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Germline biallelic \textit{PIK3CG} mutations in a multifaceted immunodeficiency with immune dysregulation

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The PI3K-AKT-mTOR signaling axis is a critical molecular pathway in humans, regulating multiple cellular processes. Phosphatidylinositol-3-kinases (PI3Ks) represent key signaling hubs for signal propagation, driving cell-activation, cell-polarization and morphological adaptations. Studies on PI3K in human disease have highlighted PI3K-gamma (PI3Kγ) as an appealing drug target for treatment of human disorders. Murine PI3Kγ studies showed its importance in regulating innate immune functions and development and activation of T cells, revealing its role in controlling inflammation. However, its role in the human immune system and diseases remains to be investigated.

We here investigated a 14-year old female index patient, born to non-consanguineous healthy Austrian/Italian parents, who was hospitalized with severe hypotonia and prolonged fever. She had neither lymphadenopathy nor hepatosplenomegaly, and no infectious agent was found. Initial laboratory findings showed a mild thrombocytopenia, hypertriglyceridemia, increased lactate dehydrogenase (LDH) and markedly elevated ferritin (Table 1, Fig. 1A), prompting work-up for hemophagocytic lymphohistiocytosis (HLH). Hemophagocytosis was indeed visible in the bone marrow aspirate (Fig. 1B). Soluble CD25 was mildly elevated at 2204 U mL⁻¹ (Table 1) but below the levels typically seen in HLH. NK-cell activity as measured by CD107a expression upon stimulation was in the low normal range in the initial diagnostic (Table 1). The presence of fever, hypertriglyceridemia, hyperferritinemia and hemophagocytosis, did not allow the diagnosis of HLH, but gave evidence of macrophage activation in the context of a hyperferritinemic inflammatory syndrome (Table 1). We initiated treatment with dexamethasone, leading to clinical improvement and normalization of LDH and ferritin levels. Tapering of dexamethasone resulted in clinical deterioration and rise in ferritin (Fig. 1A) and was accompanied by the development of autoimmune neutropenia as documented.
by HNA-1b antibodies. As the disease was distinct from classical HLH, we decided to treat the patient with recombinant human anti-IL-1β (Anakinra, 100 mg twice daily) in combination with dexamethasone, rather than using the etoposide-based HLH-94 protocol. We discontinued dexamethasone treatment after 8 weeks, and one month later, we reduced the Anakinra dose to a maintenance dose of 100 mg daily. The patient has remained clinically stable and is currently receiving Anakinra (decreased to 60 mg once daily) without any inflammatory manifestations. Immunological characterization of patient peripheral blood in the asymptomatic phase after ceasing dexamethasone revealed reduced absolute NK-cell counts and low frequency of monocytes, and slightly low absolute lymphocyte counts (Table 1).

To elucidate a potential genetic etiology of the disease, we performed whole exome sequencing on patient DNA. We identified compound heterozygous variants in \textit{PIK3CG} encoding for p110γ, the catalytic subunit of the PI3Kγ complex. Variants were validated by Sanger sequencing and both parents were identified as heterozygous carriers, each for one of the two variants (Fig. 1C). The patient inherited a variant within the adaptor binding domain of p110γ (c.145C>A, p.R49S) and (c.3254A>G, p.N1085S) near the end of the kinase domain (Fig. 1D). Both variants are rare or absent in ExAC and gnomAD databases (Table S1), and affect evolutionary conserved residues (Fig. S1A). Probability of loss-of-function tolerance was unlikely, CADD and PolyPhen-2 scoring suggested the variants as probably pathogenic/damaging (Table S1). We assessed the expression of mutated p110γ in T cells derived from patient peripheral blood mononuclear cells (PBMCs), and found comparable expression levels in the patient, mother and healthy donors (HDs) (Fig. 1E).

To investigate whether the identified \textit{PIK3CG} mutations potentially cause the HLH-like disease due to defects in PI3Kγ-dependent mechanisms, we first studied patient-
derived NK- and T-cell functions. Diminished NK-cell function is part of the diagnostic criteria for HLH.\textsuperscript{6} Pik3cg\textsuperscript{-/-} mice display reduced NK-cell numbers, defective NK-cell development and consequently, decreased cytotoxicity.\textsuperscript{3} We also observed reduced frequency, absolute counts and degranulation of patient NK cells compared to cells derived from the mother and HD (Table 1, Fig. S1B-C). The impaired NK cell compartment may have contributed to the observed clinical HLH-like phenotype.\textsuperscript{3}

We further hypothesized that a defect in PI3K\textgamma signaling would affect TCR-driven activation of T cells.\textsuperscript{7} Patient T-cell subsets were comparable to HD (Table S2). However, we observed functional defects in patient T cells, particularly a poor activation and proliferation in response to anti-CD3 or combined anti-CD3/CD28 stimulation (Fig. 2A-B, S1D). As expected, TCR-dependent T-cell activation was impaired in PIK3CG-mutated T cells. In contrast, patient T-cell proliferation was intact upon stimulation with PHA (Fig. 2A, S1D) which can be explained due to the bypassing of TCR/PI3K\textgamma-dependent activation mechanisms. To prove the causative role of mutated PIK3CG for the observed phenotypes, we performed a gene-rescue experiment on primary patient cells using GFP-labeled wild-type p110\gamma and showed that the T-cell activation defect could be restored (Fig. 2C). Additionally, we utilized the PI3K\textgamma inhibitor IPI-549 to prove the causative role of PI3K\textgamma loss-of-function for the observed phenotypes. T-cell activation and proliferation defects upon TCR stimulation were phenocopied by the addition of IPI-549 to HD cells (Fig. 2D-E).

