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ORIGINAL ARTICLE

Novel Germline *TET2* Mutations in Two Unrelated Patients with Autoimmune Lymphoproliferative Syndrome-Like Phenotype and Hematologic Malignancy

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Abstract

Somatic mutations in the ten-eleven translocation methylcytosine dioxygenase 2 gene (*TET2)* have been associated to hematologic malignancies. More recently, biallelic, and monoallelic germline mutations conferring susceptibility to lymphoid and myeloid cancer have been described. We report two unrelated autoimmune lymphoproliferative syndrome-like patients who presented with T-cell lymphoma associated with novel germline biallelic or monoallelic mutations in the *TET2* gene. Both patients presented a history of chronic lymphoproliferation with lymphadenopathies and splenomegaly, cytopenias, and immune dysregulation. We identified the first compound heterozygous patient for *TET2* mutations (P1) and the first ALPS-like patient with a monoallelic *TET2* mutation (P2). P1 had the most severe form of autosomal recessive disease due to TET2 loss of function resulting in absent TET2 expression and profound increase in DNA methylation. Additionally, the immunophenotype showed some alterations in innate and adaptive immune system as inverted myeloid/plasmacytoid dendritic cells ratio, elevated terminally differentiated effector memory CD8 + T-cells re-expressing CD45RA, regulatory T-cells, and Th2 circulating follicular T-cells. Double-negative T-cells, vitamin B12, and IL-10 were elevated according to the ALPS-like suspicion. Interestingly, the healthy P1's brother carried a TET2 mutation and presented some markers of immune dysregulation. P2 showed elevated vitamin B12, hypergammaglobulinemia, and decreased HDL levels. Therefore, novel molecular defects in TET2 confirm and expand both clinical and immunological phenotype, contributing to a better knowledge of the bridge between cancer and immunity.

Keywords ALPS · ALPS-like · Immune dysregulation · Germline mutation · Lymphoma lymphoproliferation · Malignancy · Somatic mutation · TET2

Abbreviations

5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
AITL	Angioimmunoblastic T-cell lymphoma
ALK	Anaplastic lymphoma kinase
ALPS	Autoimmune lymphoproliferative syndrome
AML	Acute myeloid leukemia
BV-CHP	Brentuximab vedotin + cyclophosphamide therapy
BV-ESHAP	Brentuximab vedotin + etoposide, methylprednisolone, cytosine arabinose, cisplatin therapy
CHIP	Clonal hematopoiesis of indeterminate potential
CHOP	Cyclophosphamide + hydroxydaunorubicin + oncovin + prednisone therapy
CMA	Chromosomal microarray
cTfh	Circulating follicular helper T-cells

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DCs	Dendritic cells
DHAP	Cisplatin, cytosine arabinoside and dexamethasone therapy
DLBCL	Diffuse large B-cell lymphoma
DNMT3A	DNA methyltransferase 3 Alpha
DNT	Double-negative T-cells
EBER	Epstein-Barr virus-encoded small RNAs
FAS	Fas cell surface death receptor
FASL	Fas ligand
FDG	18F-Fluorodeoxiglucosa
gnomAD	Genome Aggregation Database
HDL	High-density lipoprotein
HSCT	Hematopoietic stem cell transplant
IEI	Inborn error of immunity
IgG	Immunoglobulin G
IL-10	Interleukin 10
KMT2D	Lysine methyltransferase 2D
LOF	Loss of function
NGS	Next-generation sequencing
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
PET-CT	Positron emission tomography-computed tomography
SD	Standard deviation
sFASL	Soluble Fas ligand
STAT3	Signal transducer and activator of transcription 3
TEMRA	Terminally differentiated effector memory T-cells re-expressing CD45RA
TET2	Ten-eleven translocation-2
Tregs	Regulatory T-cells
VAF	Variant allele frequency
VOUS	Variant of uncertain significance

Introduction

Novel monogenic causes of immune dysregulation have been identified in the last years, some of them overlapping clinical and immunologic features with the autoimmune lymphoproliferative syndrome (ALPS). ALPS is characterized by benign and chronic lymphoproliferation, autoimmunity and increased risk of lymphoma due to mutations in the Fas-FasL apoptotic pathway [1–4]. Expanded CD3 + TcRαβ + CD4-CD8- double-negative T-cells (DNT) and elevated serum ALPS biomarkers are a hallmark of the disease [3, 5]. Disorders that mimic the ALPS phenotype but differ in the molecular cause and do not always fulfill laboratory criteria are known as ALPS like [5, 6]. To date, up to 24 distinct genes have been identified as molecular causes of ALPS like [3, 5, 6].

Malignancy is common in human inborn errors of immunity (IEI) and especially in the primary immune regulatory disorders. Although the underlying mechanism is not well known, both intrinsic genetic and extrinsic factors, such as chronic viral infections and loss of immune surveillance, are suggested [7]. Several ALPS-like related genes have been associated with susceptibility to hematological malignancy, one of them is the recently described loss of function (LOF) of the ten-eleven translocation-2 (TET2) [6, 8].

TET2 is a methylcytosine dioxygenase belonging to the TET family (TET1, TET2, TET3) that acts as a key regulator for gene expression through DNA demethylation, oxidizing the methyl group of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), among others. TET2 is mainly expressed in hematopoietic cells where plays important roles including cell self-renewal, lineage commitment, and monocyte differentiation [9]. Recent studies have evidenced the importance of TET2 also during immune cell differentiation, activation, and immune homeostasis [10–12].

Aberrant methylation process results in silencing of tumor suppressor genes and it is therefore a hallmark of cancer. Somatic LOF mutations in the *TET2* gene have been largely identified in myeloproliferative disorders and hematologic cancers in the last years [13–16].

Conversely, germline monoallelic mutations in *TET2* are less common and have been recently described in patients with hematological diseases [17]. Although there were not clinical features associated to immune system alteration, some *TET2* mutations carriers presented high percentages of activated B-cells and low percentages of effector memory T-cells with increased proportions of TEMRA cells [18, 19]. Furthermore, Spegarova et al. reported in 2020 for the first time two novel germline LOF homozygous mutations in *TET2* in three ALPS-like patients causing an autosomal recessive immune dysregulation syndrome with marked predisposition to lymphoma [8].

Mutations in *TET2* are considered a pre-malignant event and the co-occurrence with other cumulative somatic mutations can trigger the lymphomagenesis. In fact, the presence of somatic mutations in several genes as *STAT3*, *DNMT3A*, *KMT2D*, *KRAS*, *ERBIN*, *RHOA*, *BRAF*, *ZRSR2*, *SRSF2*, *JAK2*, or *GATA2* have been described in patients with other somatic and germline molecular alterations of *TET2* [8, 16, 19–23].

Here, we report two unrelated ALPS-like patients (P1 and P2) with novel previously unreported germline biallelic and monoallelic mutations in the *TET2* gene, thus confirming and expanding the recently described IEI associated with germline *TET2* LOF consisting in immunodeficiency, autoimmunity and development of lymphomas.

Methods

Patients and Control Samples

All experimental work was performed under protocols approved by the Institutional Review Board (IRB) of the

Institution (imas12), after written informed consent for publication of clinical and immunological information of the patients. The study fulfilled the IRB standards for ethical conduct of research with human subjects.

Immunophenotype

Immunophenotyping was performed on whole peripheral blood for the assessment of T, B, NK, and dendritic cells (DCs) compartments. Conjugated anti-human monoclonal antibodies are listed in Table S1. The gating strategy of the different cellular populations are shown in Figure S1. Cellular acquisition was performed using a Navios and Dx Flex flow cytometers and data were analyzed with Kaluza 2.1 software (Beckman Coulter, Miami, FL, USA).

ALPS Biomarkers

Serum levels of IL-10 (DRG, Palex, Madrid, Spain), sFasL, sCD25 (R&D, Vitro, Madrid, Spain), and HDL (Roche diagnostics, Basel, Switzerland) were measured in duplicate by enzyme-linked immunosorbent assay. Vitamin B_{12} were measured by electro-chemiluminescence immunoassay (Beckman Coulter). Serum immunoglobulin concentrations were determined by nephelometry (Beckman Coulter) [5].

