




Genetic Deficiency and Biochemical Inhibition of ITK Affect Human Th17, Treg, and Innate Lymphoid Cells

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Abstract

Purpose Interleukin-2-inducible T cell kinase (ITK) is an important mediator of T cell receptor signaling. Loss of function mutations in ITK results in hypogammaglobulinemia and CD4+ T cell loss in humans, and the patients often present with EBV-associated B cell lymphoproliferative syndrome. *Itk*-deficient mice show loss of T cell naivety, impaired cytolytic activity of CD8+ T cells, and defects in CD4+ T cell lineage choice decisions. In mice, *Itk* mutations were shown to affect Th17-Treg lineage choice in favor of the latter. In this study, we explored whether human ITK reciprocally regulates Th17-Treg balance as its murine ortholog.

Methods Whole Exome Sequencing was used to identify the mutation. ITK-deficient peripheral blood lymphocytes were characterized by FACS Aria III-based flow cytometric assays with respect to proliferation, apoptosis, cytokine production, and innate lymphoid cell (ILC) frequency. Sorted T cells from healthy donors were exposed to ibrutinib, an irreversible ITK inhibitor, to assess ITK's contribution to Th17 and Treg cell generation and functions.

Results In this study, we report a child with a novel ITK mutation who showed impaired CD3/CD28 induced proliferation in T cells. *ITK*-mutant cells were more apoptotic irrespective of TCR activation. More importantly, T cells produced less Th17-associated cytokines IL-17A, IL-22, and GM-CSF. Conversely, Th1-associated IFN- γ production was increased. An irreversible inhibitor of ITK, ibrutinib, blocked *ex vivo* Th17 generation and IL-17A production, conversely augmented FOXP3 expression only at low doses in Treg cultures. Finally, we analyzed peripheral ILC populations and observed a relative decrease in ILC2 and ILC3 frequency in our ITK-deficient patient.

Conclusions To our knowledge, this is the first report showing that both genetic and chemical inhibition of ITK result in reduced Th17 generation and function in humans. We also report, for the first time, a reduction in ILC2 and ILC3 populations in an ITK-deficient human patient.

Keywords ITK · Th17 · Treg · Foxp3 · ILC · interleukin-2-inducible T cell kinase · ibrutinib

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Introduction

Interleukin-2-inducible T cell kinase (ITK) is a member of the Tec family non-receptor tyrosine kinase critical for T cell receptor (TCR) signaling in T and NKT cells [1]. Upon activation of TCR, ITK is recruited, phosphorylated by lymphocyte-specific protein tyrosine kinase (LCK), leading to its autophosphorylation and activation, which in turn activates PLC γ . This leads to production of secondary messengers DAG and IP $_3$, consequently of Ca $^{2+}$ mobilization and activation of several signaling pathways including RASGRP1, PKC, PKC δ , MAPK, mTOR, and NF κ B as well as cytoskeleton organizers [1, 2]. The critical role of ITK in TCR signaling could readily be observed in both humans and mouse models with ITK loss of function mutations [2, 3].

In this regard, 17 ITK-deficient patients have been reported to this day [2–9]. Clinically, ITK deficiency is associated with fever, hepatosplenomegaly, lymphadenopathy, and EBV viremia. Most of these patients (13) had Hodgkin lymphoma (HL) or EBV-driven B cell lymphoproliferative disease, whereas two showed classical non-HL phenotype. At the cellular level, mostly T cells have been shown to be affected by ITK deficiency in humans. CD4 $^+$ T cells loss, (particularly CD45RA $^+$ naïve T cell) and NKT cell loss were documented [2, 3, 9, 10].

The role of *Itk* in murine T cells has been studied extensively, revealing its importance in development and function of CD4 $^+$ and CD8 $^+$ T cells, α NKT cells, and $\gamma\delta$ T cells. Thus, number of naïve CD4 $^+$ and CD8 $^+$ T cells are reduced and memory-like CD4 $^+$ and CD8 $^+$ T cells are elevated in *Itk* $^{-/-}$ mice [11]. *Itk*-deficient mice had also greatly reduced number of NKT cells in line with human data [12]. Moreover, *Itk*-deficient NKT cells are functionally defective in IL-4 and IFN- γ production [13]. On the other hand, a subset of $\gamma\delta$ T cells, CD4 $^+$ NKT-like $\gamma\delta$ T cell number, and production of Th2-associated cytokines by these cells were elevated in *Itk*-deficient mice. Felices et al. suggested that higher levels of IgE in *Itk*-deficient mice, could partly be explained by this $\gamma\delta$ T cell subset-dependent help B cells receive [14, 15]. *Itk* was also shown to be critical for activation and cytolytic activity of murine CD8 $^+$ T cells which may partly explain the susceptibility of ITK-deficient patients to EBV infections [16]. This unique role of *Itk* in regulating the degranulation, which is critical for cytotoxicity, is also encountered in mast cells, as such, *Itk*-deficient mice develop less severe allergic reactions [17].

The impact of *Itk* on CD4 $^+$ T helper subset generation and function have been extensively studied in mice in the context of Th1, Th2, Th9, Th17, and Treg [18–24]. Th2 cell generation and cytokine production was shown to be dependent on *Itk* [18, 25–27]. Thus, *Itk* $^{-/-}$ mice were resistant to allergic asthma [28]. Conversely, *Itk* $^{-/-}$ mice had impaired Th2 responses, particularly reduced IL-4 and IL-5 production, to type-2 immunity-dependent microbes including

Nippostrongylus brasiliensis and *Schistosoma mansoni* [25, 26]. *Itk* was also shown to regulate IL-17 promoter activity, thus *Itk*-deficient naïve CD4 $^+$ T cells were unable to differentiate into Th17 cells [22, 23]. Additionally, not only were *Itk*-deficient mice resistant to experimental autoimmune encephalomyelitis (EAE), but also use of an irreversible inhibitor of ITK/BTK, ibrutinib, ameliorated EAE [21, 29]. However, whether generation and functions of human Th17 cells require ITK has not yet been investigated.