Furthermore, we examined PI3K/AKT signaling and found mildly decreased AKT phosphorylation in patient cells upon stimulation (Fig. 2F, S1E), similar to a recently reported patient with PIK3CG mutations.\textsuperscript{8} To prove causality in an independent cellular system, we created Jurkat PIK3CG knockout (KO) cells (Fig. S1F) and found decreased activation as measured by CD69 upregulation upon anti-CD3 stimulation (Fig. 2G), and reduced AKT phosphorylation at the Ser473 phosphorylation site (Fig.
2H, S1F-G). These data mimic the phenotypes observed in patient T cells. Furthermore, upon reconstitution with wild-type p110γ, CD69 was upregulated on the surface of Jurkat PIK3CG KO cells (Fig. S1H). By contrast, upon reconstitution of mutant N1085S or R49S, no upregulation of CD69 was observed compared to empty vector (Fig. S1H), showing loss-of-function mechanisms for both individual variants. Altogether, the T-cell stimulation defects observed in PIK3CG-mutated patient cells recapitulate reports on Pi3ky−/− murine T-cells.5,7

For the development of familial HLH, absence of the cytotoxic activity of cytotoxic T cells (CTLs) plays a central role. The level of surface CD107a/LAMP1 was normal on patient CTLs upon stimulation (Fig. S1I). Furthermore, consistent with murine studies suggesting that PTEN activity, counteracting PI3K function, is required to maintain Treg cell stability and homeostasis,9 we observed a slightly increased frequency of CD4+CD127dimFOXP3+CD25+ Treg cells in the peripheral blood of the patient (Fig. S1J). In comparison to T cells which activate via PI3Kγ and PI3Kδ, B cells predominantly respond via PI3Kδ. This was corroborated by impaired B-cell development compared to rather intact T-cell populations in PIK3CD and PIK3R1 loss-of-function patients.10 By contrast, PIK3CG-mutated patient-derived B cells showed intact proliferation and class switch recombination upon stimulation (Fig. S1K-L), supporting data obtained in Pik3cg−/− mice.5

Since PI3Kγ is highly expressed in myeloid cells, we hypothesized that loss-of-function mutations in human PI3Kγ may compromise the function of these cells, thereby potentially contributing to the inflammatory features reported in the patient. Indeed, the observation that the patient responded well to anti-IL-1β (Anakinra) therapy (Fig. 1A) supports the notion that defects in the PI3K pathway in innate immune cells may underlie the inflammatory presentation. Preclinical studies have shown that PI3Kγ blockade leads to reprogramming of macrophages, resulting in
increased production of pro-inflammatory cytokines.\textsuperscript{11} PI3Kγ activation results in the PIP\textsubscript{3}-dependent activation of Rac and subsequently, Arp2/3-dependent actin-cytoskeleton remodeling driving cell-polarization, morphology and phagocytosis.\textsuperscript{12} We hypothesized that \textit{PIK3CG}-mutated patient cells would display abnormalities in innate cell morphology and function. We therefore differentiated freshly-isolated monocytes of patient, mother and HDs to macrophages, which showed comparable expression of macrophage differentiation markers (Fig. S2A), implying normal differentiation. Upon stimulation, the number of adhered cells per image was similar across all groups (Fig. S2B). However, patient monocyte-derived macrophages displayed dramatic reduction in cell area and total amount of F-actin per cell, as compared to HD cells (Fig. 2I-K). Moreover, cells from the mother displayed an intermediate phenotype, possibly linked to her heterozygous carrier status for one of the \textit{PIK3CG} mutations. Altogether, these data indicate that PI3Kγ deficiency is associated with a defect in actin-driven macrophage spreading upon stimulation. We also observed diminished phagocytosis in patient-derived monocytes and neutrophils (Fig. 2L). Patient neutrophils showed a pronounced apoptotic population after isolation (Fig. S2C). Loss of mitochondrial membrane potential in neutrophils is an early marker for commitment to apoptosis.\textsuperscript{13} Consistently, mitochondrial membrane potential of patient neutrophils was compromised (Fig. S2D). However, patient neutrophils displayed a normal oxidative burst upon stimulation (Fig. S2E). Studies in mice outlined the importance of PI3Kγ in neutrophils.\textsuperscript{14} However, in a transwell migration assay, patient neutrophils were able to migrate normally (Fig. S2F). Since PI3Kδ works synergistically with PI3Kγ in neutrophil migration, PI3Kδ may compensate for migration processes in human neutrophils. Collectively, we report deficiency of human p110γ underlying a previously unknown inborn error of immunity with HLH-like systemic inflammation and aberrant immune cell function. Intriguingly,
despite compromised functions of both innate and adaptive immune cells, so far the patient has not experienced serious infections as compared to a patient recently reported with *PIK3CG* mutations.\(^8\) Larger patient cohorts and longer follow-up will thus be necessary to unravel the full clinical spectrum of the disease.
ACKNOWLEDGEMENTS

The authors thank the patient and her family for participating in this study. We thank Javier Rey-Barroso (Institute of Pharmacology and Structural Biology of Toulouse) for the excellent technical advice. We thank the Superti-Furga laboratory, especially Felix Kartnig (CeMM), for technical assistance and access to the microscope. We thank Ana Krolo (LBI-RUD), Yolla German (LBI-RUD & Center for Pathophysiology of Toulouse Purpan), Arno Rottal and Ulrike Körmöczi (Institute of Immunology, Medical University of Vienna) for technical assistance.

