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GERMLINE TET2 LOSS-OF-FUNCTION CAUSES CHILDHOOD IMMUNODEFICIENCY AND LYMPHOMA

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Key points:

- We report autosomal recessive germline deficiency of the methylcytosine dioxygenase TET2 in three immunodeficient children.
- Their phenotype of immunodeficiency, autoimmunity and lymphoproliferation highlights requisite roles of TET2 in the human immune system.

ABSTRACT

Molecular dissection of inborn errors of immunity can help to elucidate the nonredundant functions of individual genes. We studied three children with an immunodysregulatory syndrome of susceptibility to infection, lymphadenopathy, hepatosplenomegaly, developmental delay, autoimmunity and lymphoma of either B- (n=2) or T-cell (n=1) origin. All three showed early autologous T-cell reconstitution following allogeneic hematopoietic stem cell transplantation.

By whole exome sequencing, we identified rare, homozygous, germline missense or nonsense variants in a known epigenetic regulator of gene expression, Ten-Eleven Translocation methylcytosine dioxygenase 2 (*TET2*). Mutated TET2 protein was either absent or enzymatically defective for 5-hydroxymethylating activity, resulting in whole blood DNA hypermethylation. Circulating T-cells showed an abnormal immunophenotype including expanded double-negative but depleted follicular helper T-cell compartments, and impaired Fas-dependent apoptosis in 2/3 patients. Moreover, TET2 deficient B-cells showed defective class-switch recombination. The hematopoietic potential of patient-derived induced-pluripotent stem cells was skewed towards the myeloid lineage.

These are the first reported cases of autosomal recessive germline TET2 deficiency in humans, causing clinically significant immunodeficiency and an autoimmune lymphoproliferative syndrome with marked predisposition to lymphoma. This disease phenotype demonstrates the broad role of TET2 within the human immune system.

INTRODUCTION

Inborn errors of immunity (IEI) are rare inherited diseases caused by aberrations in the genome [1,2], leading to functional abnormalities in the immune system that range from susceptibility to infection to immune dysregulation. Predisposition to blood cancers is a relatively frequent manifestation of IEI, in many cases directly attributable to the effect of the disease-causing mutation on lymphocyte behavior [3]. One such example is provided by autoimmune lymphoproliferative syndrome (ALPS), in which uncontrolled non-malignant lymphoproliferation results from impaired apoptosis of lymphocytes and results in a high risk for development of lymphomas as well as autoimmune phenomena such as cytopenias [4-7]. Not surprisingly, there is an overlap between those genes that harbor germline mutations causing immunodysregulatory disorders and genes seen commonly to acquire somatic variants in the context of sporadic blood cancers.

Here we report for the first time two novel germline loss-of-function point mutations in Ten-Eleven Translocation methylcytosine dioxygenase 2 (*TET2*), causing an autosomal recessive syndrome of immunodeficiency with lymphoproliferative disease in three children from two unrelated consanguineous families. Consistent with a tumor suppressor role, somatic *TET2* loss-of-function mutations are frequently observed in hematopoietic disorders, myeloid and lymphoid malignancies, as well as clonal hematopoiesis of indeterminate potential (CHIP) [8-14]. TET2, along with TET1 and TET3, is a member of the TET family of epigenetic regulators, whose enzymatic function is to convert 5-methylcytosine (5mC), an important epigenetic modification of the mammalian genome, to 5-hydroxymethylcytosine (5hmC) and further oxidation products in a pathway of active DNA demethylation [15,16].

Furthermore, TETs interact with histone-modifying enzymes and transcription factors to exert additional epigenetic effects. Thus TET2 activity shapes the local chromatin environment, especially at active enhancers and cell-type specific regulatory regions, influencing their accessibility to master transcription factors involved in hematopoiesis [17-19].

TET2 is highly expressed in hematopoietic stem/progenitor cells and plays important roles in hematopoiesis, including regulating the self-renewal of stem cells, lineage

commitment, and differentiation of monocytes [20-22]. Effects on the transcriptional programme and function of lymphocytes have been proposed in several murine models of TET2 deficiency that are viable, fertile and develop normally, but demonstrate myeloproliferation, splenomegaly, monocytosis, myeloproliferative diseases and lymphomagenesis [23-28].

In this study, we define the molecular and clinical consequences of homozygous germline TET2 impairment in three immunodeficient children, which we demonstrate leads to altered DNA methylation and B-cell maturation, skewed T-cell differentiation and hematopoiesis, and development of lymphomas in childhood.

METHODS

Detailed protocols including antibodies used are provided as supplemental material, available on the *Blood* website.

Patient cohort

The parents of all patients and healthy donor volunteers provided written informed consent to participate in research protocols approved by the local Research Ethics Committee.