Group 3 innate lymphoid cells (ILC3) are a recently defined population of innate cells, with remarkable similarity to Th17 cells in their requirement for similar transcription factors in their function and development, such as Rorc and AhR [30–32]. Both cell populations produce IL-17A, IL-22, and GM-CSF and are enriched at the mucosal surfaces and skin. A number of studies suggest that ILC3s, like Th17 cells, may be involved in protective immunity to extracellular pathogens, and also may contribute to the pathogenesis of inflammatory diseases [33]. Group 2 innate lymphoid cells (ILC2) are the innate counterpart of Th2 cells which require similar transcription factors and cytokines for their ontogeny and functions [34, 35]. ILC2 produce Th2-associated IL-4, IL-5, and IL-13. Similarly, ILC2 are important in protective immunity to parasites, and are implicated in allergic reactions. Whether *Itk* deficiency impacts ILC population in mice or humans has not been explored.

In this study, we identified a novel loss of function mutation in *ITK* gene in a patient. We report reduced Th17 cell-associated cytokines and reduced ILC2 and ILC3 frequency in the patient. Importantly, an irreversible inhibitor of ITK, ibrutinib, also blocked *ex vivo* generation of Th17 cells and IL-17A production by human Th17 cells. Lastly, ibrutinib augmented FOXP3 expression at low dose but blocked Treg generation at high doses. To our knowledge, this is the first study revealing the role of ITK in human Th17, Treg cell function, and cytokine production.

Methods

Sequencing

Next Generation Sequencing (NGS) was performed at the Dr. von Hauner Children's Hospital NGS facility, Munich, Germany. Genomic DNA was extracted from whole blood of the patient and the first-degree relatives in order to generate whole exome libraries using the SureSelect XT Human All Exon V5+UTR or V6+UTR kit (Agilent Technologies, USA). Barcoded libraries were sequenced on a NextSeq 500 platform (Illumina, USA) with an average coverage depth of 100 \times . After bioinformatics analysis and subsequent filtering, rare sequence variants were identified. *ITK* mutations were confirmed by Sanger sequencing.

Flow Cytometry

PBMCs from healthy mother and the patient prior to transplantation were purified from blood via Ficol-Paque gradient according to manufacturer's instructions. Cell suspension was prepared with RPMI 1640 supplemented with 10% FBS. The cells were activated via PMA (50 ng/ml)/Ionomycin (1 µg/ml) and Golgi Plug for 4 h in 96-well plates. Cells were surface stained for anti-human TCRab-FITC (Biolegend# 306712). Intracellular cytokines staining was performed with anti-human IFN-γ-APC-Cy7 (Biolegend#505849), anti-human GM-CSF-PE (Biolegend# 502306), anti-human IL-22-PercpCy5.5 (Biolegend# 366710), and anti-human IL-17A-APC (Biolegend#512334). The cells were analyzed with FACS Aria III. ILC staining was performed with antibodies below: Alexa Fluor® 700 anti-human CD3 (clone: HIT3a), FITC anti-human TCRαβ (clone: IP26), FITC anti-human TCRγδ (clone: B1), APC/Cy7 Anti-Human CD127 (I-7Ra) (clone: A019D5), PE anti-human CD161 (clone: HP-3G10), Brilliant Violet 421™ anti-human CD117 (c-kit) (clone 104D2), PE/Cy7 anti-human CD294 (CRTH2) (clone: BM16), APC anti-human CD336 (NKp44) (clone: 325110), Alexa Fluor® 488 anti-human CD19 (clone: HIB19), FITC anti-human CD94 (clone:DX22), FITC anti-human CD1a (clone: HI149), FITC anti-human CD11c (clone: 3.9), FITC anti-human CD123 (clone: 6H6), anti-human CD303 (BDCA-2) (clone:201A), FITC anti-human CD14 (clone: 63D3), FITC anti-human FcεRIα (clone: NP4D6), FITC anti-human CD34 (clone: 561), and APC/Cy7 anti-human IFN-γ (clone: 4S.B3) all from BioLegend.

Murine ILC3s were sorted from IL-23RGFP reporter mice (kindly provided by Dr. Mohamed Oukka [36]) as CD45+CD90.2+CD3-CD11b-NK1.1-IL-23RGFP+ cells. CD45+CD90.2+CD3-CD11b-NK1.1-IL-23RGFP- fraction is sorted as (ILC2+ILC1).

Proliferation and Apoptosis Staining

PBMCs were stimulated in anti-CD3/anti-CD28 coated (1 µg/ml) 96-well plates for 4 days following labeling with Tag-it-violet (Biolegend#425101) according to manufacturer's instructions. The cells were analyzed by FACS Aria III (BD Biosciences). CD3+ cells were gated after surface staining.

For apoptosis staining, PBMCs were stimulated in soluble anti-CD3/anti-CD28 (1 µg/ml) or plain medium in 96-well plates for 24 h. The cells were blocked with Fc-block 5 min on ice. Then, samples were stained with BioLegend's FITC Annexin V Apoptosis Detection Kit with 7-AAD.

Th17 and Treg Differentiation

Naïve or total CD4+ T cells were magnetically selected using Miltenyi human Naive CD4+ T Cell Isolation Kit II or CD4+ T Cell Isolation Kit. Sorted cells were cultured in 96-well plates pre-coated with anti-human CD3 (1 µg/ml) plus soluble anti-human CD28, TGF-β (2.5 ng/ml), and IL-2 (10 ng/ml) for 5 days with or without ibrutinib.