Purification of Cell Populations

Isolated PBMCs from healthy carrier HC and healthy donor were stained with CD56/CD16-FITC, CCR7-PE, CD8-APC-A700, CD45RA-APC-A750, and CD45-KO at room temperature for 20 min. Cells were washed with PBS + EDTA 1 mM and finally adjusted at 2×10^7 cells/mL. NK cells (CD45 + CD3-CD56/CD16 +), CD8 naïve (CD45 + CD3 + CD8 + CD45RA + CCR7 +), and CD8 + TEMRA (CD45 + CD3 + CD8 + CD45RA + CCR7 -) T-cells were gated and isolated using MoFlo XDP high-speed and CytoFLEX SRT flow cytometry sorter (Beckman Coulter) with a purity of isolated cells above 95%.

Fas-Induced Apoptosis

Functional studies for Fas-mediated apoptosis was performed as previously described [5] for screening of ALPS patients.

DNA Sequencing and Comparative Genomic Hybridization Array

Genomic DNA was extracted from EDTA blood samples using the Reliaprep Blood gDNA Miniprep system (Promega, Madison, WI, USA). For molecular germline studies, NGS were done by targeted gene sequencing with an in-house designed panel of 192 genes involved in IEI (Ampliseq, Life Technologies, Carlsbad, CA, USA) and by whole exome sequencing (WES) using an xGen Exome Panel v1.0 kit (Integrated DNA Technologies, Coralville, IO, USA). Paired-end sequencing (2 × 75 bp) was conducted on a NextSeq 550 (Illumina, San Diego, CA, USA). WES data analysis was filtered on a 452-gene custom panel targeted at the clinical suspicion of IEI. The human genomics community VarSome was used for in silico pathogenicity prediction and for a comprehensive interpretation of the variants [24].

For copy number variants (CNVs), a 60 K chromosomal microarray (CMA) (60 K KaryoNIM®, NIMGenetics, Madrid, Spain) was carried out. Analysis and interpretation of the results were performed using Cytogenomics (v.4.0.3.12, Agilent) software. A threshold of ≥ 5 consecutive probes was established to consider a CNV. CNVs detected were classified following previous CMA recommendations for clinical practice [25, 26]. Molecular germline variants found through targeted NGS gene panel and WES were confirmed by Sanger sequencing using the appropriate specific primers.

PCR-sequence specific primer (PCR-SSP) strategy was used for *cis* or *trans* allelic discrimination [27] (see supplementary methods and Figure S2).

T-cell lymphoma-related somatic variants were detected from paired normal-tumor samples through a targeted NGS gene panel (Thermo Fisher Scientific, Waltham, MA, USA). The genes included are listed in supplementary methods.

Gene and Protein Expression Assays

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) with the ReliaPrep™ RNA Cell Miniprep System (Promega). RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and gene expression was analyzed by qPCR using a TaqMan Fast Universal PCR Master Mix and specific TaqMan probes (TET2: Hs00325999_m1; GAPDH: Hs02786624_g1) (Thermo Fisher Scientific) in accordance with the manufacturer's instruction. Relative quantification using GADPH as endogenous control were evaluated with the comparative CT method (ΔΔCT).

Cell extracts for immunoblotting were prepared by incubating PBMCs in RIPA lysis buffer (Sigma-Aldrich, San Luis, MO, USA) with Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) in accordance with the manufacturer's instruction. Western Blot were performed on 4–20% precast polyacrylamide gel and proteins were transferred onto a nitrocellulose membrane (Bio-rad, CA, USA). Antibodies used were TET2 (clone 21F11, Active

Motif, Carlsbad, CA, USA) and β-actin (Merck Life Science, Darmstadt, Germany). Signal was detected in a Gel Doc XR + Imager (Bio-Rad).

DNA Methylation Assay

DNA methylation was evaluated through an ELISA-based assay (Global DNA Methylation Assay–LINE-1) (Active Motif) for the detection of 5-methylcytosine (5-mC) levels of Long Interspersed Nucleotide Element 1 (LINE-1) repeats. DNA was first enzymatically digested with the *MseI* enzyme to allow the hybridization of fragmented DNA to a biotinylated LINE-1 consensus sequence. Methylated CpG residues in LINE-1 regions were identified using a 5-mC antibody, HRP-conjugated secondary antibody, and colorimetric detection reagents (Active Motif). The relative 5-mC levels in each DNA sample were obtained interpolating the optic density (OD) absorbance in a standard curve with known LINE-1 methylation levels.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism v8.0.2 (GraphPad software, San Diego, California). Significant differences were determined by using non-paired Student *t*-test. A *p*-value of $< *0.05, **0.01$, and $***0.001$ were considered statistically significant.

Results

Case Reports

A 27-year-old woman (patient 1, P1) born to non-consanguineous Caucasian parents was diagnosed in 2010, at the age of 16 years, of diffuse large B-cell lymphoma (DLBCL) with an aggressive clinical behavior. She achieved complete remission after treatment with intensive chemotherapy schema according to the French American British/ LMB protocol for high-risk disease. Along the follow-up period, few pulmonary nodules were identified, with inconclusive biopsy results for diagnosis and with resolution after corticosteroids treatment. Five years later, in 2015, supra and infradiaphragmatic lymphadenopathies, hepatosplenomegaly, terminal ileum, and bone marrow pathological FDG uptakes were detected in a PET-CT. A lymph node biopsy described a reactive/inflammatory cellular pattern without evidence of malignancy. Similar findings were reported in another PET-CT study in 2017. Analytical results showed moderate thrombocytopenia and polyclonal IgG hypergammaglobulinemia with IgA deficiency. In October 2020, the patient was admitted in the hospital for diagnosis of abdominal pain, diarrhea, profuse sweating, and fever. Grade 3 thrombocytopenia, widespread lymphadenopathies, greater hepatosplenomegaly than previously reported, and a cecum/terminal ileum mass were detected on CT scan. The clinical and analytical phenotype presenting with lymphoproliferation, cytopenias, and immune dysregulation led us to suspicion of an ALPS syndrome [5, 6]. Right hemicolectomy piece revealed diffuse infiltration by ALK-negative anaplastic large T-cell lymphoma (CD3-, CD30 + , CD2 + , CD4-, CD103-, CD45 + , 70%Ki67 + , CD27-, CD33-, CD7-, CD14-, HLA-DR +). She received subsequent treatments with BV-CHP and BV-ESHAP due to progressive disease. A match unrelated donor hematopoietic stem cell transplant (MUD HSCT) was performed in March 2021. Unfortunately, the patient died early due to transplant related toxicity.

Patient 2 (P2) is a 56-year-old woman with a medical history of chronic mild peripheral cytopenias (neutropenia and thrombocytopenia) for 8 years that progressively developed tonsillitis, adenoiditis, and cervical adenopathies. Biopsies of tonsil and lymph node were performed during a period of evolution of 2 years with a diagnosis of bilateral hypertrophic chronic tonsillitis with lymphoid reactive hyperplasia. Finally, one year later, one of the cervical adenopathies was pathological with the final diagnosis of angioimmunoblastic T-cell lymphoma (AITL). Lymph node showed architectural distortion, expansion of follicular dendritic cells, and abundant presence of T lymphocytes, with slight cytologic atypia and PD1 expression. There were also abundant large and small CD30 + cells and numerous EBV-positive cells (EBER). Atypical cells were surface CD3-, cytoplasmic CD3 + , CD5 + + , CD7 + , CD2 + , CD45 + + , and CD4 and CD8-negative expression. In the imaging study at diagnosis, splenomegaly of 17 cm was described. She also showed persistent polyclonal hypergammaglobulinemia (IgG and IgA). Patient did not achieve complete remission after treatment with CHOP followed by DHAP, she remained with persistent splenomegaly and cytopenias not attributable to chemotherapy, so she underwent haploidentical HSCT. She suffered several post-HSCT complications as thrombocytopenia, asymptomatic graft versus host disease with eosinophilia and CMV reactivation. Six months after HSCT she was in complete remission. The clinical phenotype of the patient with autoimmunity and lymphoproliferation also led to suspicion of an ALPS disorder [5, 6].

