supplementary tables I assume this is two different collection dates for the mosaic patient – it would be good to make this clearer in the figure legend or figure itself.
- In S2b I think the label on the left graph should read “pSTAT1 (Y701)” instead of STAT3?

Reviewer: 2

Comments to the Author

This revised manuscript has increased functional and lymphocyte phenotyping showing a paucity of memory B cells, as would be expected with STAT3 deficiency. However, I still find the causality between the phenotype seen in the patient and the mutation being restricted to B cells very confusing and keep wondering if there is some other process at work. Most confusing is the rash, which sounds very atypical for AD-HIES. I understand the limitations posed by the patient not wanting to be biopsied, but it is very hard to understand the atypical rash, some of the somatic features and a mutation really restricted to B cells- as if prior to B cells, T cells would be involved due to common progenitors. Somehow would be good to have more explanation.

Small comment as well- in the scoring, it does seem that the mosaic patient has had IgE >2000, so would adjust accordingly. Also for the tables with the eosinophil and IgE levels, I think a range in the text is likely adequate and all the dates are not necessary.

But, mainly I think it would be important to have more speculation on the phenotype and how this relates to a defect of just the B cells.

Second Revision – authors' response

22-Jun-2016

Reviewer: 1

Comments to the Author

“The authors have now addressed my concerns and the paper is now acceptable for publication pending a few minor corrections”.

Comment

Minor points

- The change in line 44 of the introduction to “as a” has introduced an error. I think this should read “as a combined immunodeficiency with associated syndromic features” or “as one of a group of combined immunodeficiencies with associated syndromic features” as AD-HIES is not a group of

immunodeficiencies on its own.

Response

Thank you for your observations, we corrected this paragraph in the main manuscript (line 44).

Comment

- The text states the mosaic patient had “low eosinophil count” when in fact it was within normal ranges but low compared to the classical AD-HIES – please clarify this in the text.

Response

The manuscript was corrected in the lines 86 and 87 as follows “normal eosinophil count range (58 - 700 cell/ μ l), but low compared to the classical HIES patient”.

Comment

- The text states “the HIES mosaic patient did not have the characteristic face” but the table says it was present.

Response

Table 6 in supporting information was corrected and now match with the main manuscript.

Comment

- “The phosphorylation of STAT3 and STAT1 was reduced is increased, respectively, in B cells” – I think this would be clearer stated “The phosphorylation of STAT3 was reduced and that of STAT1 increased, in B cells” or something similar.

Response

We corrected the subtitle in the current manuscript in the line 119. “The phosphorylation of STAT3 was reduced and that of STAT1 increased, in B cells”.

Comment

- Line 174 states that the mosaic cells had “poor proliferation and reduced differentiation of plasma cells (see supporting information)” but the plasma cell data is not shown in this supporting figure.

Response

Thanks for observation we change plasma cells by plasmablast cells. In the manuscript you can read “poor proliferation and reduced differentiation of plasmablast cells” (see supporting information figure S3 and S1B respectively). Figure S1B shows CD38+ plasmablast cells.

Comment

- Supplementary figure 1 – it is unclear what the two samples are, based on the supplementary tables I assume this is two different collection dates for the mosaic patient – it would be good to make this clearer in the figure legend or figure itself.

Response

To clarify, we added some sentences to figure legend.

Supplementary Figure 1. Memory compartments in HIES mosaic patient. The gates for memory T and B cells are from the leucocyte gate; subsequently, CD3+ or CD19+ gates were selected. Two different samples from HIES mosaic patient were collected at April 7 and April 13.

S1A) Representative dot plot of CD4+ T cells from HIES mosaic patient: (CD45RO+ memory) 56 %, (CD45RA+ Naive) 34 % in HIES mosaic patient.

S1B) Representative dot plot of B cells from HIES mosaic patient, left panel (Memory CD27+IgD-) 6.4 %; naïve B cells (CD27- IgD+) 83 %. Center panel, CD21low, 1.4 %. Right panel, transitional cells (CD24+ CD38+), 4.7 %, and plasmablast (CD38+), 0.28 %.

Comment

- In S2b I think the label on the left graph should read “pSTAT1 (Y701)” instead of STAT3?

Response

Thank you for the observation, you're absolutely right, the figure has been corrected with STAT1.

Third Editorial Decision

04-Jul-2016

Dear Dr. Santos-Argumedo,

Thank you for submitting your revised manuscript ID eji.201546275.R2 entitled "Somatic mosaicism in B

cells of a patient with autosomal dominant hyper IgE syndrome" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

You should also pay close attention to the editorial comments included below. **In particular, please convert the format of your manuscript to the format of a Short Communication, reformat your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

If the revision of the paper is expected to take more than three months, please inform the editorial office.