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KEY WORDS

PIK3CG mutations, PI3Kγ deficiency, p110γ deficiency, hyperferritinemic syndrome, hemophagocytic lymphohistiocytosis (HLH)-like disease, systemic inflammation
AUTHOR CONTRIBUTIONS

MT conceptualized, designed, and performed experiments, analyzed data, and wrote the manuscript. BH performed and analyzed neutrophil assays and edited the manuscript. AK performed macrophage morphology assays and image analysis. FP, CH and AA took care of the patient, provided patient samples and interpreted clinical data. SKB and JH provided experimental help. MC analyzed macrophage morphology data. RJH conducted and analyzed NGS data, and identified PIK3CG mutations in the patient. WFP performed immunophenotyping and analysis. MG established and performed initial NK-cell degranulation assays. SE, CLL, JM, and LD gave important intellectual input. KB supervised the study, reviewed clinical and experimental data, and wrote the manuscript. All authors reviewed and approved the manuscript.

Disclosure of conflict of interest

The authors declare no conflict of interest.
REFERENCES


Table 1: Immunological features of the patient.

<table>
<thead>
<tr>
<th>Features</th>
<th>Initial presentation (July 2018)</th>
<th>After ceasing dexamethasone (Dec 2018)</th>
<th>After reducing Anakinra (June 2019)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hb (g/dL)</strong></td>
<td>12.3 (12.3-16)</td>
<td>12.6 (12.3-16)</td>
<td>13.1 (12.3-16)</td>
</tr>
<tr>
<td><strong>Absolute lymphocyte</strong></td>
<td>0.95 (1.1 – 4.5)</td>
<td>1.07 (1.1 – 4.5)</td>
<td>0.95 (1.1 – 4.5)</td>
</tr>
<tr>
<td><strong>Absolute neutrophil</strong></td>
<td>3.48 (1.9-8.0)</td>
<td>1.95 (1.9-8.0)</td>
<td>1.05 (1.9-8.0)</td>
</tr>
<tr>
<td><strong>Absolute monocyte</strong></td>
<td>0.24 (0.15-1.4)</td>
<td>0.15 (0.15-1.4)</td>
<td>0.15 (0.15-1.4)</td>
</tr>
<tr>
<td><strong>CD3-CD16-CD14+ monocytes (%)</strong></td>
<td>NA</td>
<td>7.59 (24.1-11.8)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Absolute thromocyte</strong></td>
<td>119 (140-400)</td>
<td>334 (140-400)</td>
<td>296 (140-400)</td>
</tr>
<tr>
<td><strong>Absolute CD3+ T cells</strong></td>
<td>0.71 (0.75 - 2.51)</td>
<td>0.99 (0.75 - 2.51)</td>
<td>0.75 (0.75 - 2.51)</td>
</tr>
<tr>
<td><strong>Absolute CD4+ T cells</strong></td>
<td>0.37 (0.43 - 1.69)</td>
<td>0.53 (0.43 - 1.69)</td>
<td>0.43 (0.43 - 1.69)</td>
</tr>
<tr>
<td><strong>Absolute CD8+ T cells</strong></td>
<td>0.3 (0.22 - 1.21)</td>
<td>0.4 (0.22 - 1.21)</td>
<td>0.28 (0.22 - 1.21)</td>
</tr>
<tr>
<td><strong>Absolute CD56+CD3- NK cells (x10^9/L)</strong></td>
<td>0.06 (0.12 - 0.60)</td>
<td>0.06 (0.12 - 0.60)</td>
<td>0.07 (0.12 - 0.60)</td>
</tr>
<tr>
<td><strong>Absolute CD19+ B cells</strong></td>
<td>0.13 (0.12 - 0.64)</td>
<td>0.09 (0.12 - 0.64)</td>
<td>0.08 (0.12 - 0.64)</td>
</tr>
</tbody>
</table>