Genetic analysis

Patient genomic DNA was subjected to whole exome sequencing (WES), and analysed by standard methods. This revealed candidate disease-causing variants in *TET2*, which were confirmed by Sanger sequencing. The patients' lymphoma tumour samples were analysed for additional somatic mutations by WES.

TET2 protein expression and enzyme activity

Expression of TET2 protein was determined by immunoblotting. Enzymatic activity was assessed by immunofluorescence staining of 5hmC after transfection of HEK293T cell line with either wild type or mutated $TET2^{H1382R}$.

DNA methylation assay and 5hmC staining

Methylation and 5-hydroxymethylation of DNA was analysed by enzymatic assay, involving glucosylation followed by enzyme restriction with MspI (R0106S) and HpaII (R0171S) at the DNA sequence '5 CCGG 3'. MspI cleaves both 5mC and 5hmC, but not 5ghmC, whereas any modification with 5mC, 5hmC, or 5ghmC at either cytosine will prevent cleavage by HpaII.

B-cell differentiation assay

Isolated peripheral blood B-cells were stimulated with CD40L and $F(ab')_2$ anti-IgG/M, mimicking a T-dependent immune response. The immunophenotype of differentiating cells was analysed by flow cytometry and the secretion of immunoglobulin IgG and IgM was in parallel detected by ELISA.

iPSC derivation and hematopoietic differentiation

Patients' fibroblasts were reprogrammed into induced pluripotent stem cells (iPSC) using Yamanaka factors, characterized for their pluripotent potential, and differentiated to hematopoietic precursors *in vitro*. The proportion of erythro-megakaryocytic and myeloid progenitors in culture and their clonogenic potential were determined by flow cytometry and colony forming unit assay, respectively.

FasL-induced apoptosis assay by Annexin V/ PI staining

Peripheral blood mononuclear cells (PBMC) blasted with PHA and IL-2 were stimulated with a soluble Fas Ligand set, stained with Annexin V-FITC and PI, and analysed by flow cytometry.

Statistical analysis

Data are shown as mean ± SD of at least two independent experiments. Nonparametric One-Way ANOVA Kruskal-Wallis with Dunn's multiple comparisons test, two-tailed t-test, and

unpaired t-test were used to calculate statistical significance with $p<0.05$ using GraphPad Prism 7.02 software.

RESULTS

Case descriptions

Patient 1 (P1), was the second-born child of related parents. He presented to hospital at 4 weeks of age with lower respiratory tract infection (LRTI) associated with Respiratory Syncytial Virus (RSV) and Cytomegalovirus (CMV), treated with ganciclovir. Subsequently, P1 showed failure to thrive and developmental delay, and was frequently hospitalized for LRTI. From 18 months of age, he developed autoimmune cytopenias requiring frequent transfusions, accompanied by hepatosplenomegaly, chronic lymphadenopathy, hypergammaglobulinemia and persistent EBV viremia. The diagnosis of ALPS-U was confirmed with the demonstration of defective Fas-mediated apoptosis (**Figure 1A**) and raised double negative (CD4-CD8-) TCRαβ T-cells (DNT, 20%) (**Table 1**), in the absence of known genetic causes of ALPS [29].

P1 initially responded well to immunomodulation with high dose (2g/kg) intravenous immunoglobulin, rituximab (anti-CD20 antibody) and corticosteroid. However, at 4 years his condition deteriorated markedly with massive hepatosplenomegaly, liver dysfunction and worsening lymphadenopathy reflecting the development of EBV-positive Hodgkin-like polymorphic B-cell lymphoproliferative disorder (**Figure 1B, Supplementary Figure S1A**). Despite hyperhydration, treatment with low intensity chemotherapy (vincristine and rituximab) led to tumor lysis syndrome with acute renal failure, requiring prolonged intensive care, and further complicated by *Stenotrophomonas* pneumonia and sepsis. He received four doses of adoptive EBV-specific cytotoxic T-cells with therapeutic benefit and underwent splenectomy in preparation for hematopoietic stem cell transplant (HSCT).

Patient 2 (P2) was the younger brother of P1. His health problems began with transient hematuria and nephrotic range proteinuria in the first 4 weeks of life. Subsequently he was noted to have hypothyroidism and hypogammaglobulinemia that were attributed to renal losses

and treated with thyroxine and immunoglobulin supplementation; lymphocyte numbers were normal. He developed CMV viremia around 8 weeks of age with evidence of respiratory involvement, treated with ganciclovir. At around the same age he began to develop hepatosplenomegaly and lymphadenopathy. He was thrombocytopenic but no autoantibody tests were documented and Coomb's test was negative. The clinical diagnosis of ALPS was however supported by the demonstration of defective Fas-mediated apoptosis (**Figure 1A**), variably increased DN TCRαβ T-cells (1.9 %) and raised soluble Fas ligand, 0.96 ng/ml (**Table 1**). In addition there was a very low fraction of IgM memory (0.33%) and class-switched memory B-cells (0.03%). A lymph node biopsy showed a nodal peripheral T-cell lymphoma of T follicular helper (Tfh) phenotype and clonal *TCRG* gene rearrangement (**Figure 1B, Supplementary Figure S1B**). He was treated with cyclophosphamide and methylprednisolone for his lymphoma; only one dose of vincristine was given due to deranged liver function.