Once again, thank you for submitting your manuscript to European Journal of Immunology. We look forward to receiving your revision.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Hans-Martin Jack

Dr. Nadja Bakocevic
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com

Third Revision – authors' response

16-Jul-2016

Dear Dr. Nadja Bakocevic:

Attached you will find the revised version of our manuscript entitled "**Somatic mosaicism in B cells of a patient with autosomal dominant hyper IgE syndrome**" (ID eji.201546275.R2) by Julio C. Alcántara-Montiel, Tamara Staines-Boone, Gabriela López-Herrera, Laura Berrón-Ruiz, Carlos R. Borrego Montoya and myself, which we submitted to the European Journal of Immunology for its consideration.

In this revised version of the manuscript we took into account the all comments. We converted the format of the manuscript to the format of a Short Communication, we reformatted the figure legends to follow Journal standards as outlined in the editorial comments and we attended the editorial comments included in your letter from July 4, 2016. The language of the manuscript was fully reviewed by Editage (<https://www.editage.com>), a company we use very often to improve the English language from our manuscripts.

We hope you find our revised version of the manuscript ready for its publication. We would like to acknowledge your help to improve our work.

Hope to hear from you soon.

Sincerely,

Leopoldo Santos-Argumedo

Fourth Editorial Decision

18-Jul-2016

Dear Dr. Santos-Argumedo,

It is a pleasure to provisionally accept your manuscript entitled "Somatic mosaicism in B cells of a patient with autosomal dominant hyper IgE syndrome" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal

of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Hans-Martin Jack

Dr. Nadja Bakocevic
Editorial Office
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European Journal of Immunology

