Not enough by half: NFAT5 haploinsufficiency in two patients with Epstein-Barr virus susceptibility

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Research Article

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Abstract

INTRODUCTION: The transcription factor Nuclear factor of activated T cells 5 (NFAT5), pivotal in immune regulation and function, can be induced by osmotic stress and tonicity-independent signals.

OBJECTIVE: We aimed to investigate and characterize two unrelated patients with Epstein-Barr virus susceptibility and no known genetic etiology.

METHODS: After informed consent, we reviewed their electronic charts, extracted genomic DNA, performed whole-exome sequencing, filtered, and prioritized their variants, and confirmed through Sanger sequencing, family segregation analysis, and functional assays, including lymphoproliferation, cytotoxicity, and natural killer cells characterization.

RESULTS: We describe 2 cases of pediatric Mexican patients with rare heterozygous missense variants in *NFAT5* and EBV susceptibility, a school-age girl with chronic-active infection of liver and bowel, and a teenage boy who died of hemophagocytic lymphohistiocytosis.

DISCUSSION: NFAT5 is an important regulator of the immune response. Haploinsufficiency has been described as an immunodeficiency syndrome affecting both innate and adaptive immunity. EBV susceptibility is another manifestation in the spectrum of this disease.

Introduction

Over 7,000 individually rare diseases afflict around 8% of the global population (1, 2). Often, rare diseases are congenital (Mendelian and/or monogenic) and manifest themselves early in life with a range of symptoms and signs (syndromic) that may be traced to a single defect in a cell, protein, or pathway (1). Inborn errors of immunity (IEI) are a group of nearly 500 congenital rare diseases with a predisposition to unusual infections and/or inflammation, autoimmunity, atopy, malignancy (3).

The Epstein-Barr virus (EBV) is an ancient gamma herpesvirus that has co-evolved with mammals for as long as they exist (4). In *Homo sapiens*, EBV is nearly ubiquitous and innocuous, successful at establishing persistent latent infections despite a wide array of immune system components that participate in the defense against herpesviruses (4). A few patients with IEI, experiments of Nature, are susceptible to chronic, severe, or lethal EBV infections (*e.g.*, fatal infectious mononucleosis, chronic-active infection, lymphoma, and hemophagocytic lymphohistiocytosis). In those patients, at least 30 genes have been identified as causing isolated or combined susceptibility to EBV (5), due to genetic lesions affecting pathways of cytotoxicity, apoptosis, MAPK, JAK-STAT, and calcium signaling; in lymphocytes (mainly CD8+ T cells, but also Natural killer (NK), NKT, B cells, CD4+), and macrophages.

The nuclear factor of activated T cells 5 (NFAT5), also known as Tonicity enhancer-binding protein (TonEBP), is a transcriptional regulator that belongs to the Rel family, which also includes other NFATs (1 through 4) and NFκB. The similarities, however, are mainly structural, limited to the DNA-binding domain.

NFAT5 shares an N-terminal Rel-homology domain for DNA-binding and nuclear localization, but lacks the interface (docking sites) for the phosphatase calcineurin that are present in other NFAT proteins, and can thus be activated independently of this calcium/calcineurin signaling cascade (6, 7). It was initially identified as a tonicity-responsive transcription factor, as it is induced upon hyperosmotic stimulus. However, the expression of NFAT5 mRNA in a wide variety of tissues, including but not limited to hypertonic stress, suggested transcription regulation activity besides that induced by osmotic stress (7, 8).

NFAT5/TonEBP can be induced by diverse signaling pathways such as osmotic stress, as well as by tonicity-independent (isotonic) receptor-mediated signals, such as TLR-activated macrophages and TCR-stimulated T lymphocytes, for distinct transcriptional responses and regulation of immune and cell function (9–11). NFAT5, thus, is also sensitive to ischemia, hypoxia, heat shock, viral/mycobacterial infection, cytokines, and biomechanical stretching, all of which result in activation, upregulation, and nuclear accumulation (12).

In 2015, one patient with early-onset sinopulmonary infections and autoimmune enterocolopathy was found to have a *de novo* heterozygous large genomic deletion in locus 16q22.1 that included NFAT5 and 7 other genes (13). Here, we describe two pediatric patients from Mexico with Epstein-Barr virus (EBV) susceptibility and suspected NFAT5 haploinsufficiency.

OBJECTIVE

We aimed to investigate and characterize two unrelated patients with Epstein-Barr virus susceptibility and no known genetic etiology.

Methods

Cross-sectional descriptive study: case series and literature review. After obtaining written informed consent, we evaluated two pediatric patients with increased susceptibility to EBV and no known diagnosis.

Genomic DNA was obtained from whole blood by the salting-out method. Whole-exome sequencing (WES) was performed with an Illumina HiSeq platform (Admera Health, New Jersey and Psomagen, Inc., Maryland), aiming for a 90% coverage of the IDT Xgen/SureSelect V6 library, human genome version 38 (hg38, December 2013), minimum average read depth 50x-100x; processed and analyzed at the National Institute of Pediatrics using Galaxy on the cloud (v21.09), Ensembl Variant Effect Predictor, release 104 (14), and Integrative Genome Viewer (IGV) browser (v2.4, Broad Institute) (15).