Patient 3 (P3) was the second child born to related parents from the same community as P1 and P2. Her problems with infection emerged at around 18 months, with frequent LRTIs requiring multiple courses of antibiotics and, on at least 2 occasions, ventilatory support. There was also a history of loose stools and relatively poor weight gain. Immunologic investigations suggested impaired humoral immunity: she was IgA-deficient, with reduced levels of IgM and IgG2 subclass and impaired response to pneumococcal vaccination. Subsequent investigations showed essentially normal lymphocyte subsets, except for absent class-switched memory B-cells. Despite immunoglobulin replacement, antibiotic prophylaxis and physiotherapy, she progressed to bronchiectasis with an overnight oxygen requirement. Furthermore she showed chronic hepatosplenomegaly and lymphadenopathy as well as increased DNT cells in peripheral blood (9% aged 8 years). A lymph node biopsy showed EBV-associated follicular hyperplasia (**Supplementary Figure S1C**) while Fas-dependent apoptosis as well as T-proliferative responses were normal (**Figure 1A, Table 1**). Although there was no definite evidence of autoimmunity either clinically or serologically, moderate

thrombocytopenia was evident over several years. During this time she developed two benign skin tumors: a cellular dermatofibroma and a pilomatrixoma (**Supplementary Figure S1C**).

These immunological features occurred against a background of significant global developmental delay, for example P3 walked at 4 years of age and was not able to attend mainstream school. She had feeding problems and there was a suspicion of recurrent aspiration, managed by fundoplication and creation of a gastrostomy for enteral feeding.

At the age of 12 years, P3 presented with a mediastinal mass and pericardial effusion and investigations revealed a primary mediastinal large B-cell lymphoma (**Figure 1B, Supplementary Figure S1C**). She tolerated R-CHOP chemotherapy and went into remission. At around this time she developed worsening headaches and idiopathic intracranial hypertension was detected. Imaging revealed skull thickening consistent with extramedullary hematopoiesis.

Early autologous T-cell reconstitution after allogeneic hematopoietic stem cell transplantation

After extensive discussions with the family of each patient, and in view of the ongoing risks to health and quality of life, allogeneic HSCT was attempted. Reflecting donor availability and changing clinical practice, each patient received a different conditioning regimen and stem cell source and dose, but outcomes were universally poor (**Supplementary Table S2**). Remarkably, all three patients showed autologous T-lymphoid reconstitution within six weeks of HSCT despite a T-depleting conditioning regimen containing full dose serotherapy (**Figure 1C**). P2 rejected his haplo-identical maternal transplant and died of sepsis, while his elder brother P1 developed mixed chimerism after a sibling donor transplant, followed by symptomatic relapse of ALPS and disordered hematopoiesis (**Supplementary Figure S2**). P3 tolerated myeloablative conditioning poorly and developed multi-organ failure requiring intensive care (respiratory, renal, circulatory and gut). Although she survived this phase to receive peripheral blood stem cells from an 11/12 matched unrelated donor, she showed very early reconstitution of autologous T-cells with progressive loss of her graft despite clinical

evidence of graft-versus-host disease of skin and liver (grade III). Care was shifted to a palliative footing at home, where she died.

The clinical and laboratory phenotypes of all three patients are outlined in **Table 1 and Supplementary Tables S1 and Supplementary Figure S3**. Details of their HSCT are provided as **Supplementary Table S2.**

Homozygous loss-of-function mutations of *TET2*

In view of the striking combination of immunodeficiency, lymphoma and developmental delay in the setting of consanguinity, an autosomal recessive inborn error was suspected. WES excluded genes already implicated in ALPS (such as *FAS, FASLG, CASP10, CTLA4, KRAS, MAGT1, NRAS, PIK3CD, STAT3)* [1] and other known disorders, but revealed homozygous, predicted damaging mutations within the same gene, *TET2*, in the affected children of both kindreds (**Supplementary Table S3**). Sanger sequencing confirmed that each healthy family member carried at least one wild type allele of *TET2*, consistent with autosomal recessive inheritance (**Figure 2A**). This gene encodes the epigenetic regulator Ten-Eleven Translocation 2, TET2, a 2-oxoglutarate- and Fe^{2+}-dependent methylcytosine dioxygenase. Somatic mutations in *TET2* are strongly linked with CHIP and hematological malignancy, making it a strong candidate disease-causing gene in our patients (reviewed in [30]).