**Supporting Information
for**

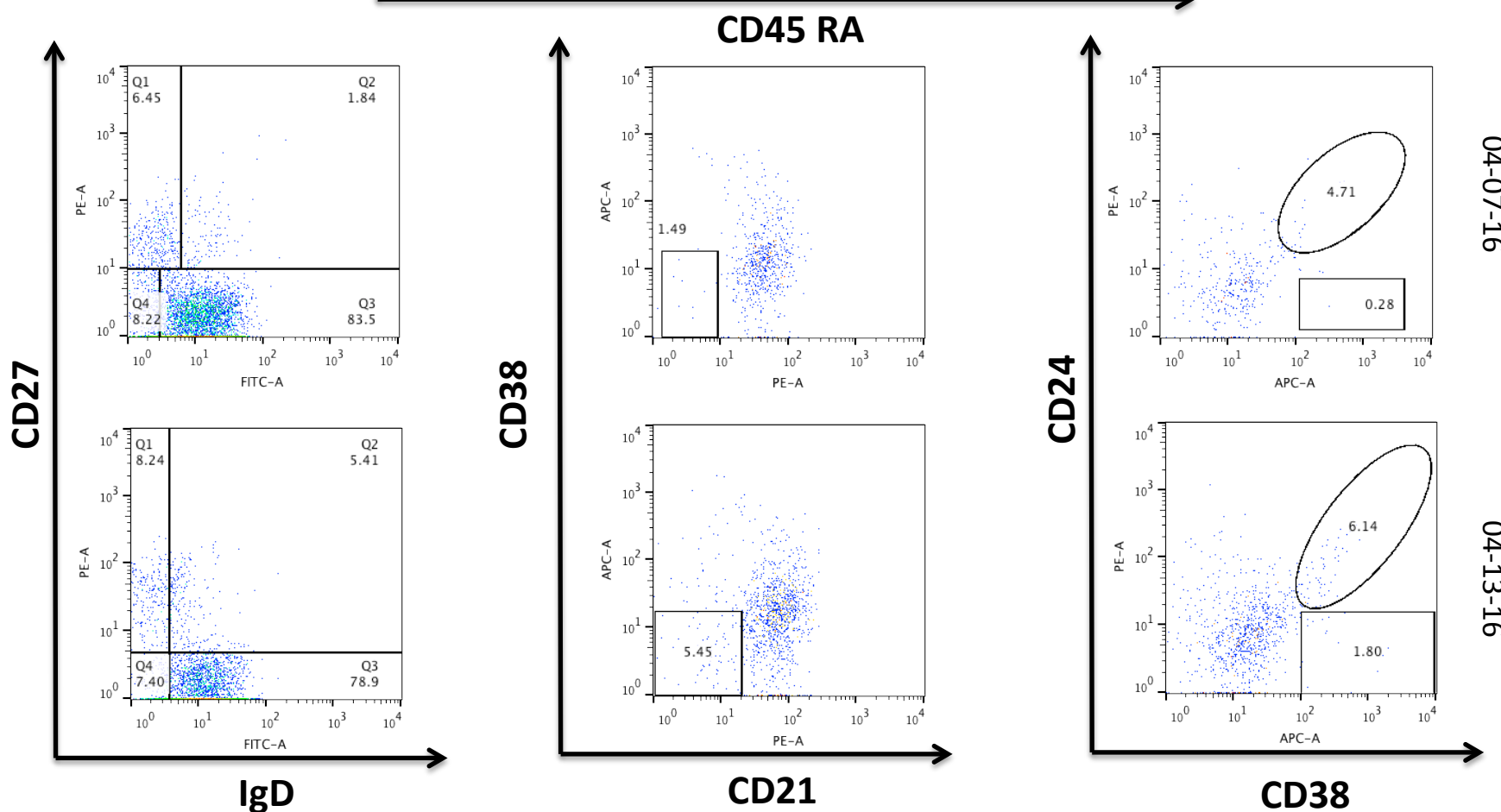
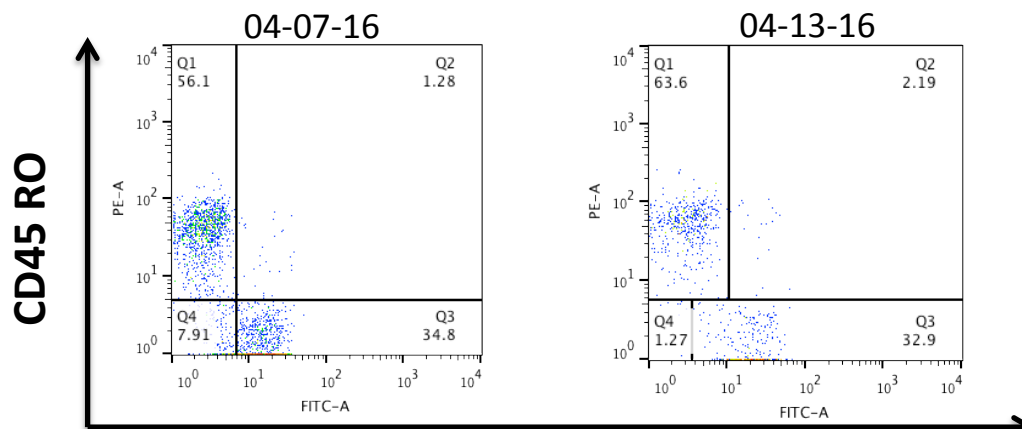
DOI 10.1002/eji.201546275

Julio C. Alcántara-Montiel, Tamara Staines-Boone, Gabriela López-Herrera,
Laura Berrón-Ruiz, Carlos R. Borrego-Montoya and Leopoldo Santos-Argumedo

**Somatic mosaicism in B cells of a patient with autosomal dominant hyper IgE
syndrome**

S1

Memory compartments in HIES mosaic patient



S1A) Memory T cells CD4+

S1B) Memory B cells CD19+

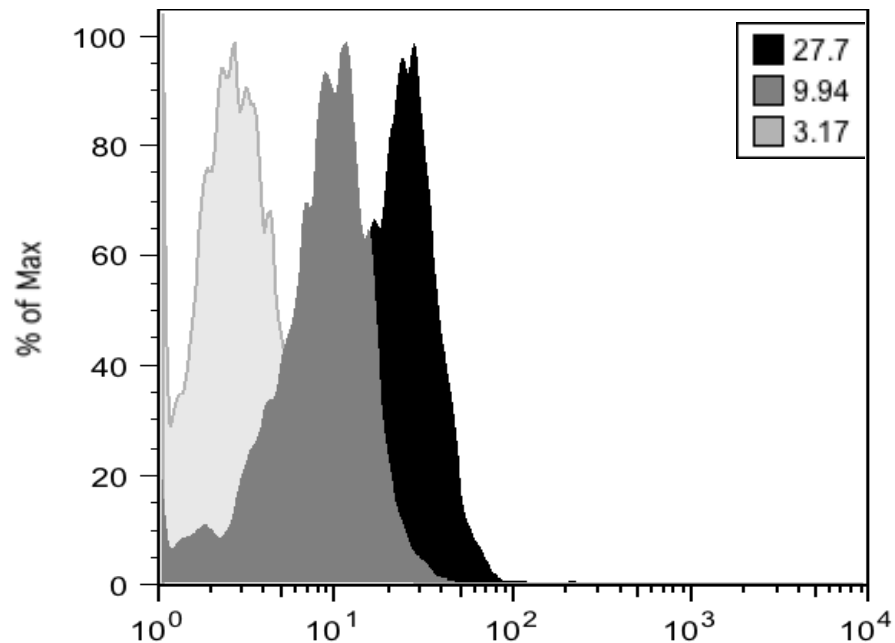
04-07-16

04-13-16

S2A)