**HLH determinants [criteria for familial HLH]**

<table>
<thead>
<tr>
<th>Test</th>
<th>Initial presentation (July 2018)</th>
<th>After ceasing dexamethasone (Dec 2018)</th>
<th>After reducing Anakinra (June 2019)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin (ng/mL)</td>
<td>13898 [&gt;500]</td>
<td>33 (7-150)</td>
<td>22 (7-150)</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>242 [&lt;150]</td>
<td>236 (150-450)</td>
<td>NA</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>265 [&gt;265]</td>
<td>41 (0-150)</td>
<td>NA</td>
</tr>
<tr>
<td>NK-cell degranulation§</td>
<td>10.64 (&gt;10)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>sCD25 (U/mL)</td>
<td>2204 (158-623)</td>
<td>1320 (158-623)</td>
<td>NA</td>
</tr>
<tr>
<td>IgG (g/L)</td>
<td>11 (4.9 – 16.1)</td>
<td>6.16 (6 – 16)</td>
<td>NA</td>
</tr>
<tr>
<td>IgM (g/L)</td>
<td>1.27 (0.5 - 1.9)</td>
<td>0.28 (0.5 - 1.9)</td>
<td>NA</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>0.98 (0.4 – 2.0)</td>
<td>0.27 (0.8 - 2.8)</td>
<td>NA</td>
</tr>
<tr>
<td>HiB IgG (µg/mL)</td>
<td>8.15</td>
<td>1.46</td>
<td>NA</td>
</tr>
<tr>
<td>Diphtheria IgG (IU/mL)</td>
<td>0.15</td>
<td>0.05</td>
<td>NA</td>
</tr>
<tr>
<td>Tetanus IgG (IU/mL)</td>
<td>1.25</td>
<td>0.25</td>
<td>NA</td>
</tr>
</tbody>
</table>
Reference ranges are indicated in round brackets. Values out of reference ranges are indicated in bold font. Criteria ranges for familial HLH are indicated in squared brackets. Hb: Hemoglobin; HiB: Haemophilus influenzae B antibody; HLH: hemophagocytic lymphohistiocytosis; Ig: immunoglobulin; NA: not applicable; sCD25: soluble CD25; # %CD107+ cells, according to the standard diagnostic measurement conducted in Freiburg, Germany.
FIGURE LEGENDS

Figure 1. Compound-heterozygous PIK3CG mutations in a patient with systemic inflammation. (A) Patient response to dexamethasone treatment (purple) as evidenced by the decrease of ferritin level in the patient’s peripheral blood. An immediate relapse of ferritin level upon decreasing dose of dexamethasone. Reinitiation of dexamethasone normalized ferritin level, and treatment with Anakinra (blue) was initiated and well-tolerated. Treatment was initiated at 14 years of age, and ferritin level was monitored over 7 months. Colored bars indicate drug dosage. (B) Patient bone marrow biopsy showing engulfment of erythroblast (black asterisk) and mature erythrocyte (white arrow) by a macrophage. The nucleus is indicated by the black arrow. Scale bar: 25 μm. (C) Compound-heterozygous PIK3CG base pair substitutions in the affected patient (filled symbol) segregate with parents. Sanger sequencing confirmed presence of a heterozygous variant in each parent (half-filled symbols). (D) Schematic representation of chromosomal position (top) and protein domains (bottom) of the identified PIK3CG/p110γ mutations, introducing two distinct missense mutations within the adaptor-binding domain (ABD) and near the kinase domain of the protein (bottom). RBD: Ras-binding domain; het: heterozygous. (E) Expression of p110γ protein in expanded T cells of the patient, compared with cells of mother and two healthy donors (HDs). HSP90 was used as a housekeeping loading control.
Figure 2. *PIK3CG* mutations affect adaptive and innate immune functions. (A) Reduced proliferative capacity of patient-derived T cells upon stimulation with anti-CD3 antibody or combined anti-CD3/CD28 antibody. PHA stimulation was not impaired, in agreement with TCR/PI3K-independent T-cell activation. Cells were stained with violet proliferation dye (VPD450), and dye dilutions were monitored three days post-stimulation. (B) Impaired activation of patient T cells. Peripheral blood mononuclear cells (PBMCs) were isolated and stained for the appearance of activation markers, one day (CD69 upregulation, left) or three days (CD25 upregulation, right) after stimulation with anti-CD3/CD28. Cells were gated on lymphocytes and CD8^-CD4^+ (left) or CD4^-CD8^+ (right) populations. (C) Rescue of T-cell activation via CD69 expression on day 2 post-stimulation with anti-CD3 in patient cells by exogenous expression of wild-type (WT) *PIK3CG* or empty vector (EV). Gated on GFP^+ transfected cells (left). Mean fluorescence intensity (MFI) of CD69 expression on GFP^+ transfected cells (right). (D) PI3Kγ inhibition with IPI-549 phenocopies T-cell proliferation defects. Cells were stimulated with anti-CD3/CD28 and monitored for VPD450 dye dilution as in (A). (E) Addition of PI3Kγ inhibitor IPI-549 (1 μM) phenocopies the T-cell activation defect, compared to DMSO control. Cells were stimulated as in (B) and gated on CD4^-CD8^+ (left) or CD8^-CD4^+ (right) lymphocytes. (F) Impaired activation of AKT signaling in *PIK3CG*-mutated patient PBMCs, gated on CD4^+CD8^- T cells. PBMCs were stimulated with anti-CD3/CD28 for 15 min. Phospho-AKT signal was reduced in patient cells compared to HDs (left). Normalization to unstimulated control is shown (right). (G) Decreased activation of *PIK3CG* knockout (KO) Jurkat cells compared to Renilla KO control upon anti-CD3 stimulation (**** *P*<0.0001, Two-way ANOVA). (H) Reduced MFI of AKT Ser473 phosphorylation in *PIK3CG* KO Jurkat cells compared to Renilla KO control and unstained control. (I) Representative immunostaining images of 40x magnification.
after 5 h incubation with PMA/ionomycin in HD, mother and patient monocyte-derived macrophages. Images show reduced cell spreading and total F-actin intensity in patient macrophages upon stimulation. Scale bars: 100 μm (top), 20 μm (middle, bottom). The lookup table (bottom right) indicates a color code for pixel values. (J) Patient macrophages show lack of cell spreading as indicated by significantly smaller cell area compared to mother and HD cells (**** $P<0.0001$, Mann-Whitney $U$ test).