For patient 1, we performed flow cytometry for lymphocyte subsets and carboxyfluorescein succinimidyl ester lymphoproliferation assay as part of her immunological workup. In Cuernavaca, Sanger sequencing of the exons involved was performed for confirmation and family segregation analysis, as well as cytotoxicity and NK cell maturation assays.

ETHICAL APPROVAL:

The study received institutional review board approval at the National Institute of Pediatrics as projects 2013/049 and 2021/054.

Results

CASE 1

A 7-year-old girl from Tijuana with two healthy younger siblings from non-consanguineous parents. At age 5, she began with recurrent episodes of hepatitis, presenting with high-grade fever, abdominal pain, nausea, vomiting, diarrhea, and weight loss, associated with increased transaminases and bilirubin. Serology was negative for hepatitis virus (HBV, HCV, HAV, and HEV). During the second episode, she was found to have IgG antibodies (IgM-negative) to antiviral capsid antigen (VCA), Epstein-Barr nuclear antigen (EBNA), and early antigen. Quantification of EBV DNA by polymerase chain reaction (PCR) in plasma was persistently positive over several months (328,107 copies/ml), and she was diagnosed with chronic active EBV (CAEBV) infection.

The abdominal ultrasound was normal. Cryptosporidium and clostridium were ruled out. A hepatic biopsy showed active diffuse inflammation with lymphocytes, plasmacytes, neutrophils, and eosinophils expanding into the portal space and producing centrilobular necrosis; EBV early RNA (EBER) was positive, predominantly in CD3+CD8+ T cells (**Figure 1**). Colonoscopy revealed pancolitis with ulcers, and histopathology showed chronic active enterocolitis associated with EBV, with T-cell predominant EBER; quantification of EBV DNA in the gastrointestinal tract was above 7’000,000 copies per mL. EBV-associated hepatitis and enteropathy were diagnosed.

Blood counts were normal, with 1,500 peripheral lymphocytes, and 400 monocytes. Immunological workup revealed an impaired lymphoproliferation of phytohemagglutinin (PHA)-stimulated CD3+ T cells, (**Figure 2A**). Moreover, the patient lymphocyte subsets were shown to have decreased central (CD45RA-CCR7+) and effector (CD45RA- CCR7-) memory CD8+ T-cells, with expanded senescent CD4+ CD57+ T-cells (**Figure 2B and 2C**). NK cell function was normal based on degranulation assays (**Figure 3A**). In contrast, the expression of various cell surface markers was abnormal in NK cells from the patient as compared to healthy control, suggesting impaired NK cell differentiation (**Figure 3B**). All these assays were performed while the patient was receiving immunomodulatory treatment. Flow cytometry for B cell subsets and Tregs found decreased plasmablasts, memory B cells, and low Tregs (**Figure 4**).

Whole exome sequencing (WES) identified a novel (gnomAD allele count 0), heterozygous (MAB 0.51, DP 74x) missense variant in exon 4 (between the transcription activating TAD1 and auxiliary export AED domains) of *NFAT5* (c.335C>T, p.Ser112Phe or p.Ser94Phe), likely pathogenic (SIFT 0, PolyPhen2 0.986, CADD Phred 25.8), at a position highly conserved across species (GERP++ RS 5.67). **See figure 5**. MutPred Top5 features predict a loss of glycosylation (p=0.007) and loss of phosphorylation at S94

(p=0.019), with a gain of a sheet (p=0.047). Family segregation through Sanger sequencing confirmed the variant to be *de novo*, as both parents had wild-type alleles.

She received treatment with ursodeoxycholic acid, cholestyramine, mesalazine, high-dose intravenous immunoglobulin, enteral immunoglobulin, rituximab, corticosteroids, azathioprine, and cyclosporine, with little improvement; she was later started on tocilizumab with clinical and EBV viral load improvement.

The patient underwent allogeneic hematopoietic stem cell transplantation (HSCT) from her haploidentical father, after cyclophosphamide Treg depletion and reduced-intensity conditioning; initially with control of the EBV infection (undetectable, down from 10,232cp/ml pre-HSCT). She received 4 donor lymphocyte infusions, despite which she evolved to secondary graft failure by day +220. She is currently 8 months post-transplant with mixed micro-chimerism (2.74%) and EBV viral load reactivation (1,531cp/ml), without clinical signs of disease.

CASE 2.

A previously healthy adolescent male from Mexico City, who at 16 presented with hemophagocytic lymphohistiocytosis (HLH) associated with EBV infection. He had two healthy sisters, with no family history of consanguinity. At ages 3 and 6 years, he suffered fissured clavicle, rotula, and forearm fracture associated with traumatisms. He also had allergies to penicillin, fava beans, and some fruits. When he was 15 years old, he developed a urinary tract infection.