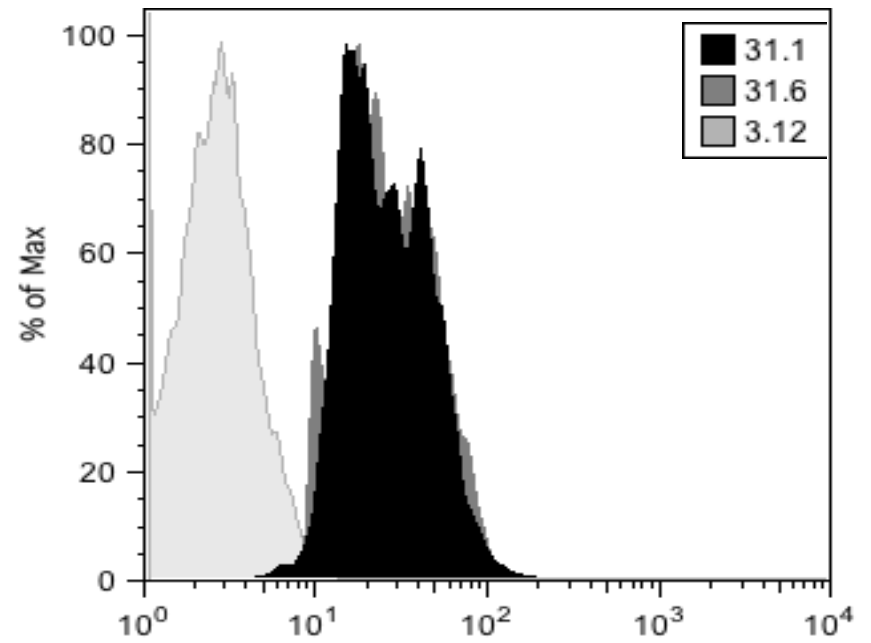
Phosphorylation of pSTAT-3 (Y705)
CD19+, CD3+ cells after 15 min of stimulation with IL-21

IL-21



pSTAT-3 (Y705)
CD19+

IL-21

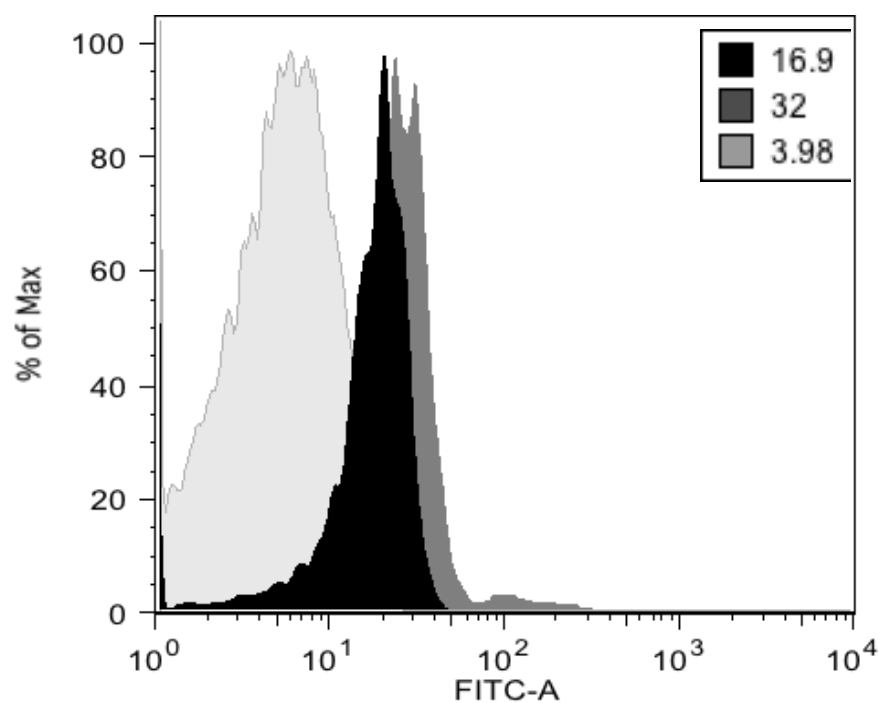


pSTAT-3 (Y705)
CD3+

S2B)