(K) Reduced total F-actin intensity of patient macrophages compared to mother and HD cells (**** $P<0.0001$, Mann-Whitney $U$ test). (L) Reduced phagocytosis ability of patient-derived monocytes (left) and neutrophils (right). Whole-blood samples were incubated with pHrodo Red E. coli and phagocytosed bacteria were evaluated by flow cytometry. Populations were gated based on forward/side scatter characteristics of monocytes and neutrophils, respectively. All error bars indicate ± standard error of mean (SEM).
Supplementary Materials for

Germline biallelic PIK3CG mutations in a multifaceted immunodeficiency with immune dysregulation

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MATERIALS AND METHODS

Whole Exome Sequencing and analysis

Whole exome sequencing was performed using a TruSeq Rapid Exome kit as well as the Illumina HiSeq3000/4000 system and the cBot cluster generation instruments as previously described,\textsuperscript{1,2} with minor changes. Briefly, reads were aligned to the human genome version 19 by means of the Burrows-Wheeler Aligner (BWA). VEP was used for annotating single nucleotide variants (SNVs) and insertions/deletions lists. The obtained list was then filtered according to the presence of variants with a minor allele frequency (MAF) >0.01 in 1,000 Genomes, gnomAD, and dbSNP build 149. After further filtering steps for nonsense, missense, and splice-site variants using VCF.Filter software,\textsuperscript{3} an internal database was used to filter for recurrent variants. Moreover, variants were prioritized using tools, such as SIFT, Polyphen-2 and the combined annotation dependent depletion (CADD) score,\textsuperscript{4,5} that predict the deleteriousness of a present variant.

Sanger sequencing

Sanger sequencing was used to validate the two variants found in \textit{PIK3CG}:

variant 1, ENST00000359195.3:c.145C>A, p.Arg49Ser; and variant 2, ENST00000359195.3:c.3254A>G, p.Asn1085Ser, in the affected patient and her family members. This was done by designing specific primers for the two variants.

For variant 1,

Fw1: 5’- CATGTACGCCGCTATACCT -3’,
Rv1: 5’- TACCACTGCCCCTTCTTCTG -3’;

and for variant 2,

Fw2: 5’- TCCTGTTTCTCCATGATGCTG-3’,
Rv2: 5’- AACAATCAGCAATGCCAACA-3’.
Cell Culture

Human PBMCs were isolated by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation, washed twice in PBS, and resuspended in complete RPMI-1640 media (Gibco) containing 10% heat-inactivated FBS, 1% penicillin and streptomycin (Invitrogen) and 1% HEPES buffer solution 1 M (Invitrogen). CTL and NK-cell degranulation was assessed by CD107a surface staining for 3 h in presence or absence of dynabeads human T-activator CD3/CD28 (Thermo Fisher Scientific) stimulation or K562 target cells respectively. The erythroleukemic cell line K562 (ATCC, CCL-243) was used as target cell line. NK cells were cultured in complete RPMI-1640 media with or without 600 U mL⁻¹ IL-2 (Peprotech) for 48h to assess NK-cell degranulation of activated or non-activated NK cells respectively. Proliferative response was measured by labeling PBMCs with 1 mM Violet Proliferation Dye 450 (BD Biosciences) according to manufacturer’s instructions. For T-cell activation and proliferation assay, PBMCs were stimulated with 2 μg mL⁻¹ anti-CD3 (OKT3, Thermo Fisher Scientific) or with 1 μg mL⁻¹ anti-CD28 (Thermo Fisher Scientific) or 5 μg mL⁻¹ PHA (Sigma-Aldrich) for 1-3 days. PI3Kγ inhibitor IPI-549 was used at 1μM. For B cell proliferation and class switch recombination assays, PBMCs were stimulated with 200 ng mL⁻¹ CD40 ligand (R&D Systems) and 100 ng mL⁻¹ rhIL4 (R&D Systems) for 5 days. For T-cell expansion, PBMCs were stimulated with feeder cells (gamma-irradiated PBMCs) and 1 μg mL⁻¹ PHA (Sigma-Aldrich) with 100 U mL⁻¹ IL-2 (Peprotech) for 14 days. Expanded T cells were starved by serum deprivation 2 h prior to 200 ng mL⁻¹ SDF1 (Peprotech) stimulation. Jurkat E6-1 cells were cultured in complete RPMI-1640 media and stimulated with 2 μg mL⁻¹ anti-CD3 (OKT3). Reconstitution with GFP-labeled wild-type PIK3CG or empty vector GFP control was performed on a NEPA21 electroporator (Nepagene), according to manufacturer’s recommendations, with a poring pulse of 175 V for 5 ms.
Monocyte isolation and differentiation

Primary monocytes were isolated from PBMCs using human Pan Monocyte Isolation Kit (Miltenyi Biotec) according to manufacturer’s instructions. To generate human monocyte-derived macrophages, isolated primary monocytes were cultured in complete RPMI-1640 medium (Gibco) with 50 ng mL^{-1} recombinant human M-CSF (Peprotech) for 7 days. Surface markers for human macrophages such as CD163, CD206 were used to stain human monocyte-derived macrophages by flow cytometry. For morphology imaging, human monocyte-derived macrophages were plated on 384-well-plate (PerkinElmer) and stimulated with 250 ng mL^{-1} PMA (Sigma-Aldrich) and 1 μg mL^{-1} ionomycin (Sigma-Aldrich) for 5 h.