For Th17 cultures, sorted cells were cultured at 37 °C in 96-well plates pre-coated with anti-human CD3 (1 µg/ml) plus soluble anti-human CD28, TGF-β (5 ng/ml), IL-23 (20 ng/ml), IL-1β (10 ng/ml), and IL-6 (25 ng/ml) for 5 days with or without ibrutinib. On day 5, cells were transferred to new uncoated wells and new IL-23 (20 ng/ml), and IL-2 (0.5 ng/ml) was added and culture was maintained 7 more days. For short-term ibrutinib assays, Th17 cells after 12 days of differentiation were cultured for 4 h in the presence of ibrutinib with CD3/CD28 (1 µg/ml) and Golgi Plug.

Real-Time qPCR

Human and murine ILC subsets and T cells were sorted, and RNA extraction was performed via Qiagen RNeasy. RNA from formalin fixed paraffin embedded lymph node biopsies (of *ITK*-mutant and *ITK*-sufficient patients) was extracted via Roche High Pure FFPE RNA Isolation Kit (06650775001). cDNA synthesis was done using iScript cDNA Synthesis kit. *Itk* expression was quantified by SYBR Green method on a Roche 480 Lightcycler. Analyses were done by deltaCt method. 18S and RPL19 genes were used as housekeeping genes for human and mice, respectively. Human *ITK* forward primer: 5'-GAAGATCGTCATGGGAAGAAGC-3', reverse primer: 5'-CGGGTATTTATAGTGGCATGGG-3'. The *ITK* primers amplify the region between nucleotides (207–318) before the mutation (328). Human *IL17A* forward primer: 5'-ACCAATCCCAAAAGGTCCTC-3', reverse primer: CACTTTGCCTCCCAGATCAC; human *RORC* forward primer: 5'-CTGCAAAGAAGACCCACACC-3', reverse primer: 5'-GGTGATAACCCCGTAGTGGA-3'; human *FOXP3* forward primer: 5'-TTCTGTCAGTCCACTTCACC A-3', reverse primer: 5'-AGGTCTGAGGCTTTGGGTG-3'; human *LCK* forward primer: 5'-TGCCATTATCCCAT AGTCCA-3', reverse primer: 5'-GAGCCTTCGTAGGT AACCAGT-3'; human *THY1* forward primer: 5'-ATCG CTCTCCTGCTAACAGTC-3', reverse primer: 5'-CTCGTACTGGATGGGTGAAC-3'; murine *Itk* forward primer: 5'-TGGCCTACTTTGAGGAC CG-3' reverse primer: 5'-CACACACTTGATTCTGGAGAGTT-3'. Murine RPL19 forward: 5'-GCATCCTCATGGAGCACAT-3', reverse:5'-CTGGTCAGCCAGGAGCTT-3'. Human and murine 18S forward: 5'-GTAACCCGTTGAACCCCAT-3', reverse: 5'-CCATCCAATCGGTAGTAGCG-3'.

Results

Case

A previously healthy (5-year-old) girl, from a consanguineous family (Fig. 1a) was admitted to the hospital with complaints of unilateral swelling on of the neck. Biopsy was compatible with Hodgkin lymphoma and she was treated with ABVD/COPP protocol, but she experienced an early relapse just 1 year after completing chemotherapy. ICE regimen followed by autologous transplantation was performed. After transplantation, she had hypogammaglobulinemia and persistent and ultimately fatal EBV infection. She died at the age of five due to respiratory failure following pneumonia.

Next generation sequencing revealed a homozygous frame-shift variant in *ITK* (LRG_189t1: c.328delA; p.Thr110ArgfsTer1551.) (Fig. 1b). The variant was confirmed by Sanger sequencing and segregated with the disease. The father and the brother of the proband are heterozygote carriers for the mutation, the sister is wild type, and the mother was not

tested, but in line with autosomal recessive inheritance is expected to be heterozygote (Fig. 1a). This variant has not been previously described in databases of healthy individuals. The variant changes the reading frame after the 110th amino acid so that the essential tyrosine kinase domain as well as the protein-protein interaction domains Bruton's tyrosine kinase pleckstrin homology (PH) domain (BTK), SH3, and SH2 will be none functional. The observed variant is likely to be an *ITK* loss of function allele. Real-time PCR from a lymph node biopsy of the patient showed reduced levels of *ITK* mRNA compared to an unrelated control sample. Suggesting that the observed *ITK* mutation may lead to nonsense mediated decay of *ITK* mRNA (Fig. 1c).

Impaired Proliferation and Increased Apoptosis of *ITK*-Mutant PBMCs

Previous studies showed that mutations in *Itk* or *ITK* confer a proliferative defect to murine or human T cells respectively [10, 25, 37]. We confirmed this lymphoproliferative defect in