Patients P1 and P2 bore the homozygous missense mutation c.4145A>G, p.H1382R in exon 9 of *TET2*. This was predicted to be highly damaging since it affects the Fe^{2+}-binding motif, known to be critical for TET2 enzymatic activity ([31], **Figure 2B**). Furthermore, we identified the same variant in heterozygosity in patients with myeloid malignancy (3/1221 patients with AML and 1/286 patients with CMML; **Supplementary Table S4**, [32]). The expression of $TET2^{H1382R}$ protein was not impaired relative to $TET2^{wt}$ either in primary cells (**Figure 2C**) or in a recombinant system (**Figure 2D**). We compared the enzymatic activity of $TET2^{wt}$ and $TET2^{H1382R}$ by immunofluorescence microscopy analysis of transfected HEK293T cells stained for 5hmC [22,31]. In contrast to $TET2^{wt}$-overexpressing cells, there was no increase

of 5hmC staining in cells expressing mutated TET2^{H1382R}, thus confirming the predicted loss of its 5-hydroxymethylating enzymatic activity (**Figure 2D**).

The homozygous variant borne by P3 was a nonsense mutation, c.4894C>T, p.Q1632* (**Figure 2A**). Again, this variant was represented among a cohort of patients with myeloid malignancy (2/1221 AML, 2/286 CMML; **Supplementary Table S4**, [32]). Immunoblotting of peripheral blood mononuclear cells (PBMC) confirmed loss of TET2 protein expression in the proband, whereas heterozygous relatives showed intermediate levels relative to wild type control (**Figure 2C**).

TET2 loss-of-function results in DNA hypermethylation

We hypothesized that the loss of TET2 enzymatic activity would manifest as an alteration in global DNA methylation/hydroxymethylation status within the hematopoietic compartment. To test this, relative quantification of genomic 5mC and 5hmC was performed in DNA extracted from PBMC. All patients showed a profound increase in DNA methylation, especially relative to 5hmC. Heterozygous carriers of pathogenic mutations showed a smaller but measurable increase in global methylation associated with a reduction in 5hmC (**Figure 2E, Supplementary Figure S4**). We conclude that the *TET2* alleles associated with a novel inherited immunodeficiency syndrome produce wide-scale alterations of DNA methylation and hydroxymethylation of circulating leukocytes, with a gene dosage effect.

Altered T-cell homeostasis in TET2 deficiency

All three patients showed features of ALPS in the form of variable combinations of hepatosplenomegaly and lymphadenopathy (progressing to lymphoma/lymphoproliferative disorder; n=3), clinically significant autoimmunity (n=2), and raised proportion of double negative (CD4-CD8-) T-cells on one or more occasions (DNT, n=3). In P1 and P2 this suggestive clinical picture was moreover accompanied by clearly impaired T-lymphoblast apoptosis *in vitro* (**Figure 1A**), together with serologic markers of ALPS, such as elevated levels of sCD25, FasL and IL-10. In contrast, P3 did not show serum markers of ALPS or

disordered apoptosis *in vitro* despite grossly elevated DNT cells (**Figure 3A**). Surface expression of Fas receptor was normal (data not shown).

To examine the effect of TET2 loss-of-function on the differentiation of CD4+ T-cells into distinct effector subsets, we performed immunophenotyping of patients' (P2 and P3) and their heterozygous relatives' PBMC using an extensive panel of surface markers. Patients showed decreased helper Th17 (defined as CD3+CD4+CCR6+CXCR3-) and Th1 (CD3+CD4+CCR6-CXCR3+) cell counts with relatively higher Th2 (CD3+CD4+CCR6-CXCR3-) cells, while proportions of regulatory T-cells (Treg, CD3+CD4+CD25+CD127low) were normal (**Figure 3B**). Intriguingly, circulating Tfh cells (defined as CD3+CD4+CXCR5+CD45RO+) were almost absent from TET2-deficient patients. Such a finding is particularly striking given that TET2-mutated T-cell lymphomas, including that of our patient P2, are typically of Tfh type [33]. An explanation for this apparent paradox may be that TET2-deficient Tfh accumulate in secondary lymphoid tissues, as observed in the spleen of TET2-deficient mice prior to the development of lymphoma [34]. Parallel immunophenotyping of PBMC from heterozygous family relatives did not reveal any consistent abnormalities.

TET2 loss-of-function impairs human B-cell terminal differentiation

Although abnormalities of humoral immunity were difficult to disentangle from exogenous immunosuppression in P1, P2 and P3 showed evidence of a primary defect of humoral immunity with a lack of class-switched memory (CSM, CD19+CD20+CD27+IgD-) B-cells despite normal overall numbers of B-cells and a tendency towards hypergammaglobulinemia (Supplementary Table S1). Such a pattern has been observed in primary immune disorders such as deficiencies of DOCK8 or TPP2 [35,36]. The lack of surface immunoglobulins IgA and IgG detected by flow cytometry confirmed this defect in B-cell differentiation *in vivo* (**Figure 4A**).