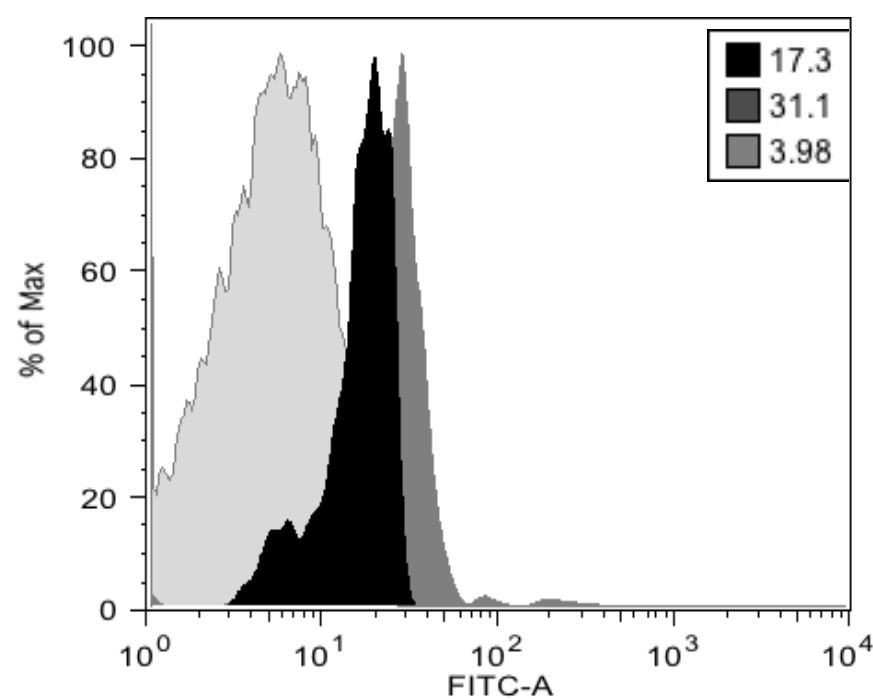
Phosphorylation of pSTAT-1 (Y701) after 15 min of stimulation with IL-21 or IFN- γ