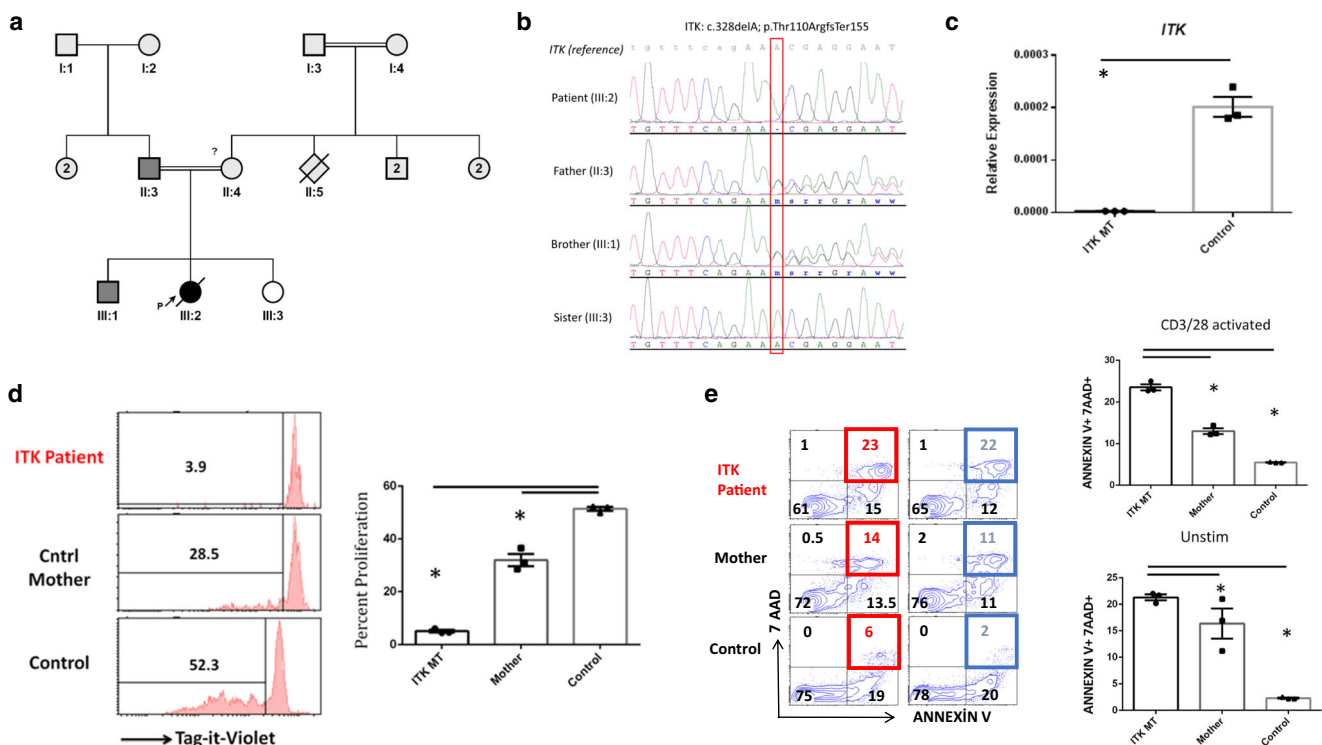


Fig. 1 *ITK*-mutant patient LNs have reduced *ITK* message, and peripheral T cells show impaired proliferation and higher apoptosis. **a** Pedigree of the patient (black circle). Father and brother are carriers (dark gray), whereas the sister is wildtype (white). The mother and additional family members were not sequenced so the carrier status is unknown (light gray). Consanguinity is known for parents and maternal grandparents of index patient. **b** Sanger sequencing confirmed the results of Whole Exome Sequencing. The patient is homozygous for *ITK* c.328delA; p.Thr110ArgfsTer1551 mutation. **c** Lymph node biopsies of index patient and unrelated lymph node samples as control were tested for *ITK* mRNA amounts by quantitative real-time PCR. Three (3)

technical replicates were used. **d** PBMCs from *ITK*-deficient patient, healthy mother, and healthy control were stained with Tag-it-violet and then, stimulated with CD3/CD28 (1 μ g/ml) for 4 days in triplicate. Percentages of proliferating cells were indicated above the gate in the flow plot (left) and quantified as bar graph (right). **e** PBMCs from *ITK*-deficient patient, healthy mother, and healthy control were either stimulated with CD3/CD28 (1 μ g/ml) or without antibodies in triplicates overnight and the cells were stained with ANNEXIN V and 7AAD. A representative flow plot was shown (left), and the combined technical replicates were shown as bar graph (right). **P* value < 0.05

our patient with *ITK* frame-shift mutation as well (Fig. 1d). CD3/CD28-induced proliferation of T cells were drastically reduced in the patient compared with her healthy mother. *Itk* deficiency in murine T cells is associated with reduced activation-induced cell death [37, 38], whereas in *Itk* negative iNKT cells, an increased apoptosis was observed [39]. Thus, we checked apoptosis in unstimulated and anti-CD3/CD28 stimulated patient derived PBMCs and could show that, in contrast to murine T cells, the human *ITK*-deficient PBMCs showed higher apoptosis with or without stimulation compared to mother and healthy control (Fig. 1e).

Reduced Th17-Associated Cytokines

Itk^{-/-} mice have been studied in detail with regard to impact of *Itk* on CD4+ T cell lineage choice. *Itk* was shown to regulate NFATc binding to IL-17A promoter, thus production of IL-17A cytokine. Absence of *Itk* also resulted in reduction in mTOR phosphorylation downstream of TCR signaling and shifted the lineage choice toward a Treg phenotype at the expense of Th17. We assessed the production Th17-associated cytokines in human *ITK*-deficient T cells and could observe a significant reduction in the production of IL-17A, IL-22, and

GM-CSF by *ITK*-mutant T cells upon stimulation with PMA/Ionomycin compared with healthy mother (Fig. 2a, Suppl Fig.1). In contrast, IFN- γ production by *ITK*-mutant T cells were elevated compared to control T cells (Fig. 2b). Compared to healthy controls, reduction in IL-17, and increase in IFN- γ by *ITK*-mutant T cells were also significant; however, IL-22 and GM-CSF were not. Due to loss of the patient, we could not compare the Treg cell numbers or the phenotype between the patient and the controls. However, we compared gene expression levels of *IL17A*, *RORC*, and *FOXP3* in the lymph node biopsies taken from *ITK*-mutant patient and a control who did not have *ITK* deficiency (Fig. 2c). Consistent with the protein data obtained from PBMCs, we observed reduced *IL17A* and *ROC* message, more importantly, elevated levels of *FOXP3* mRNA (Fig. 2c). *LCK* and *THY1* message levels were significantly higher in the lymph node of *ITK* patient compared with the control; however, normalization of *IL-17A*, *RORC*, and *FOXP3* to *LCK* also showed a similar decrease in the Th17-associated gene expression and an increase in *FOXP3* (Suppl Fig.2). Collectively, these results suggest that in humans, *ITK* may also influence the CD4+ T cell lineage choice between Treg and Th17.