Immunologic Evaluation of ALPS-Suspected Patients

T, B, and NK compartments and ALPS biomarkers of both patients were evaluated. The immunophenotype of P1 (Table 1) remarked a profound B and NK-cell lymphopenia. CD3 + T-cells counts were normal but showed expanded

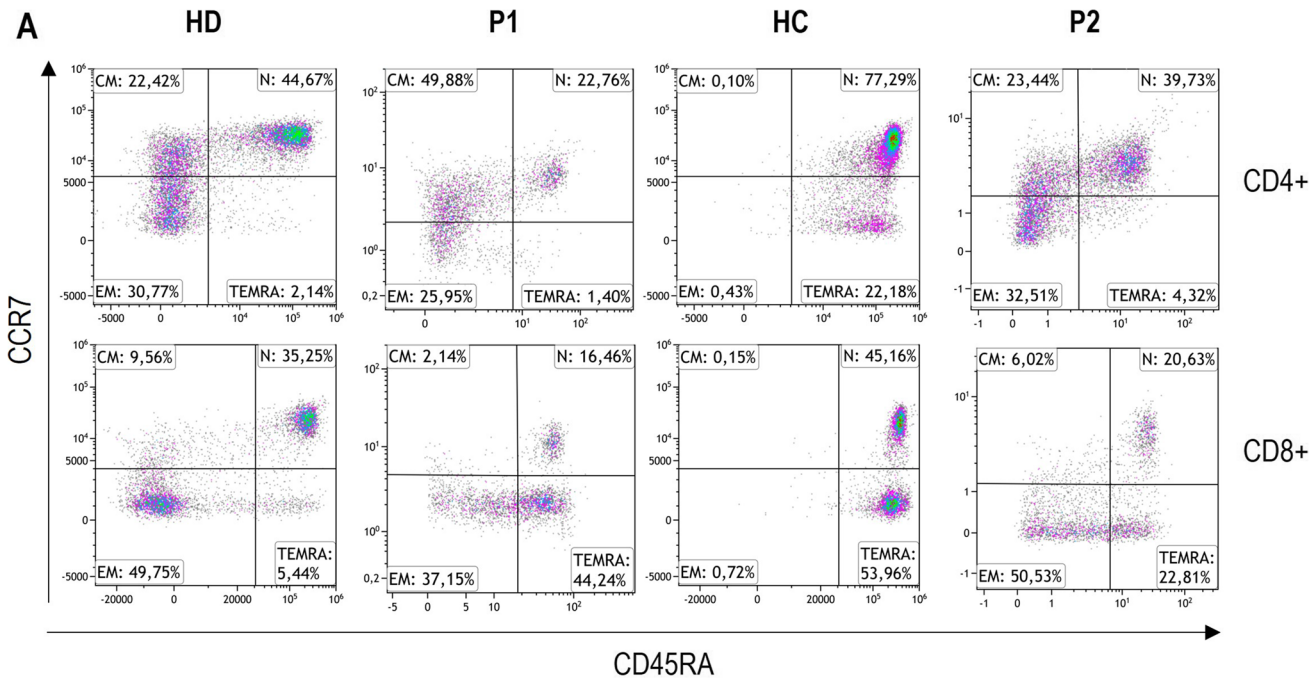
DNT, increased central memory CD4 + T-cells, and TEMRA CD8 + T-cells with an activated CD8 phenotype expressing elevated levels of HLA-DR + (Table 1, Fig. 1A, B, and Figure S3). Although the immunophenotype of P1 was studied while she was treated for lymphoma, peripheral blood immunophenotype performed between both lymphomas already showed a slight B and NK lymphopenia with a significant representation of TEMRA CD8 + T-cells (data not shown). According to the ALPS suspicion, besides expanded DNT, serum vitamin B12, IL-10, and IgG were elevated while sFASL and sCD25 levels were within the reference values range (Table 1). Fas-induced apoptosis was normal in comparison with the healthy donor assayed (data not shown). Interestingly, the healthy P1's younger brother, HC, showed an expansion of TEMRA CD4 + and -CD8 + T-cells, and CD8 + HLA-DR + T-cells with a profound decrease in the T-cell memory compartment (Table 1, Fig. 1A, B, and Figure S3). Due to the TEMRA expansion, senescence markers were analyzed. PD1 expression was evaluated in all subjects but no differences with healthy donors were shown (data not shown). The expression of CD57 could only be evaluated in HC after death of P1 and HSCT of P2. He showed a statistically significant increase in both CD4 + and CD8 + T-cells, but especially in CD8 + T-cells (Fig. 1C). Increased levels of serum IL-10 were also observed in two different samples of HC (76 and 106 pg/mL) (Table 1).

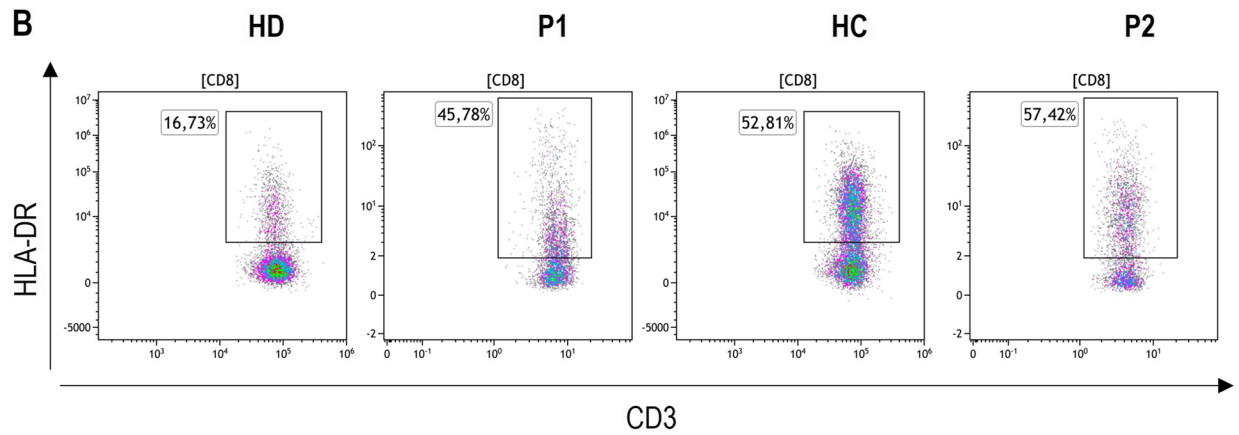
Immunophenotype at diagnosis of P2 revealed B-cell lymphopenia in peripheral blood (22 cell/μL). The T-cells subsets were not evaluated but decreased naïve CD8 + T-cell levels were noted in bone marrow (data not shown). During lymphoma treatment, whole blood was evaluated by flow cytometry (Table 1) showing lymphopenia that affected to T and B-cells counts. She showed expanded effector memory CD8 + T-cells with an activated phenotype (Table 1, Fig. 1A,

Table 1 Immunophenotype and ALPS biomarkers

Parameters	P1 (26y)	HC (16 y)	P2 (56 y)	Reference values
Total lymphocytes	1048	2110	870	1200–3000/μL
CD3 +	98 (1025)	70 (1475)	80 (697)	62–81% (850–2250/μL)
TCRαβ	95	89	98	85–99%
TCRγδ	5	11	2	1–15%
CD3 + TCRαβ + CD4-CD8- (DNT)	5	1.5	1.97	0–3.5%
CD4 +	45 (469)	40 (843)	53 (457)	32–59% (500–1450/μL)
CD4 + CCR7 + CD45RA + (N)	22.76	77.29	39.73	20–62%
CD4 + CCR7 + CD45RA- (CM)	49.88	0.10	23.44	15–35%
CD4 + CCR7-CD45RA- (EM)	25.95	0.43	32.51	20–40%
CD4 + CCR7-CD45RA + (TEMRA)	1.4	22.18	4.32	0.4–5%
CD8 +	53 (551)	23 (479)	26 (225)	15–36% (160–950/μL)
CD8 + CCR7 + CD45RA + (N)	16.46	45.16	20.63	10–50%
CD8 + CCR7 + CD45RA- (CM)	2.14	0.15	6.02	5–20%
CD8 + CCR7-CD45RA- (EM)	37.15	0.72	50.53	10–40%
CD8 + CCR7-CD45RA + (TEMRA)	44.24	53.96	22.81	5–35%
CD4/CD8	0.9	1.8	2	1–3
CD4 + HLA-DR +	20.98	15.70	15.16	0–10%
CD8 + HLA-DR +	45.78	52.81	57.42	0–10%
CD19 +	0.9 (9)	9.9 (208)	3.8 (33)	8–20% (100–500/μL)
CD56 + CD16 + CD3-	1.3 (14)	17.2 (362)	13.9 (120)	4–20% (60–450/μL)
ALPS biomarkers				
Vitamin B12	> 2000	256	> 2000	197–771 pg/mL
IL-10	189	76	12	0–20 pg/mL
sCD25	2543	1076	4264	711–4761 pg/mL
sFASL	148	142	131	0–250 pg/mL
HDL	ND	50	43	> 65 mg/dL
Immunoglobulins				
IgG	1617	1500	2185	700–1600 mg/dL
IgA	40	195	1027	40–230 mg/dL
IgM	153	142	169	70–400 mg/dL