IL-21



pSTAT-1 (Y701)
CD19+

IFN- γ

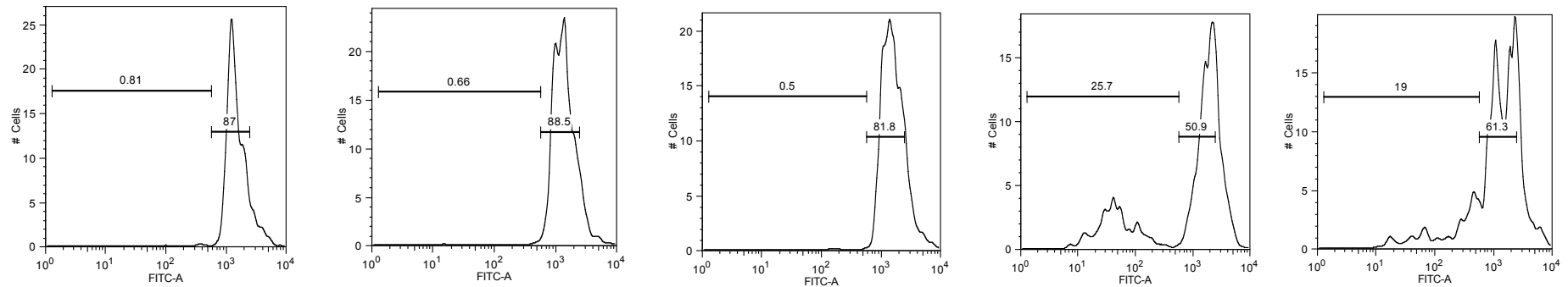


pSTAT-1 (Y701)
CD19+

S3

B cells proliferation measured by Carboxyfluorescein incorporation

HIES Mosaic patient



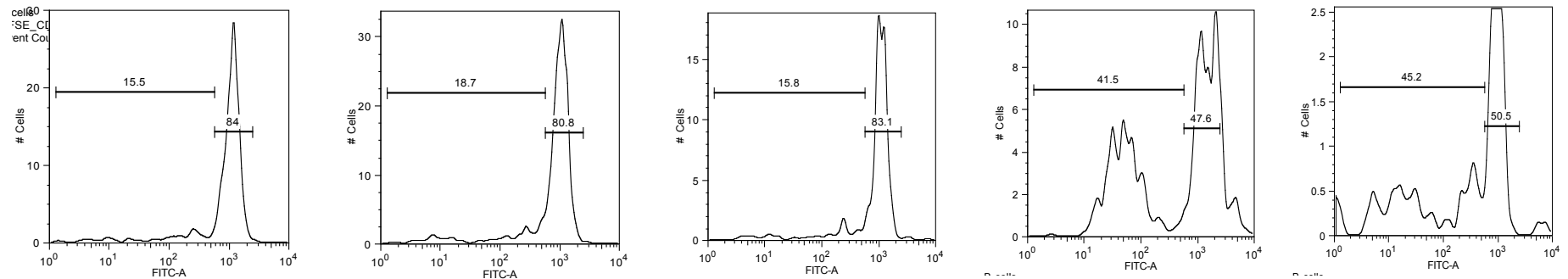
Unstimulated

IL-21
100 ng/mL

Anti-CD40 4ug

IL-21 100ng/mL
Anti-CD40 4ug/mL

CpG
PKW



Healthy control

Legends of supplementary figures

Supplementary Figure 1. Memory compartments in HIES mosaic patient.

The gates for memory T and B cells are from the leucocyte gate; subsequently, CD3⁺ or CD19⁺ gates were selected. Two different samples from HIES mosaic patient were collected at 7 and 13th day of the April.

S1A) Representative dot plot of CD4⁺ T cells from HIES mosaic patient: (CD45RO⁺ memory) 56 %, (CD45RA⁺ Naive) 34 % in HIES mosaic patient.

S1B) Representative dot plot of B cells from HIES mosaic patient, left panel (Memory CD27⁺IgD⁻) 6.4 %; naïve B cells (CD27⁻ IgD⁺) 83 %. Center panel, CD21^{low}, 1.4 %. Right panel, transitional cells (CD24⁺ CD38⁺), 4.7 %, and plasmablast (CD38⁺), 0.28 %.

Supplementary Figure 2. Phosphorylation of STAT3 and STAT1.

The representative histograms from the leucocyte gate; we then selected CD19⁺ or CD3⁺ gates to show histograms from STAT3 or STAT1. Both phosphorylation assays were performed at 15 min with IL-21 and IFN- γ . In the clear gray histogram we show unstimulated cells, the dark gray histogram represents the HIES mosaic patient, and the black histogram represents healthy controls for each condition.

S2A) Representative histograms of phosphorylation of STAT3 (Y705) after stimulation of CD19⁺ and CD3⁺ cells with IL-21.

S2B) Representative histograms of phosphorylation of STAT1 (Y701) after stimulation of CD19⁺ and CD3⁺ cells with IL-21.

Supplementary Figure 3A. B cell proliferation

Representative histograms of B cell proliferation. Each gate was obtained from the leucocyte gate; we then selected CD19⁺ cells, and the histograms from carboxyfluorescein succinimidyl ester (CFSE) were made. The histograms in the upper rows correspond to the mosaic patient. The lower row histograms correspond to the healthy control. Each column represents one condition of stimuli. The cells were stained with CFSE and cultured for 5 days in RPMI medium with or without stimuli; next the cells were harvested, stained with antibodies, and CFSE was analyzed.

Table's supplementary

Table supplementary 1.

Total and differential counts of Lymphocytes in HIES mosaic patient

HIES Mosaic patient (mm-dd-yy)	Total number of lymphocytes mm3	Neutrophils %	Neutrophils mm3	Total lymphocytes %	Total lymphocytes mm3	Monocytes %	Monocytes mm3
First determination (04-07-16)	9000	18	1620	57	5130	7	630
Second determination (04-13-16)	5585	22	1229	60	3351	8	447
Reference value in mexican population	(5000-11000)	(30 -76)	(1700-6480)	(15-49)	(990-3240)	(3-10)	(180-710)

HIES Mosaic patient (mm-dd-yy)	T Cells CD3+ %	T Cells CD3+ mm3	T Cells CD4+ %	T Cells CD4+ mm3	T Cells CD8+ %	T Cells CD8+ mm3	CD4/CD8 ratio
First determination (04-07-16)	54	2770	44	1191	35	970	1.2
Second determination (04-13-16)	62	2078	44	914	35	727	1.2
Reference value in mexican population	(55-83)	(700-2100)	(28-77)	(300-1400)	(10-39)	(200-900)	(0.9-1.9)

HIES Mosaic patient (mm-dd-yy)	B cells CD19+ %	B cells CD19+ mm3	NK cells CD16+56+ %	NK cells CD16+56+ mm3
First determination (04-07-16)	18	923	32	1642
Second determination (04-13-16)	18	603	28	938
Reference value in mexican population	(6-19)	(100-500)	(7-35)	(100-600)

Table supplementary 2.