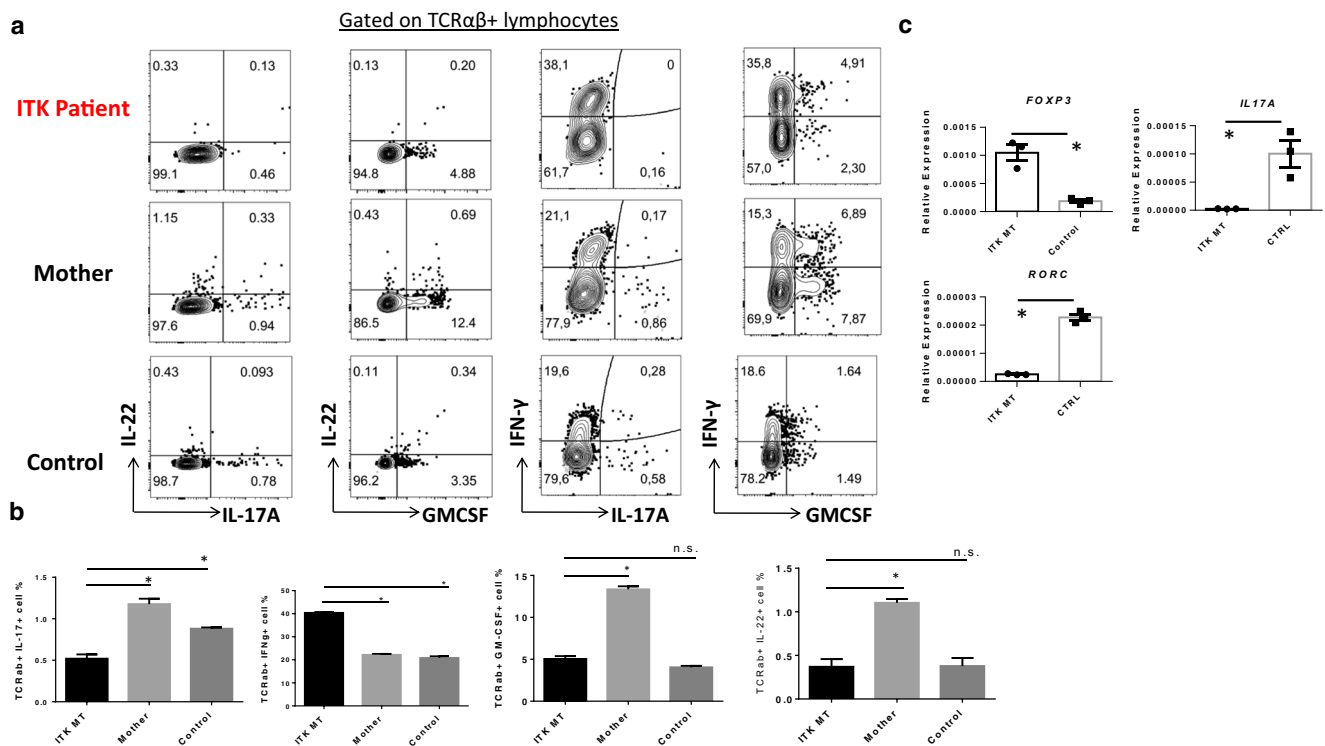


Fig. 2 Th17 cell-associated cytokine production is reduced and Th1-associated IFN- γ is increased by *ITK*-MT T cells. PBMCs from *ITK*-deficient patient, healthy mother, and healthy control were stimulated with PMA/Ionomycin/Golgi Plug for 4 h and IL-22, IL-17A, GM-CSF, and IFN- γ production was quantified by intracellular staining. **a** A representative flow plot, **b** the samples from one patient, healthy mother, and

healthy control were run in triplicates, and the percentages were charted as bar graph. **c** Relative gene expression of *FOXP3*, *IL17A*, and *RORC* was measured via real-time qPCR in the biopsy tissue of the lymph node (LN) obtained from *ITK*-mutant patient and *ITK*-WT patient. Three technical replicates were used. * $P < 0.05$, n.s., not significant

Ibrutinib Blocks *Ex vivo* Generation of Human Th17 Cells and Associated Cytokines and Elevates FOXP3 Expression

To test whether detrimental consequences of ITK deficiency for Th17 cell generation and function could be recapitulated by biochemical inhibition of ITK, we employed ITK inhibitor ibrutinib and assessed its dose-dependent impact on the generation of human Th17 cell and cytokine production in T cells from healthy individuals. We showed that ibrutinib was able to block *ex vivo* Th17 generation from naive CD4+T cells (Fig. 3a). Additionally, ibrutinib blocked production of IL-17 from PBMCs stimulated with CD3/CD28 (Fig. 3b, c). We also tested whether ibrutinib would reciprocally augment FOXP3 expression and Treg generation as previously reported for murine *Itk* deficiency (Fig. 3d, e). Ibrutinib blocked T cell proliferation in cultures as previously reported [19] (not shown), and also blocked Treg generation at and above 500 nM concentrations. However, FOXP3 positive cell frequency and expression were augmented at concentrations below 500 nM (Fig. 3e) consistent with reports in the murine models [22].

