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ORIGINAL ARTICLE

Lymphocytes Utilize Somatic Mutations, Epigenetic Silencing, and the Proteasome to Escape Truncated WASP Expression

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Abstract
Wiskott-Aldrich Syndrome Protein (WASP) deficiency causes Wiskott-Aldrich Syndrome (WAS), a sex-linked disorder characterized by combined immunodeficiency, microthrombocytopenia, and eczema. Like WASP-deficient humans, WASP-deficient mice produce normal numbers of functionally defective T cells. Here, we report a WAS patient with a novel germline frameshifting *WAS* mutation encoding a truncated form of WASP lacking the C-terminal cofilin homology (C) and the acidic region (A) domains (WASPΔCA). Although stably overexpressed in embryonic kidney cell lines, WASPΔCA was undetectable in circulating patient leukocytes. Deep sequencing, transcript profiling, and protein degradation analyses demonstrated patient lymphocytes employ an array of genetic, epigenetic, and proteasomal strategies to avoid expressing WASPΔCA.

Keywords Wiskott-Aldrich Syndrome · WAS · WASP · Proteosome

Introduction

The gene *WAS* encodes WASP, an actin cytoskeleton organizing protein critical to leukocyte and platelet function [1]. WASP deficiency causes Wiskott-Aldrich Syndrome (WAS), a sex-linked disorder characterized by combined immunodeficiency, microthrombocytopenia, and eczema [1–4]. Like WASP-deficient humans, WASP-deficient mice produce normal numbers of functionally defective T cells [5–7]. In contrast, mice with T-cell restricted expression of truncated WASP, which lacks the C-terminal verprolin homology (V), the cofilin homology (C), and the acidic region (A) domains (WASPΔVCA), demonstrate reduced TCRαβ expression, and arrest of T cell lymphopoiesis at the $CD4^+CD8^+$ "double positive" developmental stage [8]. Here, we report a T cell replete WAS patient with a novel germline frameshifting mutation in *WAS* predicted to generate a truncated form of WASP lacking the C-terminal C and A domains (WASPΔCA). Although as stable as unmutated WASP when overexpressed in embryonic kidney cell lines, patient WASPΔCA was not detectable in primary patient lymphocytes. Deep sequencing, transcript profiling, and protein degradation analyses revealed patient lymphocytes employ a variety of genetic, epigenetic, and proteasomal strategies to avoid expressing WASPΔCA.

Methods

Human Sample Collection

Peripheral blood samples and saliva samples were collected with informed consent in accordance with research protocols approved by institutional review boards of Children's Hospital of Philadelphia and Children's Health Orange County.

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Cell Preparation, Flow Cytometry, and Cell Sorting

Mononuclear cells (PBMCs) were isolated from peripheral blood samples using Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare Life Sciences). Cells were surface antibody stained for 30 min at 4 °C with the following Abs: CD4 (OKT4), CD8 (HIT8a), CD14 (HCD14), CD19 (HIB19), and TCRα/β (IP26) from Biolegend and CD3 (UCHT1, BD Bioscience). Cells were analyzed using a LSRFortessa (BD Bioscience) and/or sorted using a MoFlo Astrios EQ (Beckman Coulter). FACS data was visualized with FlowJo (TreeStar).

Genotyping

Clinical whole exome sequencing identified germline *WAS* mutations. Sanger sequencing was used to determine the presence of *WAS* mutations in the patients' family members. Buccal DNA was isolated from saliva using Gentek's prepIT L2P reagent and protocol. PBMC DNA was isolated using QIAamp Blood DNA Mini Kit (Qiagen). Genomic DNA, specifically *WAS* exon 11, was amplified using QIAGEN Fast Cycling PCR Kit and primers listed in Table S1. Sanger sequencing was performed by the University of Pennsylvania DNA Sequencing Facility. All identified *WAS* variants were annotated to cDNA transcript NM_000377. The index patient's mutation has been deposited in the NCBI ClinVar database under accession number SCV001197985 (g.12786_12787insG).