Microscopy and image analysis

Following incubation, cells were fixed with 3% Formalin (Pierce) in growth media for 15 min at 37 °C and incubated 15 min at 37 °C in permeabilization buffer (eBioscience) supplemented with 2% heat inactivated FBS (HyClone). Cells were stained first with anti-Tubulin antibodies (1:200, Sigma-Aldrich) for 1 h at RT followed by overnight staining at 4 °C with Phalloidin-Alexa488 (1:500; Thermo Fisher Scientific), DAPI (5 μg/ml, Thermo Fisher Scientific) and AF-555 secondary antibody conjugates (1:1000, Invitrogen). Finally, cells were washed and stored in PBS at 4 °C. Stained cells were imaged using PerkinElmer Opera Phenix high content screening system equipped with 20x (0.4NA) and 40x (1.1NA, water immersion) lenses, confocal unit (Yokogawa CSU-X) and solid state laser illumination (405nm, 488nm, 561nm, 640nm). Each plate was imaged twice: first with 20x lens to image all cells in each well (25x 1080x1080 pxl fields of view (FOV) per well; 640 nm/pxl resolution), followed by imaging with 40x lens to collect high-resolution images of cell footprint (9x 1080x1080 pxl FOV per well; 320 nm/pxl resolution; 3x Z stacks with dZ = 0.5 μm near the coverslip surface). All subsequent measurements were performed using the 20x dataset. 40x datasets were used to create representative images. Quantification of cell area and total amount of F-actin per cell
were done using CellProfiler software and custom written pipeline. Resulting measurements were then further processed using custom written Python scripts. Analysis of significance between donor groups was done using Mann–Whitney U test and Bonferroni correction for multiple comparisons.

Flow cytometry

Immunophenotyping was performed on a BD LSR-Fortessa. PBMC staining of surface markers was performed for 30 minutes at 4°C in the dark. The fixation/permeabilization kit for intracellular antigens or transcription factors (Affymetrix, eBioscience) were used for intracellular markers. Frozen PBMCs from patient and healthy donor controls were thawed and allowed to recover for four hours at 37°C in complete media (RPMI-1640 with 10% FBS). Following extracellular staining, cells were stimulated for 15 minutes at 37°C with Dynabeads© Human T activator CD3/CD28 (ThermoFisher) and IL2 (100 IU/mL). Cells were then immediately fixed for 10 minutes at 37°C, washed and permeabilized for 35 minutes on ice. Cells were then stained with p-AKT-PE for 1 hour at room temperature, washed again and resuspended in FACS buffer for flow cytometry analysis. All analyses were performed using FlowJo X (TreeStar Inc.) and Prism 8.0 (GraphPad Software). The following antibodies were used for flow cytometry: From Beckman Coulter: CD16-FITC (clone 3G8), CD4-PECy7 (SFCII2T4D11), CD56-PE (N901), CD56-PECy5 (N901); from eBisocience, Affymetrix: CD4-PerCPCy5.5 (RPA-T4), CD19-PerCPCy5.5 (HIB19), CD69-APC (FN50), FOXP3-APC (236A/E7); from BD Biosciences: CD19-PECy7 (SJ25C1), CD14-PECy5 (61D3), CD16-PECy7 (3G8), CD25-PE (M-A251), CD25-BV605 (2A3), CD27-V450 (M-T271), CD3-APC-H7 (SK7), CD4-BV605 (RPA-T4), CD45RA-AF700 (H1100), CD56-V450 (B159), CD8-V450 (RPA-T8), IgD-FITC (IA6-2), anti-AKT-PE (pS473); from Biolegend: CD163-BV605 (GHI/61), CD206-PE (MMR); from Miltenyi Biotec: IgA-PE (IS11-8E10); IgG-APC (IS11-3B2.2.3).
**Immunoblotting**

Whole cell lysates were prepared from control- and patient-derived expanded T cells, loaded on 10% acrylamide gel, separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated with 5% BSA in Tris-buffered saline with 0.5% Tween (TBS-T) and were probed overnight with 1:1000 dilution of anti-p110γ (4252) or anti-phospho-AKT Ser473 (D9E) or anti-AKT (pan, 40D4) from Cell Signaling, and 1:8000 dilution of anti-HSP90α/β (Santa Cruz) as a loading control. Bands were revealed using Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare).

**Generation of CRISPR/Cas9-edited Jurkat cell lines**

sgRNAs targeting PIK3CG were designed using the GPP Web portal (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) and cloned into a lentiCRISPRv2 using FastDigest Esp3I (Thermo Fisher Scientific) and standard molecular cloning. Lentivirus was generated from transfection of Lenti-X 293T cells (Takara Bio) with Polyfect (Qiagen) using the lentiviral envelope and packing vectors pMD2.g and psPAX2. Lentiviral supernatant was harvested 48 h post transfection, filtered and directly applied to Jurkat E6-1 cells. Transduction was carried out by spinfection (1 h, 37°C, 800 rcf) in the presence of 8 µg mL⁻¹ Polybrene. Transduced cells were selected with 1 µg mL⁻¹ Puromycin (Sigma). Single clones were selected by limiting dilution and editing was assessed using TIDE.⁶ The utilized knockout cells showed 84.6% total editing efficiency, with 21.5% at position +2, 42.9% at position +4 and 19.5% at position -1 after Cas9 cutting site. sgRNA and primer sequences for TIDE were as follows:

For sgRNA,
Fw: caccgAAGTATGACGTCAGTTCCA,
Rv: aaacTGGGAACTGACGTCATACTTc;

and for TIDE,
Fw: TCTAGCCGTGAAGACCCAGT,
Rv: GCATAATGCTGCTTAATTTTTCAGT.