To test the hypothesis that TET2 deficiency autonomously impaired the ability of patient B-cells to execute an appropriate differentiation programme in response to antigenic stimulation, we set up an *in vitro* culture system that enables the generation of long-lived

plasma cells from isolated primary B-cells ([37], **Figure 4B**). Phenotypic evaluation of the differentiating populations confirmed that although TET2^{Q1632*} B-cells were capable of initiating plasma cell differentiation as evidenced by the appearance of short-lived plasmablasts at day 6, these cells failed to progress to phenotypically mature plasma cells, which emerge at day 13 in healthy donors and persist during the time-frame of the assay (**Figure 4C, Supplementary Figure S5**). Moreover, assessment of immunoglobulins in the supernatant showed high levels of IgM production by the TET2^{Q1632*} plasmablasts that declined as the cells died and a complete failure to generate IgG (**Figure 4D**). The plasma cells that survive in this assay have previously been shown to be derived from memory cells that have undergone somatic hypermutation [37]. Thus, the lack of IgG is consistent with the scarcity of class-switched memory B-cells in the patient and recent data generated in a murine model of TET2 deficiency[38].

Acquired somatic mutations in genes within the RAS signalling pathway in patients' lymphoma tissue

Somatic mutations of *TET2* are prevalent within many types of malignancy, including myeloid and lymphoid neoplasms, where they are believed to represent an initiating event [39-41]; therefore, we hypothesized that lymphomagenesis would require a "second hit". To identify putative cooperating mutations within coding regions, we performed high depth WES of patients' lymphoid tumor samples and made a pairwise comparison with germline data, confirming hits by Sanger sequencing.

In the EBV-positive Hodgkin-like polymorphic B-cell lymphoproliferative disorder of P1, we detected a single point mutation p.K117N in *KRAS,* an oncogene that frequently harbors somatic variants in various solid tumor types, as well as hematological malignancies (reviewed in [42], **Supplementary Table S5**). Our observation supports the previously suggested collaboration of *TET2* loss-of-function and *KRAS* gain-of-function mutations, reported in myeloid neoplasia [8,11,43].

Another somatic variant, again within the RAS signalling pathway, was found in the peripheral T-cell lymphoma of patient P2, affecting the gene *ERBIN* (Erbb2 Interacting Protein). ERBIN acts within the RAS signalling pathway by disrupting RAS-RAF interaction [44]. Our patient's variant p.R1194H had not previously been reported in the context of neoplasia. Predictive models suggest the mutation may not be deleterious but we note that it is rare in the population (**Supplementary Table S5**). Unfortunately, we were unable to investigate the acquisition of somatic variants in the primary mediastinal large B-cell lymphoma of patient P3, due to lack of material.

TET2 deficiency skews *in vitro* hematopoietic differentiation towards the myeloid lineage

To study the effect of TET2 hypofunction on human hematopoiesis, we developed an *in vitro* disease model using patient-derived induced pluripotent stem cells (iPSC). Primary fibroblasts from patients P1 and P2 and healthy volunteers were reprogrammed into iPSC that were fully characterized (**Supplementary Figure S6**), and differentiated into hematopoietic precursors (HP) as described by Olivier *et al.* (**Figure 5A**, [45]).

TET2^{H1382R} cultures showed a higher proportion of erythro-megakaryocytic progenitors, and persistently lower fraction of myeloid progenitors, as defined by surface marker expression (**Figure 5B, Supplementary Figure S7A-F**). Yet, paradoxically, colony forming unit (CFU) assay revealed a skewed and boosted clonogenic potential of TET2^{H1382R} hematopoietic progenitors towards the myeloid lineage, whereas erythroid and megakaryocytic colony formation was severely impaired (**Figure 5C, 5D**). Our observation is in agreement with a previous study showing hyper-proliferation and impaired differentiation of TET2-deficient erythroid cells in mice [46]. This could be correlated with *in vivo* findings of marked monocytosis and variable neutrophilia in patients P1 and P3, while all three patients showed chronic thrombocytopenia (**Supplementary Figure S1**). Moreover, the surviving patient P1 has developed a transfusion-dependent anaemia over time, albeit we cannot rule out a late effect of chemotherapy on his marrow reserve.

Measurement of DNA methylation status in patients' fibroblasts, iPSC and hematopoietic progenitors revealed DNA hypermethylation in non-differentiated iPSC and early-stage hematopoietic progenitors at day 13 and day 20 (**Figure 5E, Supplementary Figure S4C**). This corresponds to the time at which TET2 expression is upregulated during hematopoietic differentiation (**Supplementary Figure S7G**), and echoes the DNA hypermethylation detected in patients' whole blood (**Figure 2E**). We saw no evidence of compensation of TET2 deficiency by upregulation of other TET family members (**Supplementary Figure S7G**), in keeping with observations in TET2 knockout mice [28,47].