Amplicon Sequencing Data Analysis

WAS and *WIP* exons, introns, promoters, and 3′/5′ untranslated regions from sorted cell subsets were amplified using AmpliSeq for Illumina. Amplicons were sequenced to 10,000X read depth. Illumina's DRAGEN Bio-IT Platform (v3.3.7) was utilized for demultiplexing, alignment, and somatic variant calling against the human reference genome (Broad Institute human_g1k_v37). Adapter sequences were trimmed from the FASTQ files with Cutadapt (v1.18) before proceeding to alignment. Somatic variant calling was limited to regions within the custom amplicon BED file. All default DRAGEN parameters were used except for the following metrics: target coverage, maximum reads per active region, and maximum reads per raw region which were increased to 10,000. VCFs were then annotated using ANNOVAR (version date: 2018–04-16). A number of tools and databases were used for variant analysis including Alamut (v2.14), HGMD (v2019.3), gnomAD (v2.1.1), and IGV (v2.7.2). Mutation frequency box plots were generated with R (v3.6.1).

Immunoblots

Whole protein lysates were isolated by lysing sorted cell subsets in ice-cold RIPA buffer (150 mM Nacl, 5 mM EDTA, 50 mM Tris pH8, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor. Protein concentrations were quantified by Bradford testing and a uniform amount of protein (5–10ug) was incubated with 4 × Laemlli sample buffer containing 2-βmercaptoethanol for 5 min at 95℃. Proteins were separated using 4–15% SDS-PAGE gel and transferred to PVDF membranes using the trans-blot turbo transfer system (Bio-Rad). Blots were probed with a rabbit anti-WASP antibody (D9C8, Cell Signaling) recognizing central residues of WASP or a mouse anti-WIP antibody (A-7, Santa Cruz) covering the C-terminus. Rabbit anti-GAPDH (D16H11, Cell Signaling) antibodies were used to ensure equal loading.

cDNA Sequencing and qPCR

RNA was isolated from cell subsets (Direct-zol RNA Microprep, Zymo Research), and cDNA was generated with ImProm-II Reverse Transcription System (Promega). For transcript sequencing, cDNA was amplified using primers spanning exon 10 through 12 (Table S3). The qPCR experiments were performed using *WAS* primers spanning the exon 7–8 boundary (Invitrogen Hs00997437) and the 7500 Fast Real-Time PCR system (Applied Biosystems). *WAS* transcripts were normalized against the β-2 microglobulin.

Protein Degradation Experiments

One million lymphoblasts were cultured with 1 μM calpeptin for 0, 2, 6, or 10 h, or 10 μM MG-132 for 0, 2, or 6 h. Whole protein lysates were isolated and immunoblots were performed, visualized, and analyzed as described above.

Plasmid Construction and HEK293 Transfections

Plasmids were constructed that contained *WAS* cDNA sequence fused to the mCherry gene under the control of a pCMV promoter. Germline and somatic mutations were introduced into the plasmid using custom site-directed mutagenesis primers listed in Table S1 and the Q5 Site-Directed Mutagenesis kit (NEB). Two hundred nanograms of plasmid was Lipofectamine (Invitrogen) transfected into 5×10^6 HEK293 cells, a line that lacks native WASP expression. Whole protein lysates were isolated and immunoblots performed as described above.

Results

Patient Information

The index WAS patient (IV.1) presented in his tenth month of life with microthrombocytopenia, eczema, and Bacille Calmette-Guérin (BCG) vaccine–associated lymphadenitis (Fig. 1A, Table S2-3). Whole exome sequencing of peripheral blood genomic DNA identified a novel, maternally inherited guanine insertion in *WAS* (g.12786_12787insG, c.1400insG, p.A469Gfs*26) that shifted the exon 11 reading frame (Fig. 1B). The mutant gene product was predicted to replace the C-terminal cofilin homology (C) and the acidic region (A) domains with 26 non-sense amino acids (i.e., WASPΔCA). Although an only child, IV.1's extended pedigree included three male relatives with congenital thrombocytopenia who all died prematurely due to bleeding and/or infectious complications (Table S2). Genotyping indicated the mothers of all affected, deceased male relatives carried the g.12786_12787insG *WAS* mutation, and that unaffected healthy male relatives did not (Fig. 1A). *WAS* cDNA generated from WAS patient IV.1 mother's PBMCs contained only wildtype sequences consistent with non-random X-chromosome inactivation (XCI) Figure S1) [9]. After 3 months of anti-mycobacterial therapy, patient IV.1 underwent a haploidentical hematopoietic stem cell transplant with an unremarkable post-transplant clinical course.