Abbreviations: *ND*, no determined; *DNT*, double-negative T-cells; *sCD25*, soluble CD25; *sFASL*, soluble FAS ligand; *N*, naïve; *CM*, central memory; *EM*, effector memory; *TEMRA*, effector memory T-cells re-expressing CD45RA; *y*, years


A
HD
P1
HC
P2
CD4+
CD8+
CCR7
CD45RA
CM: 22,42%
N: 44,67%
EM: 30,77%
TEMRA: 2,14%
CM: 49,88%
N: 22,76%
EM: 25,95%
TEMRA: 1,40%
CM: 0,10%
N: 77,29%
EM: 0,43%
TEMRA: 22,18%
CM: 23,44%
N: 39,73%
EM: 32,51%
TEMRA: 4,32%
CM: 9,56%
N: 35,25%
EM: 49,75%
TEMRA: 5,44%
CM: 2,14%
N: 16,46%
EM: 37,15%
TEMRA: 44,24%
CM: 0,15%
N: 45,16%
EM: 0,72%
TEMRA: 53,96%
CM: 6,02%
N: 20,63%
EM: 50,53%
TEMRA: 22,81%


B
HD
P1
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P2
[CD8]
16,73%
45,78%
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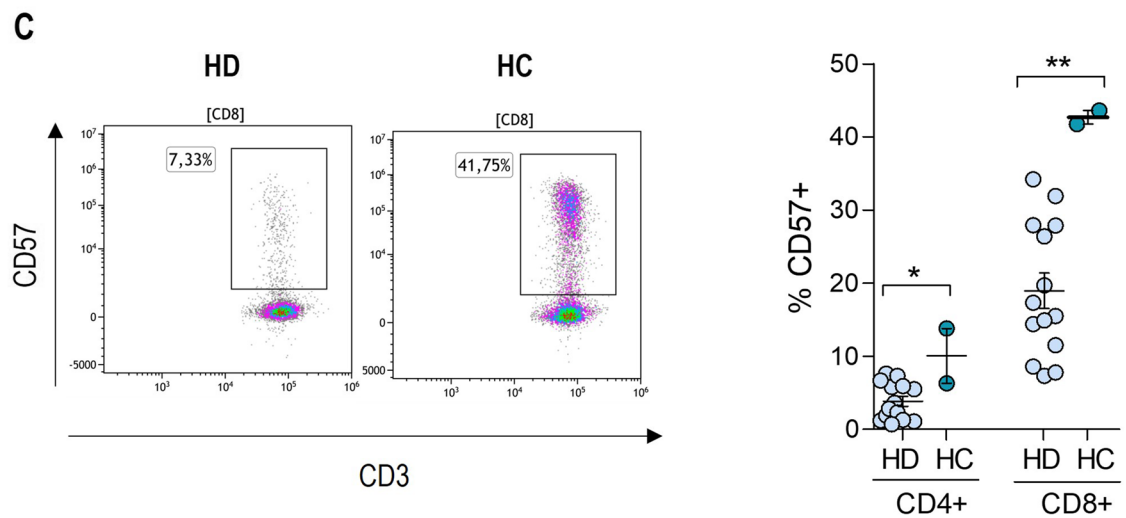
HLA-DR
CD3


C
HD
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[CD8]
7,33%
41,75%
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HD HC HD HC
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◂**Fig. 1** Flow cytometry analysis of the T-cell compartment. **A** CD4 and CD8 T-cells subpopulations analyzed in whole blood. Naïve (CD45RA + CCR7 +), central memory (CD45RA-CCR7 +), effector memory (CD45RA-CCR7-) and TEMRA (CD45RA + CCR7-). **B** HLA-DR expression in CD8 + T-cells. **C** Senescent CD57 expression in T-cells of HC and HD ($n = 14$). Two values of HC represent different samples analyzed at different times. All percentages are relative to the total of CD4 or CD8 T-cells. Abbreviations: HC, healthy carrier; HD, healthy donor; P1, patient 1; P2, patient 2

B, and Figure S3). Elevation of vitamin B12 was observed at diagnosis and still noted 3 years ago. All the others ALPS biomarkers were normal, except for the hypergammaglobulinemia and reduced HDL levels [28] (Table 1). The clinical and immune phenotype accompanied by elevation of some ALPS biomarkers led us to the diagnostic of an ALPS-like disorder [5, 6].

Identification of Novel Germline LOF Mutations in the TET2 Gene in ALPS-Like Patients

Targeted NGS gene panel of 192 genes involved in IEI was carried out to search a primary immune regulatory disorder. No pathogenic molecular candidate was found in P1 and WES study was requested. Two novel previously unreported heterozygous germline variants in *TET2* (c.277G > T, p.Gly93Ter and c.1793delA, p.Asn598IlefsTer3) were detected in WES analysis and confirmed by Sanger sequencing (Fig. 2A). Both variants had as functional consequence a premature stop codon and were predicted as pathogenic or likely pathogenic by in silico tools (Table 2). These mutations were not found in the Genome Aggregation Database (gnomAD) and the p.Asn598IlefsTer3 mutation had been previously described as somatic mutation in chronic myelomonocytic leukemia [29] (Table 2). The presence of other mutations or copy number variants (CNVs) in other known ALPS- and ALPS-like related genes were ruled out.

The familial segregation study revealed the presence of the p.Asn598IlefsTer3 mutation in the younger healthy brother (healthy carrier, HC), while no mutations were identified in her mother and older brother (Fig. 2B, Table 2). WES molecular study was also performed in HC ruling out any other pathogenic variant.

Although her father had previously died of myocardial infarction at the age of 55 years and he could not be analyzed, we assumed that the shared p.Asn598IlefsTer3 mutation between P1 and HC was of paternal origin while the p.Gly93Ter was a de novo mutation. Depending on the presence of both mutations on the same (*cis*) or opposite (*trans*) allele, P1 could suffer the monoallelic (autosomal dominant) or biallelic (autosomal recessive) form of the disease, respectively. The *trans* presence of the mutations and consecutively the autosomal recessive disorder was elucidated by using PCR-sequence specific primers (PCR-SSP) strategy (supplementary methods and Figure S2). The pathogenicity of both mutations was verified by the lack of gene and protein expression observed by qPCR and Western blot analysis, respectively (Fig. 2C, D), confirming the biallelic form of TET2 deficiency in P1.