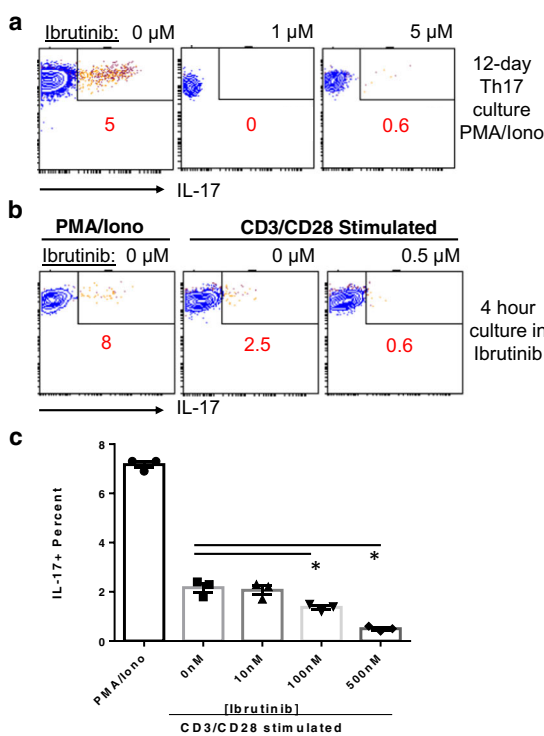
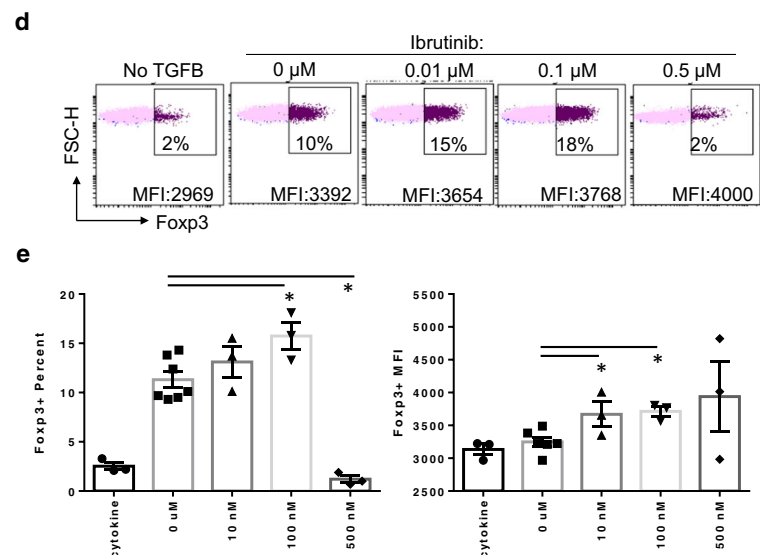


Fig. 3 Ibrutinib blocks *ex vivo* generation of human Th17 cells and IL-17A production and elevates FOXP3 expression. **a** Naïve human CD4+ T cells bead selected from healthy donor peripheral blood and were differentiated into Th17 cells for 12 days in the presence or absence of ibrutinib. On day 12, cells were stimulated with PMA/Ionomycin for 4 h and stained with anti-IL-17A. **b** Naïve human T cells bead selected from healthy donor peripheral blood were differentiated into Th17 cells for 12 days. Ibrutinib was added only for 4 h while cells are being stimulated with CD3/CD28 (1 μg/ml). A representative flow plot is shown. **c**

Reduced Group 2 and 3 Innate Lymphoid Cells in ITK-Mutant Patient

We next tested whether ITK deficiency impacted innate lymphoid cell (ILC) populations. As ILCs lack T cell receptors, the function of ITK in these ILCs is unclear. ILC3s have remarkable similarity to Th17 cells with regard to factors required for their ontogeny and function. Therefore, the observed Th17 defect would suggest that particularly, the ILC3s might be affected by ITK deficiency. The datasets retrieved from GEO database suggest that ILC subsets both in humans and mice express *ITK* at similar levels to that of NK cells. The expression levels appear to be above that of B cells or dendritic cells, for the murine dataset (Fig. 4a). Moreover, *Itk* expression in murine ILC3 was comparable to that of *Il22*, *Rorc*, and *Il23r* in intestinal Th17 cells (not shown). We could also confirm ITK expression in ILC subsets sorted from human tonsils and mouse intestine lamina propria via real-time qPCR (Fig. 4b). The gating strategy for ILC sorts are shown in Fig. 4c, d. We stained ILCs based on Mjosberg et al. After gating out Lineage+ CD3+ cells, we gated for CD161+ CD127+ ILCs population [30]. ILC3s are c-Kit+, ILC2s are



d, **e** Bead-sorted total human CD4+ T cells from healthy donor peripheral blood were differentiated into Treg cells for 5 days at increasing doses of ibrutinib, a representative flow plot was shown in (d). FOXP3 percent (e, left) and mean fluorescent intensity (e, right) of cells were shown in (e). The experiment was repeated with 3 different donors. **P* value < 0.05