He started at age 16 with fever, cytopenia, triglyceridemia, and documented hemophagocytosis, for which he was treated with cyclosporin, dexamethasone, and etoposide. Soon after discharge, he was readmitted with fever, hepatosplenomegaly, oral candidiasis, and herpetic stomatitis.

Blood counts showed pancytopenia, with Hb 7.5g/dL, white blood cells 2,200 (down to 300), neutrophils 1500 (down to 200), lymphocytes 1,100 (down to 14), monocytes 100, and platelets 19,000-29,000/mm3. Ferritin 14,724-225,300 ng/ml, Serum immunoglobulins: IgG 515-1,280 mg/dL, IgM 38, IgA 272 mg/dL. IgG1 766, IgG2 154, IgG3 28.3, IgG4 16.9mg/dl. Serum autoantibodies (anti-Ro/La, ANA, lupus anticoagulant), and serologies for HIV, HBV, HCV, syphilis, and brucellosis, were all negative. EBV serum antibodies VCA (IgG), EA, and EBNA. PCR identified 2,580 copies/ml of EBV in serum, and up to 239,411 cp/ml in bone marrow. A bone marrow aspirate (BMA) was normocellular, with megaloblastic changes and active hemophagocytosis. A second BMA found hypocellularity, low megakaryocytes, and 8 hemophagocytes, with EBER diffusely positive in numerous cells.

In addition to the HLH-2004 protocol (etoposide, dexamethasone, and cyclosporine) and high-dose intravenous immunoglobulin, the patient received treatment with blood transfusions and filgrastim. He was admitted to the intensive care unit and required mechanical ventilation, despite which he progressed to disseminated intravascular coagulation with multiorgan failure and perished. Given the rapid course of the disease, further immunologic studies could not be performed.

A *post-mortem* WES analysis revealed a very rare heterozygous missense variant, predicted as pathogenic and highly conserved, in exon 7 of 15 of NFAT5, c.1291A>T (p.Thr431Ser, or p.Thr449Ser, p.Thr355Ser), in the DNA-binding domain RHD (Rel homology domain); gnomAD exomes allele count 1, SIFT 0.04, PolyPhen2 0.996, CADD Phred 24.6 (Minimum significance cutoff 3.3). GERP++ RS 4.85. MutPred Top5 features predict a loss of sheet (p=0.014) and gain of a loop (p=0.024). See **figure 5**.

A literature review on PubMed Medline searching for: (NFAT5 AND deficiency AND patient) without filters, retrieved 5 results and identified only one previously published human patient with NFAT5 haploinsufficiency (*vide infra*).

Discussion

We present the cases of two pediatric patients with NFAT5 haploinsufficiency and EBV susceptibility: one with CAEBV infection with hepatitis and enterocolitis, and one with fatal HLH. Both patients were found to have rare and conserved heterozygous missense variants in critical domains of NFAT5. The clear limitations of this report are a small number of cases and a lack of mechanistic evidence to prove causation. However, this is the first time that we know, that NFAT5 deficiency has been linked to EBV susceptibility and HLH.

In recent years, NFAT5 has emerged as an important regulator of the immune response. An animal model with homozygous targeted deletion of exons 6 and 7 of the *NFAT5* gene, which encode a critical region of the DNA-binding domain, resulted in complete loss of function and late gestational lethality (16,17). Heterozygous mice with partial loss of function (haploinsufficiency) resulted in impaired lymphocyte proliferation under hypertonic conditions, lymphoid hypocellularity, and impaired antigen-specific antibody response (16,18). Lymphoid tissues have a hyperosmolar microenvironment, and lymphocyte-mediated immunity requires adaptation to physiologic osmotic stress; NFAT5 is thus a critical component for optimal adaptive immunity (8), and defective NFAT5 signaling results in poor thymocyte development and survival, independent of NFAT5's osmoprotective role (18).

In T lymphocytes, NFAT5 is also critical for development and subsistence. NFAT5 is expressed constitutively and abundantly in the thymus and can be induced in mature lymphocytes upon TCR activation (7). In hyperosmotic environments, NFAT5 helps proliferation and survival of T cells, promotes polarization towards Th17 cells, and attenuates excessive pro-inflammatory responses in T cells (19). NFAT5 deficiency may also contribute to the development and survival of NK cells (13).

There is one report of an NFAT5-haploinsufficient human patient with recurrent sinopulmonary infections during the first years of life, who developed autoimmune entero-colonopathy around age seven. Basic lymphocyte subsets, levels of serum immunoglobulins, and vaccine response were within normal limits, but further immunologic evaluation showed impairment of both innate and adaptive immunity. The patient had an altered distribution of B cell subsets and a selective IgG subclass deficiency. Lymphocyte proliferation to mitogens was normal but antigen-specific proliferation was impaired, and CD8+T cell

function was altered due to a reduced ability to degranulate and produce the pro-inflammatory cytokines IFNγ and TNFα. He also had a reduction of CD56+CD16+ NK cells and reduced survival of peripheral blood mononuclear cells (PBMCs) in hypertonic conditions. The genetic evaluation revealed a de novo large deletion at 16q22.1 encompassing 8 genes, NFAT5 among them. The authors, Boland *et al*, used a dominant-negative NFAT5 construct with reduced NFAT5 expression that showed decreased cell viability in hypertonic conditions and reduced production of TNFα by CD8+T cells, analogous to the immune defects seen in the patient (13).