Phagocytosis assay

The phagocytic function of neutrophils and monocytes was evaluated in 100 μL of sodium-heparinized whole blood sample to 20 μL pHrodo Red *E. coli* (Life Technologies) for 15 minutes at 37°C according to manufacturer’s instructions. Erythrocytes were lysed using reagents provided in pHrodo Red *E. coli* BioParticles Phagocytosis Kit for Flow Cytometry (Life Technologies) and analyzed using flow cytometry (PE channel). Monocytes and neutrophils were distinguished based on forward/side scatter characteristics.

Neutrophil assays

Neutrophils were isolated from whole blood by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation. After recovering of PBMCs, the remaining fraction was sequentially pulsed with Milli-Q water and 3 M KCl for erythrocyte lysis, and neutrophils were recovered by centrifugation. Isolated neutrophils were kept in complete RPMI-1640 media, spread on a 10cm culture dish, until further use. For detection of apoptotic cells, neutrophil samples were taken at the indicated time points, replaced by Annexin-V binding buffer (BD Biosciences), and stained on ice for 5 min with propidium iodide (BD Biosciences) and Annexin-V-APC (BD Biosciences), before acquisition by flow cytometry. HD neutrophils treated at 70 °C served as positive control for gate setting. Gating was based on forward/side scatter including live and dead neutrophils. For evaluation of mitochondrial membrane potential, neutrophil fractions were washed in PBS and stained with 3.5 μM JC-1 (Thermo Fisher Scientific) for 15 min at 37 °C. After PBS wash, samples were treated with 100 nM valinomycin in complete RPMI-1640 media. Samples were either taken immediately (0 h) or incubated for 2 h. Cells were gated on single-cell neutrophils by forward/side scatter, and final gates were set on comparing
mitochondrial J-aggregates (PE channel) with cytosolic JC-1 monomers (AlexaFluor488 channel) appearing from mitochondrial membrane leakage. For evaluation of oxidative burst capacities, neutrophils were resuspended in pre-warmed KRP-PBS buffer and placed in a 37 °C thermoshaker. At time point 0, Oxyburst green reagent (Thermo Fisher Scientific, used 1:20) was added and samples were immediately taken at the indicated time points, by pipetting into tubes prepared with ice-cold PBS. For flow cytometry acquisition, an unlabeled sample was taken for peak setting (FITC channel) within the neutrophil scatter gate. For neutrophil chemotaxis, 50,000 neutrophils were placed into the upper chambers of a 96-well transwell plate (Corning, 5 μm pore size), and left to migrate towards 5 nM fMLP or DMSO control in complete RPMI. After 20 min incubation, migrated neutrophils were counted by forward/side scatter detection, and normalized to sample controls of 50,000 neutrophils seeded into the lower compartments, respectively, to reflect total input.

Statistical Analysis

Statistical evaluation of experimental data was performed using Prism version 8 (GraphPad Software, USA). Probability (P) values < 0.05 were considered statistically significant. P values and statistical tests are indicated in figure legends, where applicable.
**SUPPLEMENTARY TABLES**

**Table S1:** Characteristics of the identified germline-encoded *PIK3CG* variants.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Ref.</th>
<th>Obs.</th>
<th>Substitution</th>
<th>PolyPhen-2</th>
<th>CADD</th>
<th>gnomAD allele counts</th>
<th>ExAC allele counts</th>
<th>ExAC pNull</th>
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</thead>
<tbody>
<tr>
<td><em>PIK3CG</em></td>
<td>Chr7 106545777</td>
<td>A</td>
<td>G</td>
<td>p.N1085S</td>
<td>probably damaging</td>
<td>23.8</td>
<td>---</td>
<td>---</td>
<td>4.4365 E-06</td>
</tr>
<tr>
<td><em>PIK3CG</em></td>
<td>Chr7 106508151</td>
<td>C</td>
<td>A</td>
<td>p.R49S</td>
<td>benign</td>
<td>22</td>
<td>---</td>
<td>hom: 0, het: 2 (117466), MAF: 1.703 E-05</td>
<td>4.4365 E-06</td>
</tr>
</tbody>
</table>