Somatic Mutations Prevent Expression of WASPΔCA

Prior to transplantation, patient peripheral blood mononuclear cells (PBMCs) were assessed for WASP expression with an antibody recognizing central WASP domains. Although most (~80%) patient PBMCs stained WASP negative, consistent with absolute protein deficiency, we identified a distinct subset of WASP-expressing cytotoxic T lymphocytes (CTLs) (Fig. 2, A and B). Unlike control CTLs, which were phenotypically uniform, patient CTLs could be divided into three distinct subsets (CTL#1, CTL#2, and CTL#3) based upon variable CD8, TCRαβ, and WASP expression. CTL#1 cells were most abundant and demonstrated the highest surface CD8 expression ($CD8^{hi}TCR\alpha\beta^{hi}WASP^{+}$). CTL#2 cells were rarer and distinguished by absent WASP with very low TCRαβ expression ($CD8^{int}TCR\alpha\beta^{lo}WASP^{-}$). CTL#3 cells were rarest but closely resembled the phenotype of healthy control CTLs ($CD8^{int}TCR\alpha\beta^{hi}WASP^{+}$). To determine if somatic *WAS* mutations could potentially explain WASP, TCRαβ, and CD8 expression differences, genomic DNA was extracted from patient CTL#1, CTL#2, CTL#3, $CD4^{+}$ T cells, $CD19^{+}$ B cells, EBV-transformed lymphoblasts, and monocytes. *WAS* gene exons, introns, promoters, 5′ untranslated regions (UTRs), and 3′ UTRs were subsequently amplified by PCR and then deep sequenced to at least 10,000 reads. Transcriptional and translational consequences of germline and somatic *WAS* mutations were assessed by sequencing cell population–specific *WAS* cDNA transcripts and by probing the lysates of *WAS* overexpressing HEK293 lines with an anti-WASP detection antibody.

Deep sequencing identified multiple somatic mutations in *WAS* within patient CTL#1 and CTL#3 cells. Mutations were clustered at genomic locations in and around *WAS* exon 11 (Fig. 3). Most (88%) *WAS* genes from CTL#1 cells contained both the germline g.12786_12787insG *WAS* variant and intronic exon 11 acceptor-site somatic mutations (g.IVS10-1G > C or A). Two detectable *WAS* transcripts were associated with this allele. One transcript joined exon

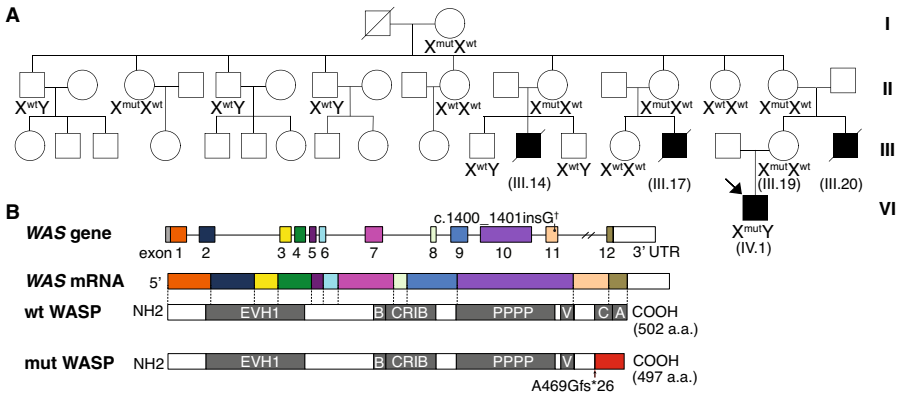

Fig. 1 Patient pedigree and genotypes. **A** The Wiskott-Aldrich Syndrome (WAS) patients' pedigree is annotated with *WAS* genotypes of affected (filled) and unaffected (unfilled) relatives. The studied index patient (IV.1) is indicated (arrow). **B** *WAS* gene, *WAS* transcript, and WAS protein (WASP) structures are depicted. The location of the germline c.1400_1401insG mutation and V469Gfs*26 altered mutant WASP are shown. Missense amino acids appear in red. † indicates germline *WAS* mutation