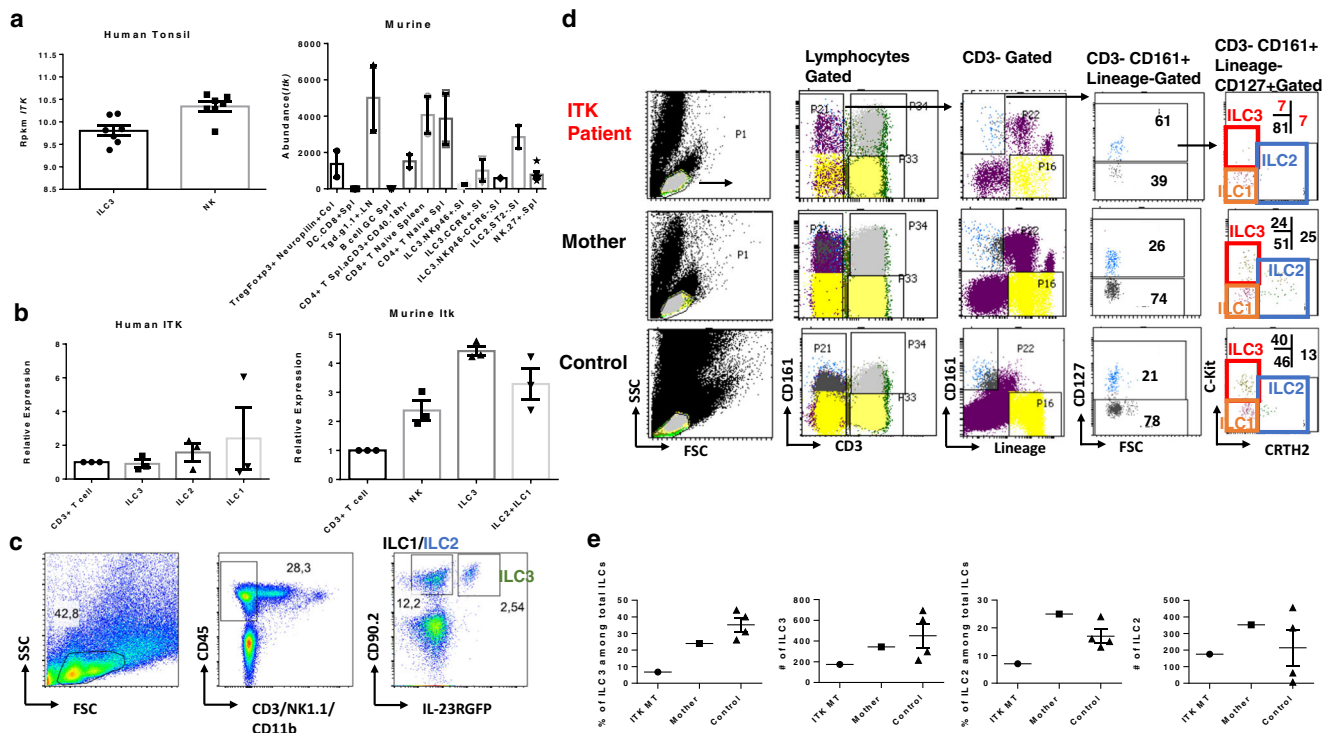


Fig. 4 ITK-mutant patient has reduced percentages of peripheral blood group 3 innate lymphoid cells. **a** *ITK* transcript abundance in human tonsil NK and ILC3s were analyzed from GEO deposited dataset GSE70596 (left). Murine *Itk* transcript abundance in various immune cell types were obtained from GSE109125 dataset and analyzed (right). **b** Relative gene expression of human *ITK* was quantified by real-time qPCR in ILC subsets and CD3+ T cells sorted from human tonsils (based on the gating in “d”). Delta Ct method was used, and the expression is normalized over 18S (left). Relative gene expression of murine *Itk* was quantified by real-time qPCR in murine ILCs subsets sorted from ileum based on the gating in “c” and CD3+ T cells (sorted by gating on CD3+

cells) or NK cells (sorted by gating on NK1.1+ cells) (left). **c** Gating strategy for murine ILC3 and ILC2/ILC1. Ileal lamina propria ILC3s were gated as CD45+CD3-NK1.1-CD11b-CD90.2+IL23RGFP+ population. CD45+CD3-NK1.1-CD11b-CD90.2+IL23RGFP- cells collected as ILC2/ILC1 fraction. **d** PBMCs from ITK-deficient patient, healthy mother, and healthy control were surface stained for lineage markers (TCRαβ-, TCRγδ-, CD34-CD123-, CD94-, CD14-, BDCA2-, FcεR1α-, CD1a-, CD11c-, CD19-, B220-) CD3, CD161, CD127, c-Kit, and CRTH2. **e** Percentages of ILC3 and ILC2 among total ILCs, or absolute number of ILC3 and ILC1 per ml blood was charted for ITK-deficient patient (*n* = 1), healthy mother (*n* = 1), and healthy controls (*n* = 4)

CRTH2+, and ILC1 are c-Kit-CRTH2-. The percentages of ILC3s and ILC2s in ITK-deficient peripheral blood was drastically reduced compared to her healthy mother or controls (Fig. 4d, e). The reduction in the absolute number of ILC2 was not as pronounced compared to four healthy controls. These data suggest that, even though upstream receptor that ITK partners with in ILCs is unclear, ITK may be important in maintenance/or functioning of human ILCs, in addition to its known function in Th17 cells.

Discussion

In this study, we identified a novel loss of function ITK mutation in a patient with HL and progressive EBV infection. Assessment of T helper cytokine profiles revealed reduced production of Th17-associated cytokines IL-17A, IL-22, and GM-CSF, and augmented Th1/IFN-γ production. The mutation leads to a proliferation defect in T cells and rendered them more susceptible to apoptosis. Additionally, we observed a

reduction in ILC3 and ILC2 subsets in the peripheral blood of the patient. Moreover, we tested whether irreversible blockade of ITK by ibrutinib mimicked genetic ITK deficiency in regulating Th17/Treg generation or function. To our knowledge, our results recapitulate for the first time in humans, the data presented previously on mice Th17 cells, and show that ITK is important in human Th17 cell generation and function.