Some other patients from Belarus, Ukraine, and the USA, with NFAT5 haploinsufficiency caused by heterozygous missense or small deletion variants, manifest early in life with diverse autoimmune diseases, and their T cells show reduced proliferation and survival under hypertonic conditions (Svetlana Sharapova, personal communication).

Our two patients from Mexico seem to be the first to present with EBV susceptibility and HLH. NFAT5 is expressed in all T cells, natural killers, and macrophages, the three protagonists of the hemophagocytic cytokine storm. Inside the nucleus, NFAT5 suppresses the induction activity of IRF3, involved in the interferon beta response to viruses (20). Both NFAT5 and EBV directly interact with IRF3: the EBV kinase BGLF4 phosphorylates residues of IRF3 to prevent DNA binding, while NFAT5 forms dimers that compete with the DNA binding of IRF3 to the IFNb enhancer consensus region (21). This suggests plausibility for the implication of NFAT5 haploinsufficiency in our patients' phenotypes. Type I interferon is a double-edged sword: unbridled production and prolonged secretion result in systemic inflammation and stem cell exhaustion. Although we do not fully understand the physiopathogenic mechanism, lymphocyte maturation/activation defects, excess interferon response, and/or disruption of the latent phase of EBV, are suitable candidates.

In the investigation of patients with increased susceptibility to EBV and HLH, physicians and researchers should also include NFAT5 haploinsufficiency as a differential diagnosis. Phenotypes that include enteropathy, sinopulmonary infections, and reduced numbers of transitional B cells, plasmablasts, and NK cells, are perhaps the most likely candidates.

Next, we want to further characterize the two heterozygous variants in cell lines through plasmid transfection, as one of our patients was successfully transplanted and the other one died. We expect to be able to perform lymphocyte proliferation assay under hyperosmotic condition, and phenotype rescue.

In conclusion, NFAT5 deficiency can impair T lymphocyte function, and NFAT5 haploinsufficiency has already been described as an immunodeficiency syndrome affecting innate and adaptive immunity. It could also predispose to EBV susceptibility and HLH, given the pivotal role it plays as a transcription factor in lymphocytes, macrophages, and NK cells.

Abbreviations

BMA, bone marrow aspirate; CAEBV, chronic active Epstein-Barr virus; CD, cluster of differentiation; DNA, deoxyribonucleic acid; EBV, Epstein-Barr virus; EBER, EBV early RNA; EBNA, Epstein–Barr virus nuclear antigen; HLH, hemophagocytic lymphohistiocytosis; NFAT5, nuclear factor of activated T cells 5; NK, natural killer; PBMC, peripheral blood mononuclear cell(s); PCR, polymerase chain reaction; TCR, T cell receptor; TLR, toll-like receptor(s); TNF, tumor necrosis factor; VCA, viral capsid antigen; WES, whole-exome sequencing.

Declarations

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Dr. Alfonso Gilberto Ramírez Ristori, MD, from the Pathology Department at the National Institute of Pediatrics (INP) stained, analyzed, and retrieved pictures of histopathology specimens from patient 1, under the supervision of CTCD. Dr Karen Rubí Ignorosa Arellano, from the Gastroenterology service at INP performed the colonoscopy on patient 1. We thank Drs Svetlana Sharapova and Vivien Béziat for helpful discussions and feedback on *NFAT5* and haploinsufficient patients. Dr. Sharapova also shared with us her data on other NFAT5 patients and the hyperosmotic assay from her lab in Belarus. The Immune deficiencies laboratory is a Jeffrey Modell Diagnostic Center. Fundacion mexicana para niñ@s con inmunodeficiencias (FUMENI) contributed with sample shipping. Funds for sequencing were obtained through CONACYT: innovation stimulus program and Frontier Science grant #10869.

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Availability of data and material: Not applicable. Further information is available upon demand.

Code availability: Not applicable.

Consent for publication: Consent for publication was given by the patients' family members.

Ethical approval: The study received institutional review board approval at the National Institute of Pediatrics as projects 2013/049 and 2021/054.

Authors' contribution statement:

LMC, MAY, and SCS provided both patients' health care and clinical information. DOLR and MECM performed additional functional immunologic and genetic testing for patient 1 and her family. RMNRU coordinated the stem-cell transplant and follow-up for patient 1. CCD stained analyzed and facilitated pictures of histopathology specimens. EAMT and LBR performed additional experiments. SEEP coordinated the diagnostic approach. SOLR performed the processing and analysis of the genetic data. LMC and SOLR wrote the initial manuscript. SCSM and MECM also designed figures. All authors provided revision to the initial manuscript, read, and approved its final version.