Targeted NGS gene panel in DNA extracted from whole blood of P2 revealed a heterozygous variant in the *LRBA* gene (c.7880C > T, p.Thr2627Ile) confirmed by Sanger sequencing (Fig. 2E). This variant was reported in 7 heterozygous in the gnomAD browser and predicted as pathogenic by in silico analysis (Table 2) since the cytosine 7880 residue is only at 3 positions from the intron sequence, probably affecting the canonical splice site [37]. Autosomal recessive LRBA deficiency is common for ALPS-like phenotype [6], so a chromosomal microarray (CMA) was performed to discard any large heterozygous deletion involving the *LRBA* gene [38–40]. Surprisingly, a large heterozygous deletion of chromosome 4 affecting the *TET2* gene was identified (4q24del, chr4:105,477,859–106,196,146) (Fig. 2F and Table 2) in DNA from whole blood. This deletion uniquely leads to exon 11 conservation of TET2 gene, losing the initial methionine codon. So, we could expect haploinsufficiency of TET2 in P2. This deletion has been previously reported in hematological malignancies [35, 36] (Table 2), and as DNA of P2 was extracted when she was suffering a T-cell lymphoma, tumor cells could have altered the NGS result. However, the 4q24 deletion was also detected in DNA from a bone marrow aspirate sample extracted when the lymphoma remitted, probably confirming the germline origin of the mutation. The biallelic form of TET2 deficiency and other molecular causes of ALPS and ALPS-like were not found in P2 through WES and CMA study. Therefore, P2 presented the monoallelic form of TET2 LOF in addition to a heterozygous variant in *LRBA* that could act as a phenotype-modifier gene.

Acquired Somatic Mutations in the T-Cell Lymphoma of Patients

Targeted NGS lymphoma-related gene panel was performed in tissue samples to identify somatic mutations restricted to the T-cell lymphoma of both patients.

In the anaplastic large-cell ALK-negative lymphoma of P1, single-point mutations in *STAT3* (c.1940A > T; p.Asn647Ile), previously reported in several T and NK-cell malignancies [30, 31, 41], and in *KMT2D* (c.5509C > T; p.Leu1837Phe), a histone methyltransferase involved in the gene transcriptional regulation were identified (Table 2). This *KMT2D* somatic variant is predicted as a variant of uncertain significance (VOUS) and has not been previously reported, but the concomitant presence of *TET2* mutations have been associated with a worse prognosis in T-cells malignancies [20].

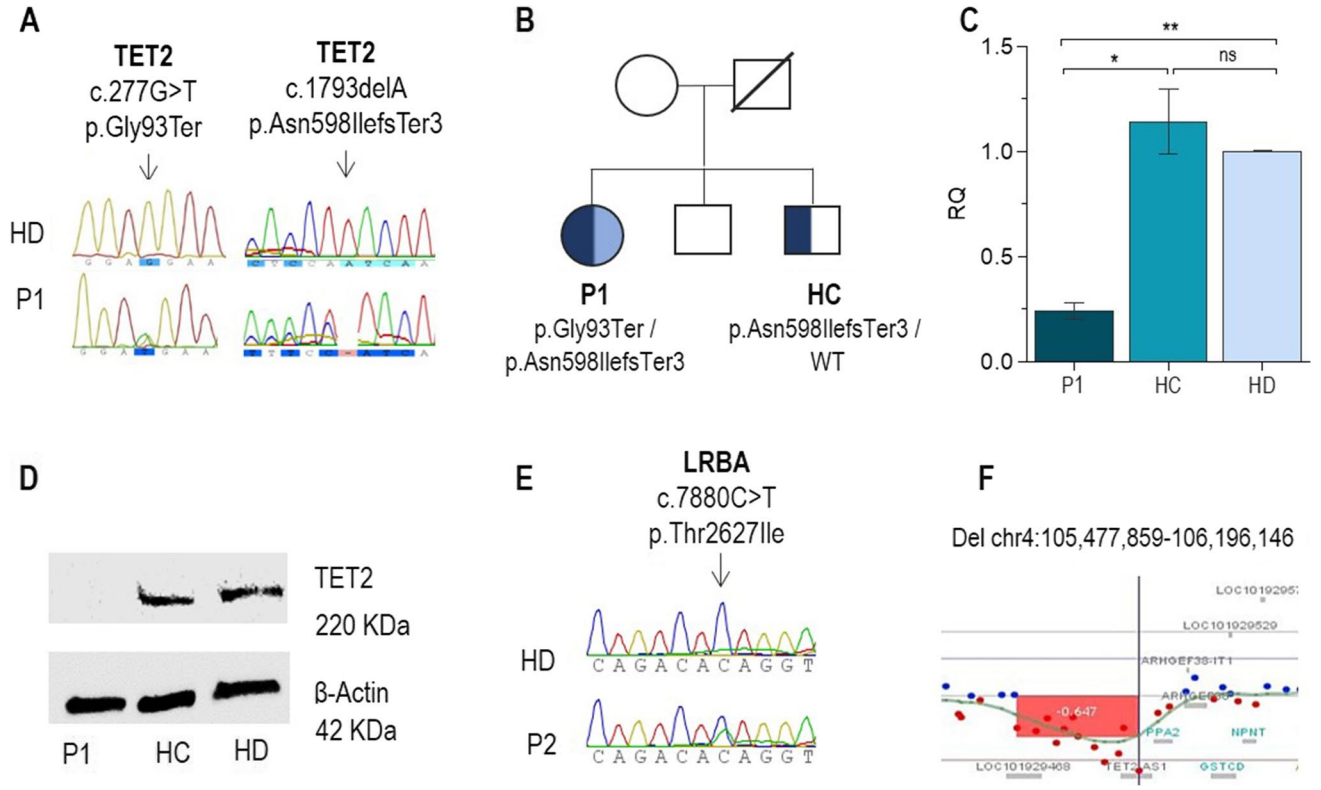

Fig. 2 Molecular diagnosis of P1 and P2. **A** Sanger sequencing of *TET2* confirming the heterozygous c.277G > T, p.Gly93Ter and c.1793delA, p.Asn598IlefsTer3 mutations identified through WES in P1. **B** Familial pedigree of P1. **C** Quantitative PCR of TET2 mRNA expression from P1, HC, and control subjects. **D** TET2 protein expression in PBMCs lysates from P1, HC, and control subjects. HC, healthy carrier; HD, healthy donor; P1, patient 1. **E** Sanger sequencing of the heterozygous c.7880C > T, p.Thr2627Ile variant identified in the *LRBA* gene through targeted NGS gene panel in P2. **F** CMA showed a heterozygous 4q24 deletion in P2 (chr4:105,477,859–106,196,146). Abbreviations: HC, healthy carrier; HD, healthy donor; P1, patient 1; P2, patient 2; RQ, relative quantification

The P2's angioimmunoblastic T-cell lymphoma carried two previously unreported acquired mutations in *TET2* (c.1617_1618insTGGGAGTGATGGGGGAGG GGAGGT; p.Glu539_Ile540insTrpGluTer) and *DNMT3A* (c.767_768dup; p.Thr257ProfsTer60) (Table 2). Unlike TET2, DNMT3A inhibits the gene transcription due to its methyltransferase activity. The co-occurrence of mutations in these genes have been reported in human T-cell lymphoma, suggesting an oncogenic cooperation between *TET2* and *DNMT3A* mutations that may involve deregulation of the methylation and demethylation processes [16].

Only Biallelic TET2 LOF Mutations Lead to Global DNA Hypermethylation But Monoallelic Carrier HC Could Present Increased Methylation in CD8 T-Cells

Considering the physiological role of TET2, LOF mutations are expected to lead to DNA hypermethylation [42]. Global DNA methylation was evaluated by relative quantification of genomic 5-mC percentage in DNA extracted from whole blood of P1, HC, and P2. Furthermore, lower activity of transcription factors involved in development, activation, cytotoxicity, and exhaustion of CD8 + T-cells and NK cells has been reported in *TET2* mutations carriers maybe implying dysregulation of cytotoxic lymphocyte maturation and function [18]. To evaluate if DNA methylation was altered in these cells, the 5-mC % was also quantified in isolated cell populations (PBMCs, NK cells, naïve, and TEMRA CD8 + T-cells) of HC and HD. P1 showed significantly increased percentage of global 5-mC in whole blood, confirming the functional consequence of TET2 deficiency, while no alteration were found in heterozygous carriers, HC and P2 (Fig. 3A). However, when methylation levels were compared between HC and HD in isolated cell populations, HC showed higher levels of 5-mC in all cell subpopulations compared with the HD, with statistically significant differences in TEMRA CD8 + T-cells (Fig. 3B).