This novel deletion mutation described here (LRG_189t1: c.328delA: p.Thr110ArgfsTer1551) results in a frame-shift in *ITK*, comparable with previously reported loss of function variants [2, 3]. We could show that the variant leads to nonsense mediated decay of ITK mRNA in the patient. Residual small amounts of mRNA would lead to expression of an ITK protein lacking important functional domains such as SH3 and SH2, and protein tyrosine kinase domains [1]. *ITK* loss of function (LOF) mutations have been reported to impair TCR-induced T cell proliferation in humans as well as in *Itk*-/- mice [2, 10, 25, 37, 40]. The consequence of the mutation in ITK defined herein parallels these previous observations. Previous studies in mice revealed that T cells became

resistant to CD3 activation-induced cell death in the absence of *Itk* [37, 38]. More recently, *Itk*^{-/-} murine iNKT cells were shown to express more Bax, Fas, in contrast, less Bcl2, consequently, they were more prone to apoptosis [39]. Our assessment of human PBMCs showed, unlike murine T cells, that the total PBMCs were more prone to apoptosis before and after stimulation with CD3/CD28. Although toxicity may be the primary reason, biochemical targeting of ITK by various inhibitors was also shown to result in apoptosis in several cell lines [41].

A number of studies which targeted ITK by genetic or biochemical means revealed that Th-lineage skewing is regulated by *Itk* in mice [25, 27, 42, 43]. In this regard, *Itk*^{-/-} mice were shown to mount defective Th2 responses, and were susceptible to *Nippostrongylus braziliensis* [25]. Along the same lines, *Itk*^{-/-} BALB/c mice became resistant to *Leishmania major* infections [25]. Importantly, CD4⁺ T cells from ibrutinib-treated patients and mice showed Th1-skewed immunity, and, thus, ibrutinib-treated mouse models infected with *L. major* or *Listeria monocytogenes* mounted better Th1 immunity [44]. At the molecular level, *Itk* was shown to promote NFATc nuclear translocation, and restrain T-bet expression [42, 43]. Whether human ILC2s express or are regulated by ITK has not yet been investigated. Our data in a single patient and the qPCR data reveal that ITK is expressed by human ILC2s, and that ITK-deficient individuals may have reduced ILC2s.

Murine Th17 cell generation and IL-17 cytokine production in the absence of *Itk* was impaired [18, 20, 22, 23]. This was also recapitulated when *Itk* inhibitors were used. However, to this day, the evidence for whether this applied to human Th17 cells has been lacking. Our data provide evidence that human Th17 cell generation and IL-17A cytokine production are regulated by ITK. We show this in two distinct ways. ITK-deficient patient peripheral blood CD4⁺ T cells produced less IL-17A, IL-22, and GM-CSF. Additionally, ITK-deficient lymph nodes had reduced *IL17A* and *RORC* mRNA message compared with ITK-sufficient lymph node. More importantly, CD4⁺ T cells sorted from healthy donors did not differentiate into Th17 when exposed to ibrutinib. In addition, when *ex vivo*-generated Th17 cells were activated by CD3/CD28 in the presence of ibrutinib, they produced less IL-17A.

The reciprocal regulation of Th17-Treg lineages manifest itself in various genetic defects favoring the former at the expense of latter or vice versa. Recently, this was suggested to be the case for *Itk*-deficient murine CD4⁺ T cells, which showed impaired differentiation into Th17, but elevated propensity toward Foxp3⁺ T reg cell lineage [22]. Although *Itk* deficiency was shown to positively impact Foxp3 expression in nTreg, its expression by nTreg is suggested to be necessary for suppressive activity in the context of T cell driven colitis [18, 19]. There is no information regarding how human ITK

deficiency or inhibitors might impact human Treg generation. We could not provide nTreg percentage in our patient's peripheral blood due to her death. Nevertheless, biopsy tissue from the LN of ITK-mutant patient showed significantly higher gene expression of *FOXP3* compared to ITK-sufficient LN. In addition, we provide evidence that *ex vivo* differentiation of human CD4⁺ T cells into Treg is improved by low-dose ibrutinib exposure, corroborating murine data. However, higher doses of ibrutinib completely blocked Treg generation.

Lastly, we also observed a reduced frequency of human peripheral ILC3s in the ITK-deficient patient compared with healthy mother. Besides T cells, ITK is also expressed by NK [45] and mast cells [46] and regulates degranulation in those cells. Which processes ITK regulates in human or murine non-NK ILC subsets requires further study.

Collectively, the data presented here describes a novel ITK mutation and explores how ITK deficiency may impact human Th17, Treg, and ILC subsets using patient PBMCs, lymphoid tissue sample, and irreversible ITK inhibitor ibrutinib.

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Author Contributions CK, IS, YM, SH, NZ, and MR performed NGS and analyses, identified the mutation, and read and revised the manuscript. AE, MC, HC, and EU conceived and supervised the study and wrote and edited the manuscript. TP, MK, AO, and SA cared the patient and provided samples, provided intellectual input, and read and revised the manuscript. FZO, SE, and AE performed the experiments.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants Informed consent for participation in this study was obtained in accordance with local regulations, with approval from the IRB. The experiments described here were performed in Turkey and in Germany. All the experiments were conducted in accordance with local regulations, and with the approval of the IRB for Erciyes University, Turkey, and were in line with the current guidelines of the Declaration of Helsinki. The ethics permit number is 2018/388.

Informed Consent Written informed consent was obtained from the patients.

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