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Figures

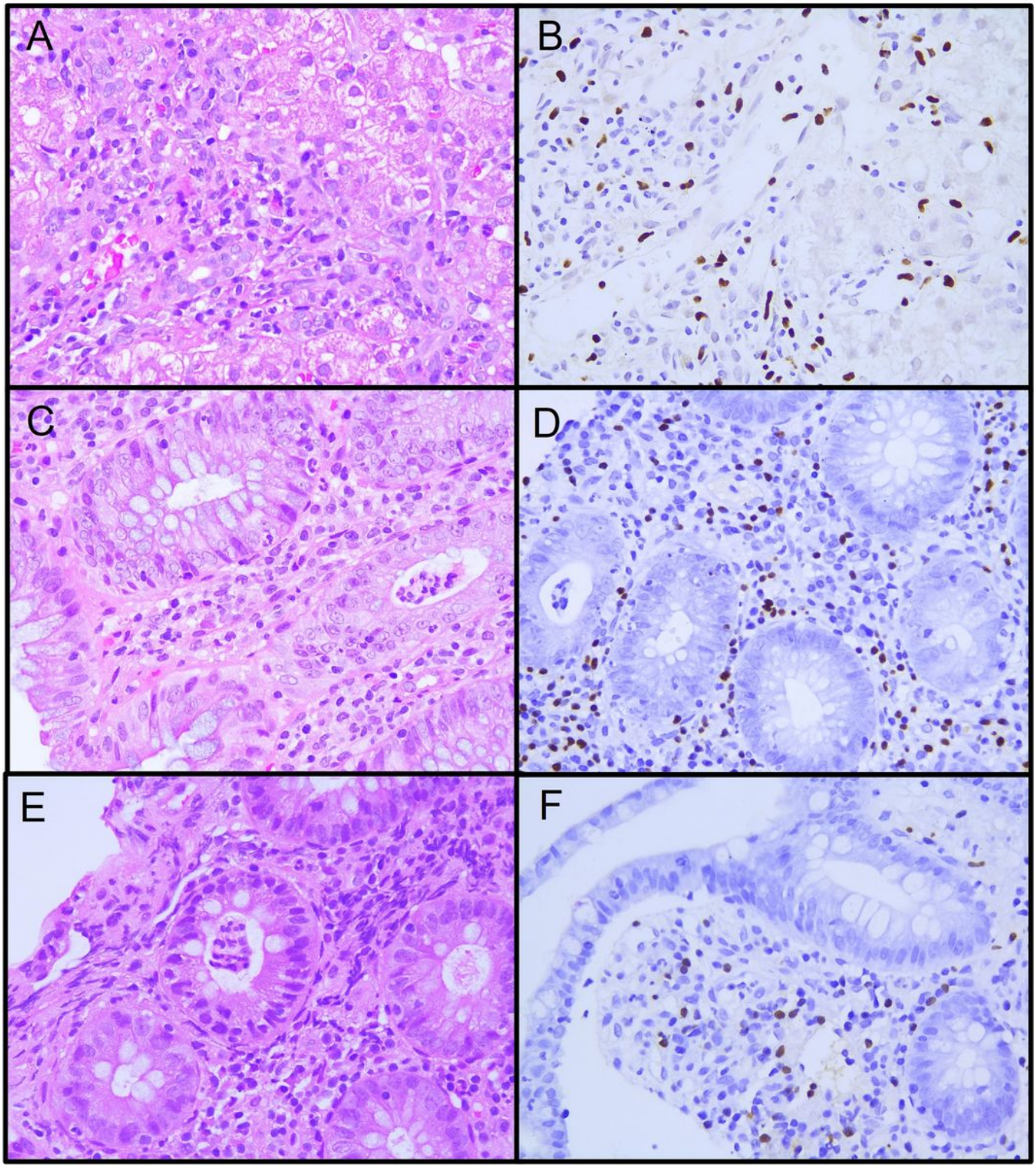

Figure 1

Histopathology specimens from patient 1. **A.** Liver biopsy showing active diffuse lymphocytic inflammation. **B.** Epstein-Barr encoding region (EBER) in situ hybridization in inflammatory lymphocytic infiltrate. **C.** Colon biopsy showing intense lymphocytic and polymorphic inflammatory infiltrate at the lamina propria and neutrophilic crypt micro-abscesses. **D**. EBER positive in inflammatory lymphocytic infiltrate in the lamina propria and crypta. **E.** Ileum biopsy showing moderate lymphocytic and

polymorphic inflammatory infiltrate at the lamina propria and neutrophilic crypt microabscesses. **F.** EBER positive in inflammatory lymphocytic infiltrate in the lamina propria.