Monoallelic and Biallelic TET2 LOF Implies the Alteration of Innate and Adaptive Immunity

Although the role of human TET2 in the immune system is barely studied, mice deficient for TET proteins have been

Table 2 Germline and lymphoma-restricted somatic variants identified in the patients

Patient	Gene	cDNA	Protein	Zygosity (VAF)	gnomAD	COSMIC	CADD	In silico prediction (P/B)
P1	TET2	c.1793delA	p.Asn598Ilef-sTer3	Heterozygous (42.7%)	NR	COSV54426182 [29]	NA	LP*
	TET2	c.277G > T	p.Gly93Ter	Heterozygous (40%)	NR	NR	26.6	P (7/2)
	STAT3	c.1940A > T	p.Asn647Ile	Somatic (17.7%)	NR	COSM1155744 [30–34]	23.7	P (11/8)
	KMT2D	c.5509C > T	p.Leu1837Phe	Somatic (13.1%)	NR	NR	16.1	VOUS (1/17)
HC	TET2	c.1793delA	p.Asn598Ilef-sTer3	Heterozygous (50%)	NR	COSV54426182 [29]	NA	LP*
P2	LRBA	c.7880C > T	p.Thr2627Ile	Heterozygous (49.5%)	7He	NR	26.4	P (12/8)
	TET2	4q24del	-	Heterozygous	NR	COSP21637 [35, 36]	NA	P*
	TET2	c.1617_1618insTGGGAGTGATGGGGGAGGGGAGGT	p.Glu539_Ile540insTrp-GluTer	Somatic (38%)	NR	NR	NA	VOUS/LP*
	DNMT3A	c.767_768dup	p.Thr257Prof-sTer60	Somatic (14%)	NR	NR	NA	P*

Abbreviations: *VAF*, variant allele frequency; *NR*, no reported; *NA*, no analyzable; *He*, number of heterozygotes reported in gnomAD browser; *COSMIC*, Catalogue of Somatic Mutations in Cancer; *CADD*, Combined Annotation Dependent Depletion; in silico prediction was evaluated in the human genomics community Varsome; (P/B): number of pathogenic versus benign predictors in Varsome; LP: likely pathogenic in Varsome; P: pathogenic in Varsome; VOUS: variant of uncertain significance in Varsome; *no pathogenicity predictors available in Varsome

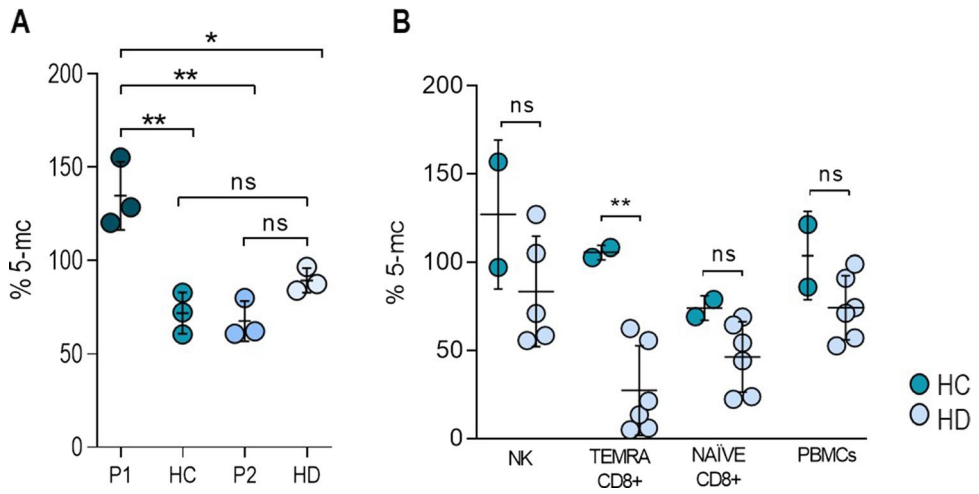

Fig. 3 DNA methylation assay. **A** The relative quantification of methylation levels in DNA extracted from whole blood is represented as percentage of 5-mC. Three different experiments with duplicated samples were done. **B** Methylation levels in DNA extracted from sorted NK, TEMRA, and naïve CD8 + T-cells and PBMCs in HC and HD ($n = 6$). Two different experiments with duplicated samples were done in HC. Error bars show mean and SD values. Abbreviations: ns, not statistically significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; HC, healthy carrier; HD, healthy donor; P1, patient 1; P2, patient 2

used to inquire their impact in immune cell development and function [10, 11]. We wanted to further evaluate the effect of the *TET2* mutations in the differentiation of the innate and adaptive immune system through the immunophenotyping of PBMCs (Figs. 4 and 5). This was only possible in P1 and HC because P2 had already undergone a HSCT.

According to the TET2-mediated DNA methylation-dependent in vitro differentiation of human monocytes [43],

both P1 and HC showed normal levels of dendritic cells (DCs) but inverted myeloid/plasmacytoid DCs ratio (mDCs/pDCs) (Fig. 4A) compared with healthy donors (Fig. 4B) [44]. Exacerbated and dysregulated monocytes and macrophages activation has been proposed to be essential for accelerated atherosclerosis due to somatic *TET2* mutations in humans and Tet2 loss in mice [45, 46].

Some groups have reported decreased levels of regulatory T-cells (Tregs) in Tet2-deficient mice due to TET2 is essential for the stabilization of FoxP3 [22, 47, 48]. Surprisingly, flow cytometry showed elevated percentage of CD4 + CD25 + FoxP3 + (Fig. 5A) and CD4 + CD25 + CD127- Tregs (data not shown) in P1. Tregs can be subdivided into two populations based on Helios expression, natural/thymic Tregs (nTregs, Helios +), and peripheral Tregs (pTregs, Helios −) [49]. An important elevation of nTregs was noted in P1 while no alteration of pTregs or nTregs was found in HC (Fig. 5B). In view of these results and the elevated serum IL-10 levels in both P1 and HC, we wanted to characterize the function of Treg cells by in vitro suppression capacity assay. Unfortunately, P1 died before she was tested and HC showed normal suppression function (Figure S4).

Mice that lack Tet2 exhibited also compromised differentiation toward CD4 + helper T-cells (Th) and follicular helper T-cells (Tfh) [50]. Spegarova et al. reported decreased Th17 and Th1 and relatively higher Th2 phenotype with complete lack of circulating Tfh (cTfh) cells [8]. In P1 and HC, cTfh were partially reduced but no absent, compared with the evaluated healthy donors (Fig. 5C). cTfh subsets were also evaluated, showing normal levels of cTfh1 and cTfh17, but decreased cTfh1/17 and an important switch to cTfh2 phenotype (Fig. 5D).

Discussion

The development of hematologic malignancies is commonly driven by somatic mutations but recent studies have shown that germline cancer predisposition may not be as rare as previously presumed [51, 52]. Somatic *TET2* mutations occur