DISCUSSION

This is the first report of human germline homozygous TET2 loss-of-function, which we identified in association with combined immunodeficiency, autoimmunity and childhood lymphoma in two unrelated kindreds. In keeping with a severe autosomal recessive trait, inborn homozygous null mutations of this gene are absent from databases of human genomic variation [48,49]. There are two recent reports of inherited heterozygous deficiency of TET2 in humans: in a family with lymphoma, Kaasinen et al. found carriers, some but not all of them affected, of a heterozygous germline mutation that had already been reported as a somatic mutation in an AML patient, and one additional unrelated case with a *de novo* variant showing mild developmental delay [17]. Duployez et al subsequently described three siblings with myeloid malignancy in the 6th and 7th decade of life and a shared heterozygous germline frameshift mutation in TET2; their mother had died of T-cell lymphoma but no DNA was available from her [50]. To our knowledge, the extensive literature on human TET2 deficiency otherwise focuses exclusively on the more frequent somatic variations, both heterozygous and homozygous, mainly in the context of CHIP, myeloid and lymphoid malignancies [8,9,51]. Our findings confirm the strong link between TET2 loss-of-function and lymphomagenesis, with early onset lymphoid tumors of diverse types in all three affected children. At present we must consider healthy heterozygous family members as being at some increased risk of hematologic malignancy, but this is difficult to quantify.

Our patients' phenotype is also consistent with observations of *TET2* knockout (KO) mice that develop normally, nevertheless demonstrate myeloproliferation, splenomegaly and lymphomagenesis. Indeed, engineered $TET2^{-/-}$ mice have earlier onsets of myeloproliferative neoplasia than $TET2^{+/-}$ mice that exhibit similar, but less severe symptoms [24,27]. The TET2 defect elevates blood DNA methylation levels, especially at active enhancers and cell-type specific regulatory regions with binding sequences of master transcription factors involved in hematopoiesis, endorsing the importance of TET2 in regulation of hematopoietic differentiation [17-19]. Furthermore, 5hmC level in DNA was reduced dramatically in homozygous TET2 mutant mice compared to heterozygotes [27], as likewise noticed in peripheral blood in our cohort, patient-derived iPSC and hematopoietic progenitors generated *in vitro*. Dominguez et al. related murine TET2 loss-of-function to specific alterations in DNA hydroxymethylation and corresponding gene expression within germinal centre B-cells [38]. Interestingly this effect was seen only when TET2 was absent at earlier stages of B-cell differentiation, implying epigenetic imprinting.

Our patients' immunodeficiency and immune dysregulation emphasizes a broader role of TET2 in homeostasis and function of the human adaptive immune system. Whereas an immunodeficiency phenotype has not been reported for *TET2* KO mice to date, recent studies imply a crucial role for TET2 in maintaining T-cell homeostasis and B-cell development [38,52,53]. In agreement with the mouse KO model, in which TET2 deficiency and subsequent 5hmC reduction affected differentiation of T-cell subsets [54], we detected relative loss of T-helper subsets Tfh, Th1 and T17 with a skew towards the Th2 fate and preservation of Treg numbers. Abnormal Treg proliferation and Foxp3 destabilization were observed only after double *TET2/TET3* knockout in mice [52,55]. Moreover, TET2 deficient B-cells in both species display deficiencies in the generation of memory populations as well as mature plasma cells, thereby compromising long-term effective humoral immunity. That this should cause an immunodeficiency phenotype in human beings in the natural environment is perhaps not surprising.

It is noteworthy that clinically relevant autoimmunity and impaired T-cell apoptosis in our TET2^{H1382R} patients co-segregated and that these abnormalities were absent from both P3 and the knockout mouse model. One attractive hypothesis is that the hypomorphic nature of the H1382R mutation dissociates the enzymatic and non-enzymatic epigenetic activities of TET2 [56,57], potentially modulating disease phenotype. However, it is by no means uncommon for individual IEI to produce a broad disease spectrum, ranging from lymphoproliferation to immunodeficiency [1]. We can conclude that impaired T-cell apoptosis shows variable expressivity in TET2 deficiency, but at present we cannot confidently ascribe this to a genotype-phenotype effect. Indeed, all of the patients reconstituted autologous T-cells strikingly early after conditioned hematopoietic stem cell transplantation, suggesting a cell-autonomous pro-proliferative phenotype. This is in keeping with the observed outgrowth of a clone of CAR-T cells with biallelic TET2-inactivation in a case of successful cancer immunotherapy [58] and expanded CD8 memory response after LCMV challenge in a mouse model [59].