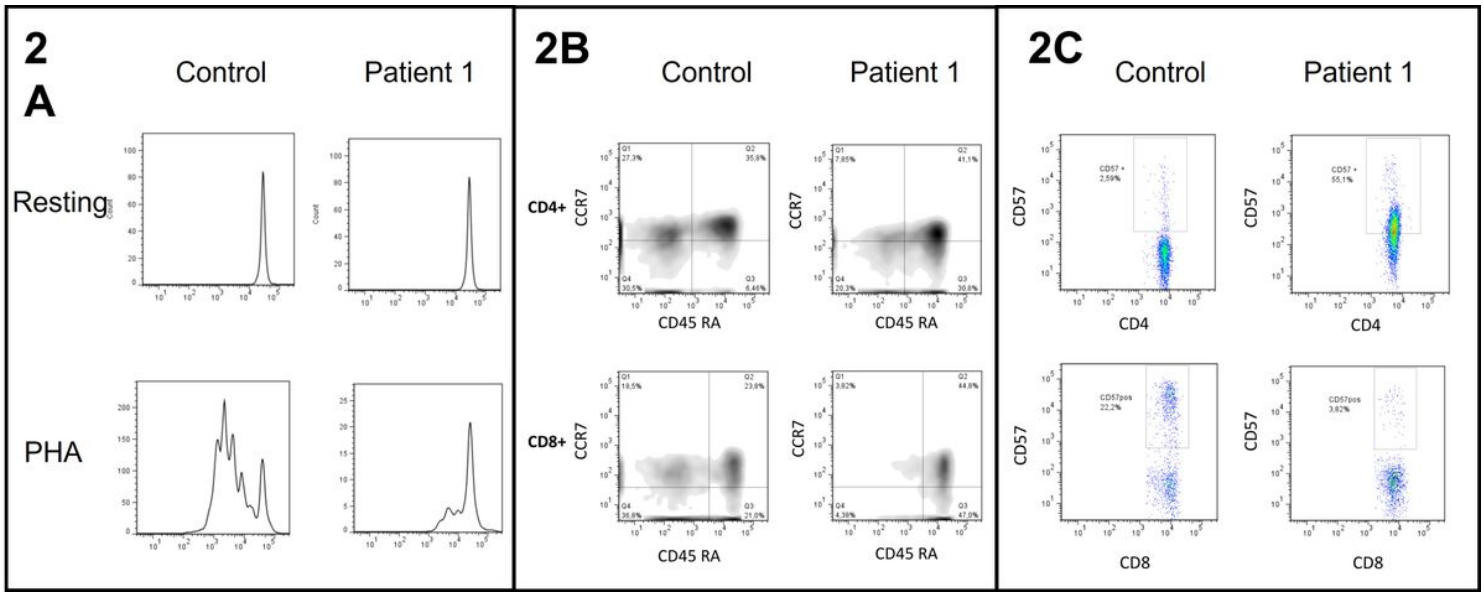

Figure 2

2A. CD3+ T-cells lymphoproliferation, resting and after phytohemagglutinin (PHA) stimulus. Patient 1 cells show an impaired lymphoproliferation of PHA-stimulated CD3+ T cells. **2B.** CD4+ and CD8+ cells subsets, naive (CD45RA+ and CCR7+), central memory (TCM, CD45RA- and CCR7+), effector memory (TEM, CD45RA-and CCR7-), and CD45RA+ effector memory cells (TEMRA, CD45RA+ and CCR7-). Patient 1 also has decreased TCM TEM CD8+ T-cells and expansion of TEMRA CD4+ T-cells. **2C.** CD57+ expression in CD4+ and CD8+ T-cells as compared to control, as well as increased senescent CD4+ CD57+ T-cells.