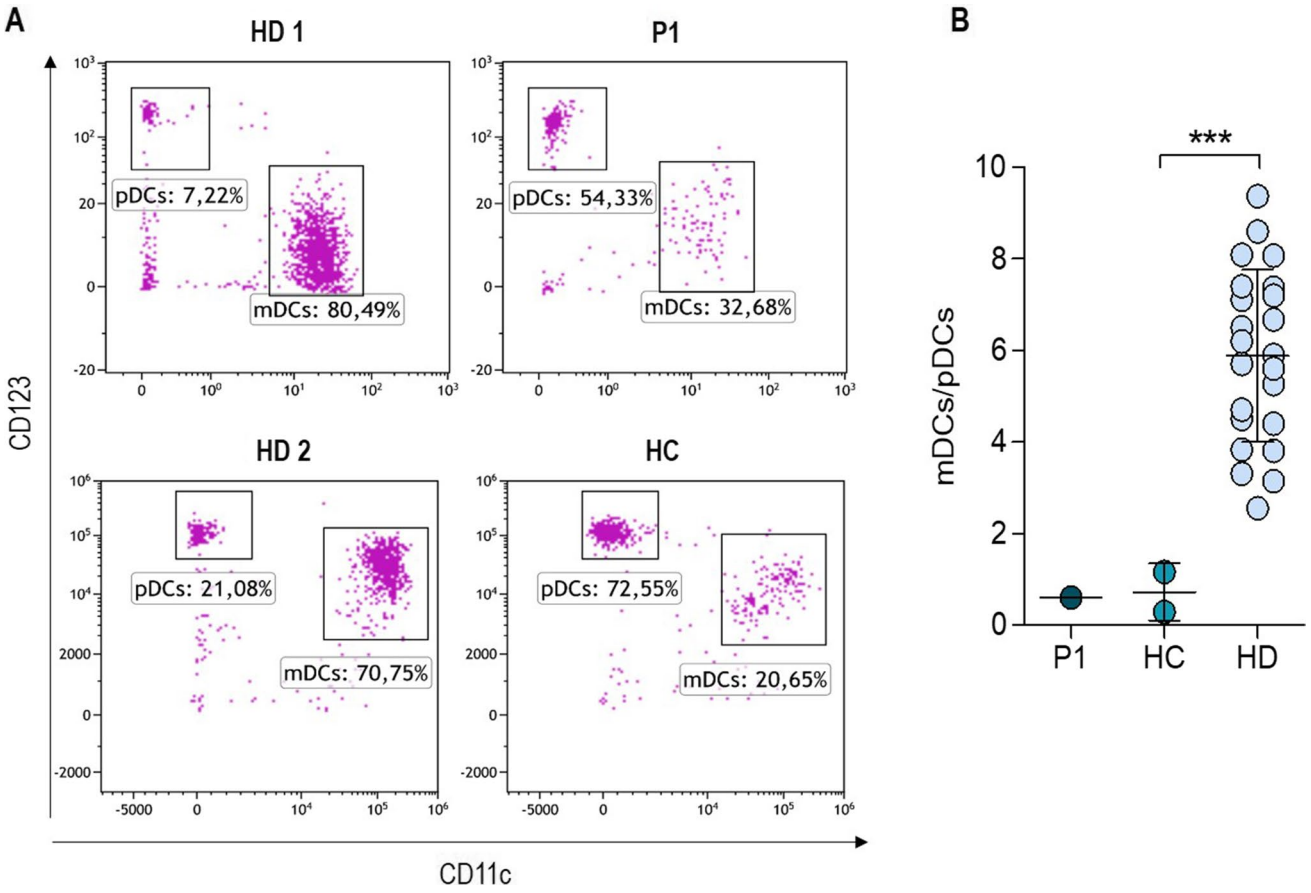

Fig. 4 Flow cytometry analysis of dendritic cells subpopulations in P1 and HC. **A** Subpopulations of dendritic cells represented as CD45 + Lin-(CD3, CD19, CD20, CD56, CD14), HLADR + CD11c-CD123 + (plasmacytoid dendritic cells, pDCs), and CD45 + Lin-HLADR + CD11c + CD123- (myeloid dendritic cells, mDCs). Two healthy donors are shown because different flow cytometers with different settings were used into samples. **B** mDCs/pDCs ratio calculated from percentages of total DCs in P1, HC, and HD ($n = 23$). Two values of HC represent different samples analyzed at different times. Abbreviations: HC, healthy carrier; HD, healthy donor; P1, patient 1. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

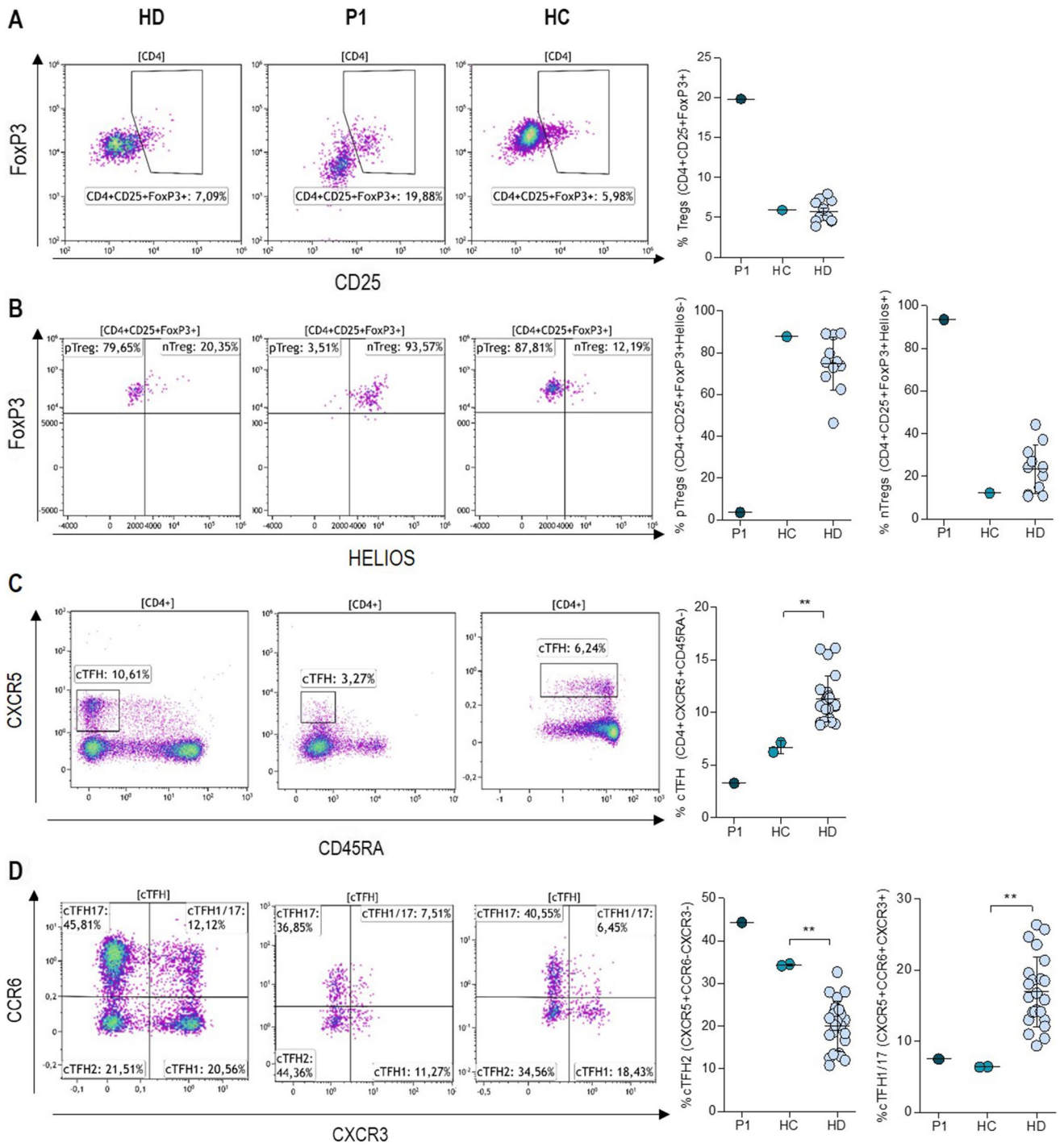

Fig. 5 Flow cytometry analysis of regulatory and follicular T-cells compartment in P1, HC, and HD ($n=23$). **A** Regulatory T-cells represented as CD45 + CD3 + CD4 + CD25 + FoxP3 + . Percentages are relative to total CD4 + T-cells. **B** Peripheral (CD45 + CD3 + CD4 + CD25 + FoxP3 + Helios-, pTreg) and natural (CD45 + CD3 + CD4 + CD25 + FoxP3 + Helios + , nTreg) subpopulations of Tregs. Percentages are relative to total CD4 + CD25 + FoxP3 + cells. **C** Percentage of circulating follicular helper T-cells represented as CD45 + CD3 + CD4 + CD45RA-CXCR5 + cells. **D** Subpopulations of cTfh, based on the surface expression of CXCR3 and CCR6 (cTfh1: CXCR3 + CCR6-; cTfh2: CXCR3-CCR6-; cTfh17: CXCR3-CCR6 + ; cTfh1/17: CXCR3 + CCR6 +). Abbreviations: HC, healthy carrier; HD, healthy donor; P1, patient 1. $*p<0.05$; $**p<0.01$; $***p<0.001$

in approximately 10–30% of myeloid malignancies and are also common in both T and B-cell lymphomas. However, *TET2* mutations are often an early event, maybe requiring a second hit to induce lymphomagenesis since clonal hematopoiesis of indeterminate potential (CHIP) due to acquired *TET2* mutations is also detected in peripheral blood cells of healthy elderly [53]. Recently, germline monoallelic and biallelic LOF mutations in the human *TET2* gene causing susceptibility to malignancy have been described [8, 17–19]. Spegarova et al. reported for the first time the autosomal recessive germline TET2 deficiency in three patients of two unrelated families with ALPS-like phenotype [8]. Here we have described two novel unrelated patients with biallelic and monoallelic alterations in *TET2*, both presenting with ALPS-like phenotype. Interestingly, neither patients with somatic mosaicism nor monoallelic disease in the *TET2* gene suffering the ALPS-like phenotype have been reported in the literature.