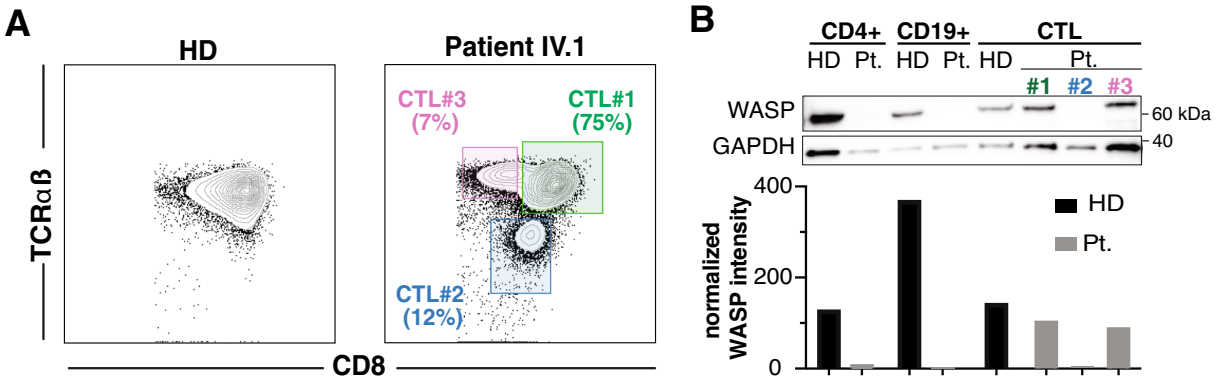

Fig. 2 Patient cytotoxic T lymphocyte (CTL) subsets variably express CD8, TCRαβ, and WASP. **A** Two-dimensional flow cytometry plots show normal and abnormal cell-surface expression of CD8 and TCRαβ on heathy donor (HD) and patient IV.1 CTLs. The gating strategy to sort-separate patient CTLs into three subpopulations (CTL#1–CTL#3) is shown. **B** WASP immunoblots of HD and patient CD4$^+$ T cells, CD19$^+$ B cells, and CTL subsets (above) and relative WASP quantification (below) are displayed

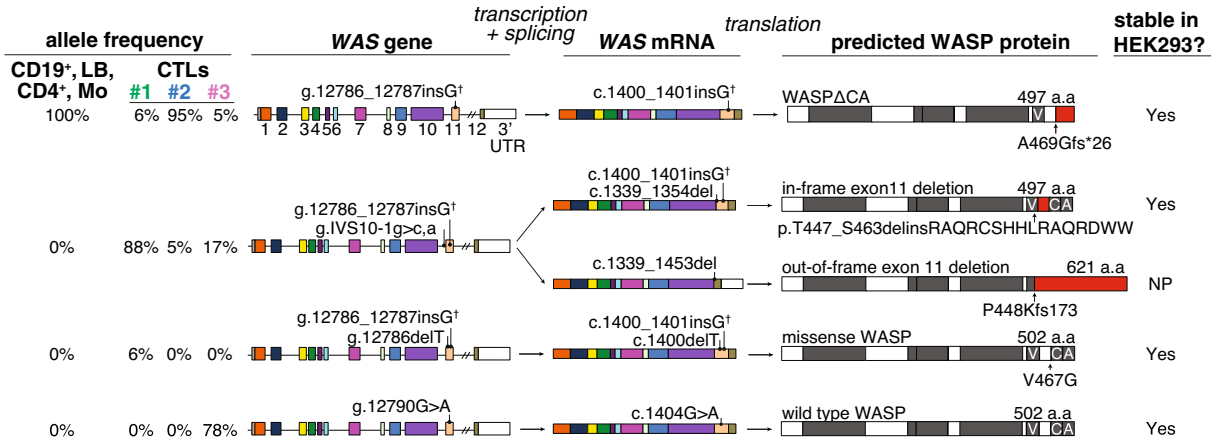

Fig. 3 Identification of somatic WAS mutations in patient IV.1 cytotoxic T lymphocyte (CTL) subsets. The frequency of mutant *WAS* alleles in patient CD19$^+$ B cells, patient-derived lymphoblasts, CD4$^+$ T cells, monocytes, and CTL subsets #1–3 are displayed. Germline (†) and somatic mutations in *WAS* genes and *WAS* transcripts are shown. Mutated WASP proteins, with missense amino acids indicated (red), are depicted. Detection of mutated proteins in transfected HEK293 cells is indicated. NP, not performed