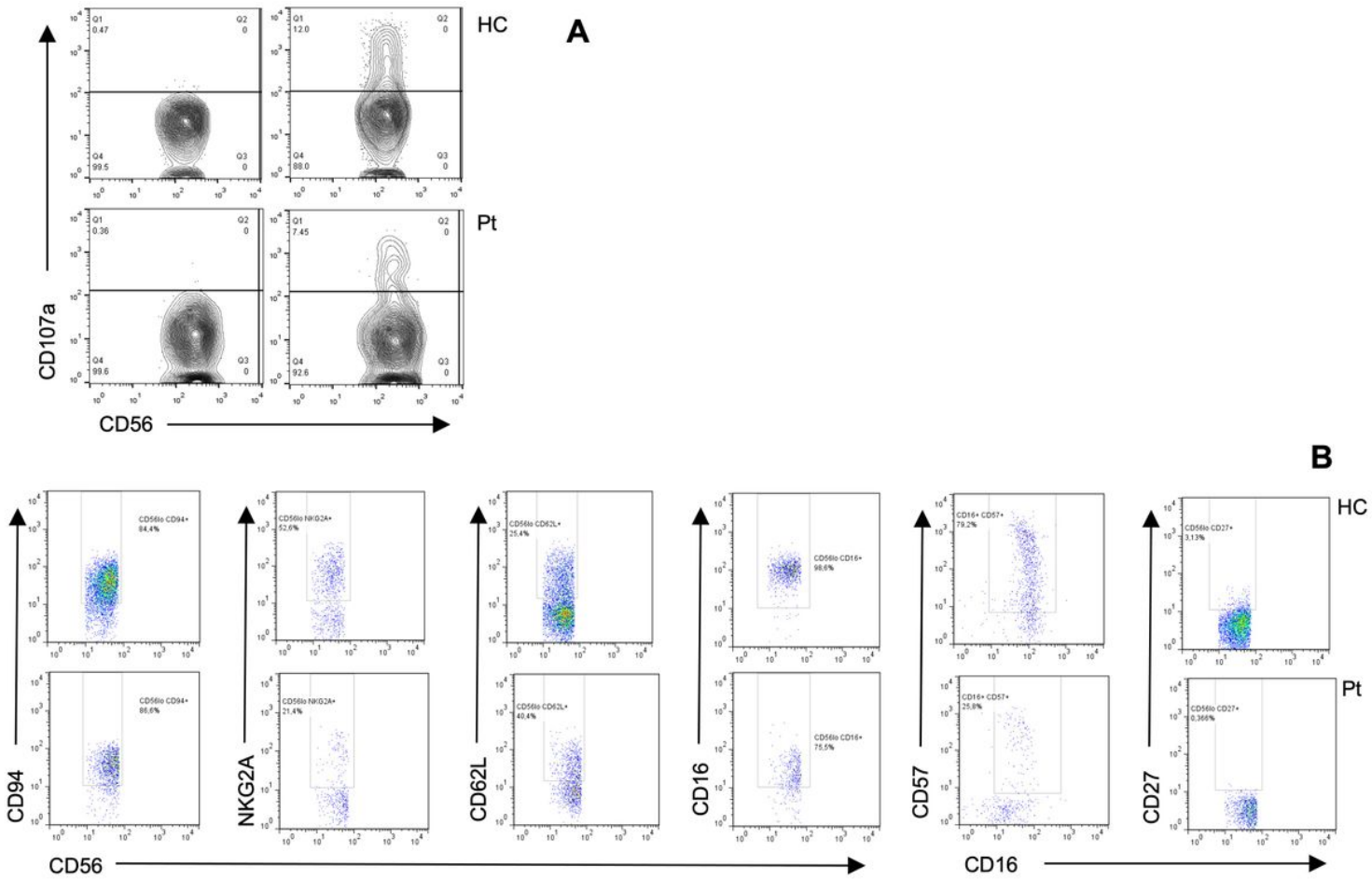

Figure 3

Flow cytometry from patient 1 showing **A:** Diminished degranulation in Natural Killer (NK) cells from the patient (P) as compared to healthy control (HC). **B:** Abnormal cell surface marker expressions in NK subsets cells from the patient as compared to healthy control, suggesting impaired NK cell differentiation.