The growing number of reports of incomplete penetrance or variable expressivity of monogenic disorders in IEI have led to consider additional genetic patterns. Somatic mosaicism, digenic inheritance, epistasis, or genetic defect accumulation as a primary cause of IEI have emerged, particularly in patients with late-onset presentation [54–58]. In ALPS, the reduced penetrance observed in some cases suggests that a second hit is required for a full clinical expression. Combination of somatic and germline mutations and involvement of other ALPS-phenotype-modifier genes that may contribute to clinical manifestations and outcome have been described [58–61]. As far as we know, P2 is the first monoallelic patient for TET2 with an ALPS-like phenotype. So, we speculate that combined *LRBA* and both germline and somatic mutations in *TET2* could have triggered the late-onset susceptibility to immune dysregulation, being the interaction of both genes which contributed to the severe clinical phenotype. Due to HSCT performed in P2, no more molecular and functional studies could be done. However, more studies are needed to determine the role of each mutation separately prior to attributing the epistatic effects to her phenotype.

Regulation of DNA expression consists in a competitive balance between methylation and demethylation process. TET2 LOF breaks that equilibrium leading to DNA hypermethylation. As expected, biallelic P1 showed significantly increased percentage of 5-mC in whole blood but by the contrary to previously reported by some authors, the monoallelic subjects studied (P2 and HC) did not shown elevation of 5-mC levels respect to the healthy donors. Interestingly, isolated NK, CD8 + T-cells, and PBMCs of HC had relatively higher 5-mC levels compared to the healthy control, with statistical differences in TEMRA CD8 + T-cells, suggesting a mild effect of TET2 LOF in some cellular subpopulations, in which TET2 has a key role for development, activation, cytotoxicity, and exhaustion [18].

Although not yet elucidated in humans, murine models with TET deficiency have been used to inquire the impact of TET2 in immune cell development and function, placing it as link between cancer and immunity [12, 62]. There is evidence that several innate and adaptive signaling pathways require TET2-dependent DNA demethylation. In particular, TET2 seems to be important in the maturation and differentiation of monocytes, B, and T-cells [43, 63]. P1 and HC showed an innate immune system alteration with inverted mDC/pDC ratio. In the case of T-cell compartment of P1, it was striking the high levels of TEMRA CD8 + T-cells and Tregs, the skewed phenotype towards cTfh2, and the high levels of serum IL-10 that could promote an autoimmune phenotype as in ALPS patients [5]. An intermediate phenotype was observed in the asymptomatic HC, with high levels of TEMRA T-cells and IL-10.

FoxP3 is a master regulator of transcription necessary for the development and differentiation of Tregs. P1 showed elevated percentage of CD4 + CD25 + FoxP3 + cells. By the contrary, Spegarova et al. reported normal Tregs but, in accordance with our results, some murine studies showed that Tet deficiency in Tregs promotes an abnormal proliferation leading to lymphoid tissue hyperplasia [64]. The functional stability of these cells has been correlated to the demethylated state of the Treg-specific demethylation region (TSDR) of the FoxP3 promoter [48, 65, 66]. Whereas Foxp3 + Helios + nTregs are demethylated at all residues in the TSDR region allowing stable FoxP3 expression, Foxp3 + Helios- pTregs display only partial demethylation [67, 68]. In this case, the switched phenotype into nTreg with absence of pTregs observed in P1 could be explained by her intrinsic alteration of methylation–demethylation balance due to TET2 LOF. Indeed, it is reported a subset of FoxP3 + IFN-γ + cells being the majority Helios- pTregs and it is thought that probably convert to an effector Th1 T-cell during an inflammatory response in vivo [67, 68]. The lack of pTregs in P1 could compromise the role of Tregs in controlling autoimmunity and suppressing inflammatory process. In the context of cancer, increased numbers of Tregs are also often observed. They are present in the tumor, promoting tumor development by dampening anti-tumor immune response. Therefore, if the elevated levels of Tregs in P1 is an early event, consequence of the TET2 deficiency or a response of the immune system during tumor progression is yet unknown [69].

Interestingly, functional relation between DCs and Tregs has been established, as well as that interactions between DCs, CD4 + T-cells, and B-cells are required for Tfh differentiation [70–72]. The increased Tregs and nTregs in P1, the decreased cTfh levels, and the skewed phenotype towards cTfh2 cells observed in P1 and HC could suggest a regulatory role of TET2 in the differentiation of these cells, as previously reported in the literature [8, 50, 73], what could be more affected by the deficiency of B-cells in P1 and the alteration of DC subsets.

TET2 is one of the earliest and most frequently mutated gene in CHIP and shows significant associations with the risk of coronary heart disease or early-onset myocardial infarction in humans and accelerated atherosclerosis in mice. A probable mechanism proposed is that an altered clonal hematopoiesis in TET2-deficient macrophages and monocytes promotes their recruitment and local inflammation in plaques in the arteries, accelerating atherosclerosis [18, 45, 46]. In this context, the father of P1 had previously died of myocardial infarction and he was an obligate carrier of the p.Asn598IlefsTer3 mutation. Considering the possible role of TET2 as a pre-malignant state, the association with risk of coronary heart disease and the outcome of these patients, the asymptomatic carriers such as HC should be periodically monitored to anticipate a malignant transformation and/or possible signs of cardiovascular disease.

In summary, the role of TET2 LOF over immune system and the reported impaired suppressor capacity of tumors in the literature point out a direct impact of germline and probably somatic *TET2* mutations over clinical, immunologic phenotype, and tumor progression. The study of TET2 LOF in patients prior to the malignant transformation would be needed to further understand the role of TET2 in controlling the innate and adaptive immune system and to clarify whether the alterations found are caused by the TET2 deficiency or a consequence of the tumor progression in these patients.

The report of these novel patients allows to confirm and expand the recently described IEI associated to germline TET2 LOF, consisting in ALPS-like syndrome and development of lymphomas.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10875-022-01361-y.

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Author Contribution M. L.-N. and L. M. A. contributed to conception, design of the study, and drafted the manuscript. M. L.-N. did the molecular and functional studies and analyzed the results and constructed the tables and figures. F. J. G.-E. contributed to the sample preparation, DNA, and protein extraction and functional studies. J. O.-M., C. S., M. A. P.-S., J. L. L.-L., E. R.-F., O. C.-M., C. M.-C., and R. A.-S. conducted the clinical and immunological follow-up of the patients and informed them about the study and collected the informed consents approved by the ethics committee. M. S. R.-P., R. M., R. N. S.-S., and A. C.-M. facilitated the molecular studies in the T-cell lymphomas. J. F. Q.-E. and M. J. G.-R. did the whole exome sequencing and chromosomal microarray studies. J. O. –M., C. S., C. M.-C., R. A.-S., P. M.-P., and E. P.-A. contributed to the critical review of the manuscript. All authors contributed to the article and approved the submitted version.

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Data Availability Not applicable.

Declarations

Ethics Approval All experimental work was performed under protocols approved by the Institutional Review Board (IRB) of the Institution (imas12), after written informed consent for publication of clinical and immunological information of the patients.

Consent to Participate All participants provided written informed consent to participate.

Consent for Publication All participants have consented to publication of their data.

Conflict of Interest The authors declare no competing interests.

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