Prediction scores were calculated with PolyPhen-2 and CADD tools. Current genomes include 10,738 (gnomAD, https://gnomad.broadinstitute.org/) and 60,706 individuals (ExAC, http://exac.broadinstitute.org/). MAF frequencies are indicated for identified heterozygotes (accession date: May 21, 2019). Gene names are indicated in italic font. (Ref.: reference; Obs.: observed; het: heterozygous; hom: homozygous; MAF: minor allele frequency; pNull: probability of loss-of-function tolerance; ---: not reported)
Table S2: Extended immunological features of patient T-cell subsets.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patient</th>
<th>Reference Ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ (%)</td>
<td>61.6</td>
<td>(25-48)</td>
</tr>
<tr>
<td>CD45RA+ CCR7+ (%)</td>
<td>45.8</td>
<td>(43.3-63.2)</td>
</tr>
<tr>
<td>CD45RA- CCR7+ (%)</td>
<td>40.5</td>
<td>(30.85-45.25)</td>
</tr>
<tr>
<td>CD45RA- CCR7- (%)</td>
<td>12.7</td>
<td>(4.2-16.25)</td>
</tr>
<tr>
<td>CD45RA+ CCR7- (%)</td>
<td>0.88</td>
<td>(0.1-2.1)</td>
</tr>
<tr>
<td>CD8+ (%)</td>
<td>12.2</td>
<td>(9-35)</td>
</tr>
<tr>
<td>CD45RA+ CCR7+ (%)</td>
<td>60.1</td>
<td>(37-69.35)</td>
</tr>
<tr>
<td>CD45RA- CCR7+ (%)</td>
<td>19.7</td>
<td>(14-36.85)</td>
</tr>
<tr>
<td>CD45RA- CCR7- (%)</td>
<td>12.2</td>
<td>(2.4-15.5)</td>
</tr>
<tr>
<td>CD45RA+ CCR7- (%)</td>
<td>7.98</td>
<td>(3.9-27.25)</td>
</tr>
</tbody>
</table>

Reference ranges are indicated in round brackets. Values out of reference ranges are indicated in **bold** font.
SUPPLEMENTARY REFERENCES


Figure S1. (A) Affected amino acid stretches of p110γ are conserved across species. (B) Reduced NK-cell frequency in patient peripheral blood. Numerical inserts depict percentages within the final gate. (C) Reduced degranulation capacity in patient-derived NK cells, as compared to mother and HD, indicative of impaired cytotoxic capacities. Peripheral blood-derived NK cells were incubated for 3 h with K562 target cells (middle panel) or NK cells activated with IL2 for 48 h prior to K562 target cells (right panel), and compared according to percentages of CD107a+CD3−CD56+ cells as a proxy of degranulation ability (* P <0.05, ** P <0.01, Two-way ANOVA). (D) Quantification of proliferated CD8+ T cells in percentage upon stimulation with various stimuli (** P <0.01, Two-way ANOVA). (E) Impaired activation of AKT signaling in PIK3CG-mutated patient cells. Patient-derived expanded T cells were stimulated with SDF-1 for the indicated time points. Phospho-AKT signal intensity was compared with total AKT signal. HSP90 was used as housekeeping loading control. (F) Abrogated p110γ and reduced p-AKT expressions in PIK3CG knockout (KO) Jurkat cells compared to Renilla KO control Jurkat cells. GAPDH was used as a housekeeping loading control. (G) Reduced normalized mean fluorescence intensity (MFI) of AKT Ser473 phosphorylation in PIK3CG KO Jurkat cells compared to Renilla KO control (** P<0.01, Mann-Whitney U test). Dotted line represents the normalization to unstained control. (H) Genetic rescue of CD69+ activation (normalized) in PIK3CG KO Jurkat cells using wild-type (WT) vector, empty vector (EV), mutant N1085S or mutant R49S vectors (P=0.1, Mann-Whitney U test). (I) Intact cytotoxic capacity in patient CTLs compared to cells from mother and HDs. Cells were incubated for 3 h with CD3/CD28 stimulation and compared according to mean fluorescence intensity (MFI) of CD107a (LAMP1) on CD3+CD8+ T cell surfaces as a proxy of degranulation ability. (J) Increased frequency of peripheral blood CD4+CD127dimFOXP3+CD25+ Treg cells in patient, compared to mother and HD. Numerical
(K) Patient B cells proliferate normally during 5-day stimulation with CpG or CD40L in combination with IL4 or IL21. (L) Class switch recombination capacity of patient B cells is intact after 5-day stimulation with CD40L with IL4.

**Figure S2.** (A) Comparable macrophage surface markers CD163 and CD206 on monocyte-derived macrophages of patient, mother and HD, after 9 days in differentiation culture. (B) Violin plots showing average cell number per image of patient, mother and HD. (C) Slightly elevated cell death in patient neutrophils. Freshly-isolated neutrophils showed a pronounced propidium iodide-positive population (top, red numerical insert). Apoptotic neutrophils persist during incubation (bottom). Numerical inserts indicate percentages. (D) Compromised mitochondrial membrane potential in patient neutrophils. Mitochondria were stained with JC-1 dye, membrane leakage was monitored during incubation with valinomycin. Presence of mitochondrial J-aggregates was compared with appearance of cytosolic JC-1 monomers. Patient neutrophils rapidly lose membrane integrity compared to mother and HD, as observed by the increased dye loss (red numerical insert). Numerical inserts indicate percentages. (E) Patient neutrophils are able to initiate oxidative burst upon Fc receptor stimulation as shown by the appearance of oxyburst green-positive cells. (F) Patient-derived neutrophils are able to migrate towards fMLP, compared to cells from HDs and mother.
[Image showing various graphs and tables related to experimental data in the fields of immunology and cell biology. The graphs illustrate the percentages of proliferated cells in different conditions, the expression levels of specific proteins, and other relevant metrics.]