Total count of memory T cells

HIES Mosaic patient (mm-dd-yy)	Memory T Cells CD4+ CD45RO+ %	Memory T Cells CD4+ CD45RO+ mm3	Naïve T cells CD4+ CD45RA+ %	Naïve T cells CD4+ CD45RA+ mm3
First determination (04-07-16)	56	667	35	417
Second determination (04-13-16)	64	585	33	302
Reference value in mexican population	(18-38)	(240-700)	(33-66)	(230-770)

Table supplementary 3.

Total count of memory B cells

HIES Mosaic patient (mm-dd-yy)	Naïve B cells %	Naïve B cells mm3	Memory B cells without isotype change %	Memory B cells without isotype change mm3	Switched memory B cells %	Switched memory B cells mm3
First determination (04-07-16)	84	775	2	18	6	55
Second determination (04-13-16)	79	476	5	30	8	48
Reference value in mexican population	(46-78)	(74-363)	(8-18)	(14-63)	(8-32)	(16-95)

HIES Mosaic patient (mm-dd-yy)	Transitional B cells %	Transitional B cells mm3	Plasmablasts %	Plasmablasts mm3	CD 21 low %	CD 21 low mm3
First determination (04-07-16)	5	46	0.3	3	1.5	14
Second determination (04-13-16)	6	36	2	12	5	30
Reference value in mexican population	(0.9-7)	(0.1-17)	(0.3-3)	(0-18)	(0.5-17)	(3-33)

References values were obtain from berron-ruiz L et al., Variations of B cell subpopulations in peripheral blood of healthy Mexican population according to age: relevance for diagnosis of primary immunodeficiencies, paper in press.

Table supplementary 4.

**Eosinophil count (cell/ μ l) in classical HIES
and HIES mosaic patients**

Classical HIES Patient (mm-dd-yy)	HIES Mosaic Patient (mm-dd-yy)
957 (01-10-05)	700 (07-08-05)
442 (07-01-10)	100 (03-05-07)
1090 (03-21-12)	113 (06-16-08)
1230 (11-04-13)	116 (02-13-09)
2572 (07-05-13)	133 (01-20-10)
600 (12-07-13)	58 (03-26-12)

Reference value eosinophil count 30-350 (cell/ μ l).

The eosinophil counts of classical and mosaic patients were obtained from hematic biometry corresponds from to a period from year 2005 to 2015.

Table supplementary 5.

**Concentrations of IgE (IU/mL) in classical
HIES and HIES mosaic patients**

Classical HIES Patient (mm-dd-yy)	HIES Mosaic Patient (mm-dd-yy)
11650 (11-11-08)	4988 (07-08-05)
8517 (01-29-09)	1798 (03-01-06)
24600 (11-24-10)	1420 (06-11-07)
44000 (03-21-12)	2487 (03-29-08)
63300 (06-17-13)	2139 (11-07-08)
42900 (06-10-14)	1165 (02-12-09)
22000 (05-11-15)	832 (06-24-09)
24900 (12-07-15)	1110 (08-04-10)
--	793 (07-04-11)
--	552 (07-30-12)

Normal <130 IU/mL

The IgE quantifications of classical and mosaic patients correspond to a period from year 2005 to 2015.

Table supplementary 6.

Clinical features of classical HIES and HIES mosaic patients

Clinical Findings	Classical HIES	Mosaic
Highest serum IgE (IU/ml) (a)	>2000	>2000
Skin abscesses	>4	>4
Pneumonia (episodes over lifetime)	3	1
Parenchymal lung anomalies	Pneumatocele	Absent
Retained primary teeth	>3	None
Scoliosis, maximum curvature	<10	10-14
Fractures with minor trauma	None	None
Highest eosinophil count (cells / μ l) (b)	>700	<700
Characteristic face	Present	Absent
Mildline anomaly (c)	Present	Absent
Newborn rash	Absent	Absent
Eczema	Severe	Moderate
Upper respiratory infections per year	>6	3
Candidiasis	Oral	Oral
Other serious infections	Severe	None
Fatal infection	Absent	Absent
Hyperextensibility	Present	Present
Lymphoma	Absent	Absent
Increased nasal (d)	>2DS	>2DS
High palate	Present	Present
Young-age correction	1-2	1-2

(a) Normal < 130 (UI/ml)

(b) 700/ μ l = 1 SD, 2 SD= 800/ μ l

(c) For example, cleft palate, cleft tongue, hemivertebrae, other anomaly

(d) Compared with age and sex matched controls

Supplementary methods

Flow cytometry for Memory compartments.