We found no impairment in the reprogramming efficiency or pluripotent potential of TET2 deficient iPSC, just as mouse embryonic stem cells (mESC) deleted for TET proteins retain pluripotency [60,61]. Increased hematopoietic repopulating capacity with skewing of cell differentiation toward monocytic/granulocytic lineages was described in *TET2* KO mice that died by 1 year of age because of the development of myeloid malignancies [27]. Our results from *in vitro* hematopoietic differentiation of TET2 deficient iPSC confirm a similar effect in humans, showing boosted clonogenic potential of myeloid progenitors at the expense of impaired erythroid and megakaryocytic progenitors. There were echoes of this *in vivo* in the two patients who survived infancy, both of whom showed coexistent monocytosis and frequent neutrophilia along with thrombocytopenia.

Had sufficient material been available, it would have been ideal to explore our observations further at a transcriptomic and epigenomic level. Such analysis awaits the identification of future cases, which might now be achieved by targeted screening among children with lymphoid malignancy, especially on a background of consanguinity and

immunodeficiency. Nonetheless, the present findings expand understanding of the critical role of TET2 within the human hematopoietic system and define a new inborn error of immunity.

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AUTHOR CONTRIBUTIONS

Jarmila Spegarova[1a] – designed and performed experiments, analysed data, participated in manuscript preparation

Dylan Lawless[2a] – designed and performed experiments, analysed data, participated in manuscript preparation

Siti Mardhiana Binti Mohamad[3] – performed experiments and analysed data

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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TABLES

Table 1. Major clinical features of three patients with immunodeficiency and immune dysregulation.

	Patient 1	Patient 2	Patient 3
Immunodeficiency			
Recurrent respiratory tract infections	++	+	++
Bronchiectasis	++	+	++
Herpes viral infection	++	+	+
Lymphoproliferation			
Lymphadenopathy	+	+	+
Hepatosplenomegaly	+	+	+
Lymphoma	+	+	+
Autoimmunity			
Autoimmune cytopenias	+	+	-
Autoantibodies	+	+	-
Laboratory Values			
Class-switched memory B-cells	↓	↓	↓
FasL-mediated apoptosis	Impaired	Impaired	Normal
Soluble Fas Ligand	Increased	Increased	Normal
DNT cells	↑	↑ / Normal	↑
Specific antibodies	Normal	ND	↓
Developmental delay			
Moderate	+	+	+
Outcome of HSCT			
Autologous T-cell reconstitution	+	+	+
	Split mixed chimerism	Rejected and died	Rejected and died

DNT: double negative T-cells, HSCT: hematopoietic stem cell transplantation, ND: not determined.

FIGURE LEGENDS

Figure 1. Patients clinical and laboratory characteristics.

(A) Fas Ligand-induced apoptosis in patients' and healthy controls' PHA and IL-2 stimulated T-blasts determined by flow cytometry using Annexin-V/PI staining showed impaired apoptosis in patient P1 and P2 before transplantation, normal response of patient P3 before transplantation, and repaired response of patient P1 after transplantation compared to healthy control cells.

(B) Histopathology of all three patients' lymphoid tumors. P1: EBV-positive Hodgkin-like polymorphic B-cell lymphoproliferative disorder; P2: nodal peripheral T-cell lymphoma with T follicular helper phenotype; P3: primary mediastinal large B-cell lymphoma. Upper panel: hematoxylin and eosin staining; lower panel: EBV EBER *in situ* hybridization, CD3 and CD20 immunohistochemical staining in P1, P2 and P3, respectively.

(C) Rapid autologous lymphocyte reconstitution after HSCT in all three patients with homozygous TET2 loss-of-function, despite full T-cell depleting serotherapy. Asterisks indicate the first measurement of T-cell chimerism in each patient: P1 (D+28) 78% recipient, P2 (D+46) 100% recipient, P3 (D+52) 91% recipient.

Figure 2. Germline homozygous loss-of-function mutations of *TET2* in three immunodeficient patients with lymphoma.

A) Pedigree of two unrelated consanguineous families, and Sanger sequencing results of patients and unaffected family members with highlighted affected nucleotide in the sequence of *TET2*. Affected patients, all with homozygous variant, are indicated by full black symbol, heterozygous family members by gray and other relatives with open symbol. Slash indicates deceased patients.

B) Schematic of TET2 protein structure with highlighted mutated residues H1382R and Q1632* within catalytic, double stranded beta-helix (DSBH) domain, predicting damaging

effect of the mutations on enzyme activity. H1382 residue is positioned in the catalytically important Fe^{2+}-binging HxD motif.

C) Left, TET2 protein expression as detected by immunoblotting in fibroblasts of an unrelated Control (C), Patient 1 and 2 (P1, P2; Family 1). Right, absent TET2 expression in patient P3 and its decreased expression in heterozygous relatives as detected by immunoblotting in PBMC of Family 2: unrelated Control (C), Mother (M), Father (F), Sibling 1 and 2 (S1, S2) and proband 3 (P3). Quantification of TET2 expression normalized to house-keeping proteins GAPDH and beta-actin, respectively. Data are shown as mean ± SD from 2 independent experiments. Numbers indicate p-values calculated by unpaired t-test comparing patients vs respective healthy control, and pooled heterozygous relatives vs control. **, $P < 0.01$; ***, $P < 0.001$.