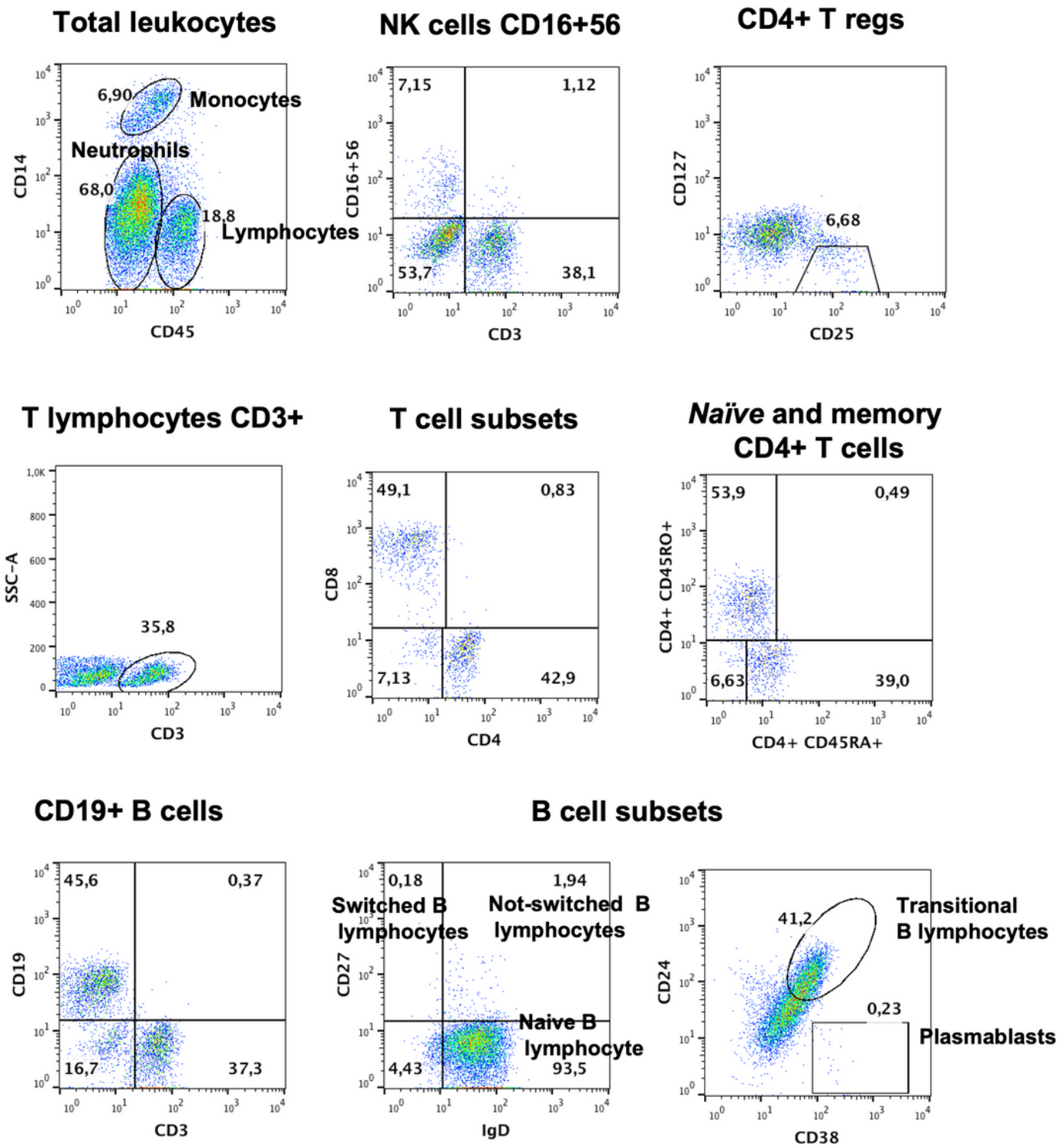

Figure 4

Lymphocyte subsets for B and Treg cells from patient 1, after failed HSCT with mixed chimerism. Plasmablasts and Memory B cells are low, as are Tregs.

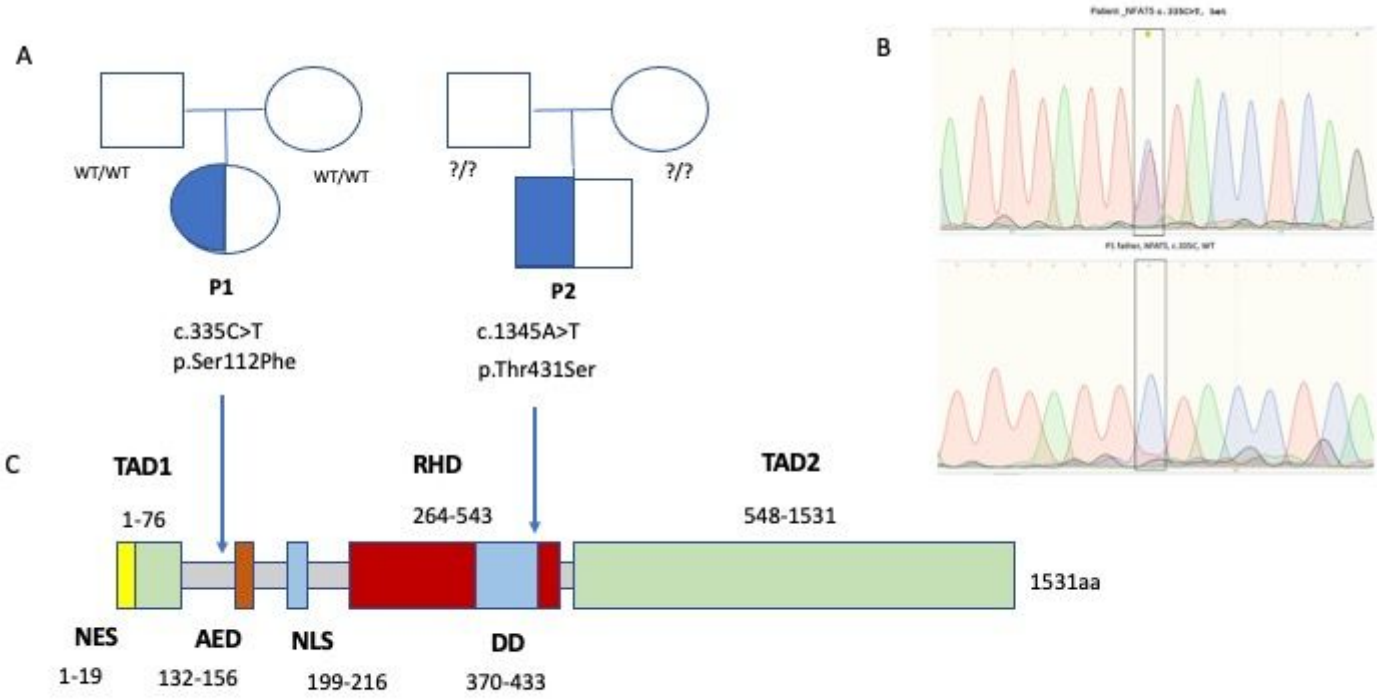

Figure 5

Family trees (A) and Sanger electropherograms (B) showing *de novo* heterozygous missense variants in exons 4 and 7 of *NFAT5*, affecting Transcription activation and DNA binding domains of NFAT5 (C), in two patients with EBV-susceptibility.