All flow cytometric analysis was performed on EDTA blood samples obtained by venipuncture. Lymphocyte subset analysis and absolute count of total lymphocytes, total T cells (CD3+), T cell subsets (CD4+ and CD8+), B and NK cells were performed using BD Multitest CD4FITC/CD8PE/CD3PerCP, CD3FITC/CD19PE/CD45PerCP, CD3FITC/CD16+56PE/CD45PerCP and Leucogate CD45FITC/CD14PE. To identify T CD4+ cell subpopulations were stained using CD45RAFITC/CD45ROPE/CD3PerCP/CD4APC. To identify all B subpopulations were stained with combinations of the following monoclonal antibodies: CD19 APC, CD19 FITC, IgD FITC, CD27PE, CD21 PE, CD24 PE, and CD38 APC (all from Becton Dickinson). B cell subsets were defined by the following markers on their surface, according to Morbach *et al*, (15): naïve B cells (CD19+CD27-IgD+), class-switched memory B cells (CD19+CD27+IgD-), non-switched memory/marginal-zone-like (CD19+IgD+CD27+), double-negative B cells (CD19+CD27-IgD-), transitional B cells (CD19+CD38++CD24++), and plasmablasts (CD19+CD38+++CD24-); and for CD21 low B cells (CD19+CD38lowCD21low). After staining, cells were washed, fixed with 1% paraformaldehyde in PBS and stored until analysis. A FACS Aria 1 flow cytometer (Becton Dickinson) was used to acquire 30,000 events and FlowJo (Treestar) was used for analysis of each B cell subpopulation. Leukocyte counts and total lymphocytes per mm³ were determined using a hematology analyzer (Coulter LH 500).

Flow cytometry Phosphorylation of STAT-1

To evaluate the IL-21 AN IFN pathways, PBMCs from the patients and healthy controls were incubated in 1 mL AIMV medium (Life Technologies, Carlsbad, CA, USA). One million cells were allowed to rest at 37°C and 5% CO₂ for 2 h. They were then stimulated with 100 ng of recombinant IL-6 or IL-21 (Miltenyi Biotec®, Auburn, CA, USA), and incubated at 37°C and 5% CO₂ for 20 min.

Next, the cells were fixed with 100 μ l of 16% paraformaldehyde (PFA) for 10 min. The pellets were resuspended, and permeabilized with cold methanol followed by incubation for 20 min at 4°C.

Cells were then washed with 3 mL of PBA. Cell staining was performed by incubating the following antibodies: 5 μ l anti-CD19 APC, 10 μ l anti-STAT1 Alexa Fluor 488 (Y701), 5 μ l mouse IgG1 (isotype control) (BD Pharmingen, San Jose, CA, USA). Finally, the cells were washed twice with 3 mL of PBA, and fixed with 300 μ l of 4% PFA.

Proliferation detection by CFSE incorporation

To detect B cell proliferation, CellTrace™ CFSE proliferation kit was used following manufacturer instructions. Briefly, PBMCs were obtained and labeled with 0.5ml of CFSE 1 μ M, cells were incubated at 37°C for 5 minutes and washed twice with PBS-FBS 8%. Labeled cells were cultured at a density of 0.5×10^6 cells/ml using RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO), penicillin and streptomycin (both from Sigma-Aldrich). Stimulation were performed with 2 μ g/ml of Pokeweed mitogen (Sigma-Aldrich) and 2 μ l of pansorbin (Merck-Millipore) or with 4 μ g/ml anti-CD40 (Clone G28.5 Enzo Life Sciences) and 100ng/ml of recombinant IL-21 (R&D Systems). Cells were incubated for a total of 5 days. After incubation, cells were stained with anti-CD19 APC (Beckton-Dickison) and 30,000 events were acquired in a FACS Aria cytometer (Becton-Dickinson). Analysis of proliferating CD19+ cells were performed using Flowjo software (Treestar).