D) Impaired TET2 hydroxymethylating activity detected by 5hmC immunofluorescence staining in HEK293T cells transfected with either empty lentiviral vector, Flag-tagged wild type TET2 or mutant $TET2^{H1382R}$. Blue: DAPI stain, green: Flag, red: 5hmC staining. Result is representative of three independent experiments.

E) Increased ratio of 5mC to 5hmC, as determined by DNA methylation assay of total blood DNA, in patients bearing homozygous H1382R and Q1632* mutations (red bars) compared to homozygous wild type controls (black bars). Heterozygous relatives (blue bars) showed significantly increased, intermediate levels. Data shown as mean ± SD from 2 independent experiments and seven healthy controls. P-values are shown for unpaired t-tests compared to healthy controls. **, $P < 0.01$; ***, $P < 0.001$.

Figure 3. Immunophenotyping of patients and heterozygous relatives.

(A) Representative flow cytometry dots plots showing increased levels of DNT ($CD4^-CD8^-$) cells in patient P3, and normal levels in healthy control, heterozygous relative and patient P2.

(B) Demonstrative flow cytometry dot plots representing normal levels of Treg (CD3+CD4+CD25+CD127low) in patients and heterozygous relative, lack of Tfh

(CD3+CD4+CXCR5+CD45RO+) cells, reduced Th1 (CD3+CD4+CCR6-CXCR3+) and Th17 (CD3+CD4+CCR6+CXCR3-) cells and differentiation preference towards Th2 (CD3+CD4+CCR6-CXCR3-) cells in both patients P2 and P3.

Figure 4. Failure of TET2 deficient B-cells to undergo class-switch recombination *in vivo*, generate mature plasma cells and produce IgG *in vitro*.

(A) Representative flow cytometry dot plots showing almost absent CSM (CD19+CD20+CD27+IgD-) B-cells and block in expression of surface IgA and IgG in patients as detected in peripheral blood B-cells compared to healthy control and family 2 heterozygous relative.

(B) Scheme showing *in vitro* B-cell differentiation strategy after mimicking a T-cell dependent immune stimulus.

(C) Flow cytometric profile of patient P3 and healthy control *in vitro* differentiating primary B-cells, indicating a defect in B-cell maturation and impaired cell survival due to loss of TET2 function.

(D) Secreted levels of IgM and IgG detected by ELISA during B-cell differentiation showed a failure of class-switch recombination in patient cells.

The data are presented as mean ± SD from two independent experiments.

Figure 5. Impaired *in vitro* hematopoietic differentiation by TET2 deficient iPSC

(A) Schematic presentation of experimental strategy to assess the hematopoietic differentiation capacity from iPSC *in vitro*[24] with representative pictures of cell culture at major differentiation stages; starting with embryoid bodies at Day 0-3, followed by presence of hematopoietic precursors and their budding at Day 5-12, culminating in proliferation and maturation at Day 13-30.

(B) Scatter box plots demonstrating the percentage of positive cells detected by flow cytometry at individual time points during differentiation; showing hematopoietic progenitors (CD34+/-CD43+), erythro-megakaryocytic progenitors (CD43+CD235a+CD41a+), erythroid

progenitors (CD43+CD235a+CD41a-), megakaryocytic progenitors (CD43+CD235a-CD41a+) and myeloid progenitors (CD43+CD235a-CD41a-CD45+). Bar represents median value from minimum 6 independent experiments. Statistical significance was calculated using non-parametric Kruskal-Wallis test. **, $P < 0.01$; ***, $P < 0.001$.

(C) Quantitative and qualitative results of colony forming unit assay (CFU) of hematopoietic precursors, plated at individual time points of differentiation into semisolid medium and incubated for 2 weeks, showing skewed differentiation towards myeloid lineage at the expense of erythroid and megakaryocytic colonies. Data are presented as mean ± SD from minimum 3 independent experiments.

(D) Representative pictures of individual CFU classified according to characteristic morphologic features. Data from three healthy control lines are presented in one Control group. CFU types: Erythroid (E), megakaryocytic (Meg), granulocytic (G), monocytic (M), and erythroid burst forming unit (BFU-E).

(E) Ratio of 5mC to 5hmC determined by DNA methylation assay in healthy control (C; black bars) and patients' (P, red bars) fibroblasts, iPSC and hematopoietic progenitors at day 13 (D13) and day 20 (D20) of hematopoietic differentiation *in vitro*. Data shown as mean ± SD from 2 biological repeats. P-values are shown for unpaired t-tests compared to healthy controls. *, $P < 0.05$.