10 to an out-of-frame cryptic acceptor splice site within exon 11. The out-of-frame joint was predicted to encode 16 consecutive non-sense amino acids between the V and the C domains before the germline c.1400insG mutation restored the native reading frame, allowing translation of fully intact C and A domains. This protein, p.T447_S463delinsRAQRCSHHLRAQRDWW, appeared to be more stable in HEK293 lines than unmutated and germline-mutated WASP (Figure S2). The other transcript associated with intronic g.IVS10-1 G > C or A somatic mutations produced an out-of-frame exon 10/12 junction that skipped exon 11 entirely (including the germline mutation c.1400insG) and moved the native stop codon 519 nucleotides downstream into the untranslated 3′ region. This much longer mutated protein (p.P448Kfs*173) was undetectable in patient CTL#1 cells (Fig. 2) and was predicted to be unstable at physiologic temperature by an in silico algorithm [10]. A second *WAS* allele identified in 6% of CTL#1 cells contained a novel thymidine deletion (g.12786delT) next to the original germline g.12786_12787insG mutation. This deletion restored the reading frame of the associated transcript which produced full-length WASP with a valine to glycine single amino acid substitution (p.V467G). WASP p.V467G appeared to be more stable in HEK293 lines than unmutated and germline-mutated WASP (Figure S2).

Unlike CTL#1 cells, CTL#3 *WAS* genes completely lacked the germline g.12786_12787insG mutation (Fig. 3). Instead, most CTL#3 cells carried a silent

guanine for adenine substitution at g.12790. The variant was not present in other patient cell types or cells from the patient's mother. The associated c.1404G > A *WAS* transcript generated a full-length, stable wild-type WASP protein in HEK293 cells. In contrast to the somatic mutations identified in patient CTL#1 and CTL#3 cells, the only mutation identified in CTL#2, $CD4^+$ T cells, $CD19^+$ B cells, EBV-transformed lymphoblasts, and monocytes was the maternally inherited germline c.1400insG variant. The associated transcript, c.1400insG, was detected in studied cell types and the predicted protein p.A469Gfs*26 (WASPΔCA) appeared similarly stable to unmutated WASP when overexpressed in HEK293 cells (Figure S2). Hence, patient CTL#1 and CTL#3 subsets, but not other cell types, utilize somatic mutations to produce forms of WASP other than WASPΔCA.

Lymphoblast Proteosomes Preferentially Degrade WASPΔCA

To determine why HEK293 stably expressed WASPΔCA but patient lymphocytes did not, we quantitatively compared c.1400insG *WAS* transcripts in patient and healthy donor-derived lymphoblasts. *WAS* transcript copies were not numerically different between patient and control cells, suggesting WASPΔCA was degraded after translation (Fig. 4A). WASP-interacting protein (WIP) is a known WASP stabilizer and WIP deficiency creates a WASP-deficient state that is clinically indistinguishable from WAS [11]. To assess for WIP altering somatic mutations, *WIP* exons, introns, promoters, and 5′ untranslated regions were deep sequenced in CTL#1, CTL#2, CTL#3, $CD4^+$ T cells, $CD19^+$ B cells, EBV-transformed lymphoblast, and monocytes. No somatic mutations were identified. Additionally, WIP protein was similarly expressed in patient and healthy control lymphoblasts (Figure S3).

Calpain and the proteosome mediate intracellular turnover of damaged proteins including WASP [12]. To determine if WASPΔCA was specifically targeted by either degradation pathway, patient and control lymphoblasts were cultured in either MG-132 (proteasome inhibitor) or calpeptin (calpain inhibitor). At assessed time points, neither inhibitor qualitatively affected WASP in control cells. In contrast, WASPΔCA became increasingly detectable in patient lymphoblasts after 2–6 h in MG-132 (Fig. 4B) while calpeptin treatment did not increase WASPΔCA detection even after 10 h in culture (Fig. 4C). Accordingly, patient lymphoblasts employ the proteasome to preferentially degrade WASPΔCA whereas somatically mutated WASP proteins were tolerated in patient CTL#1 and CTL#3 cells.

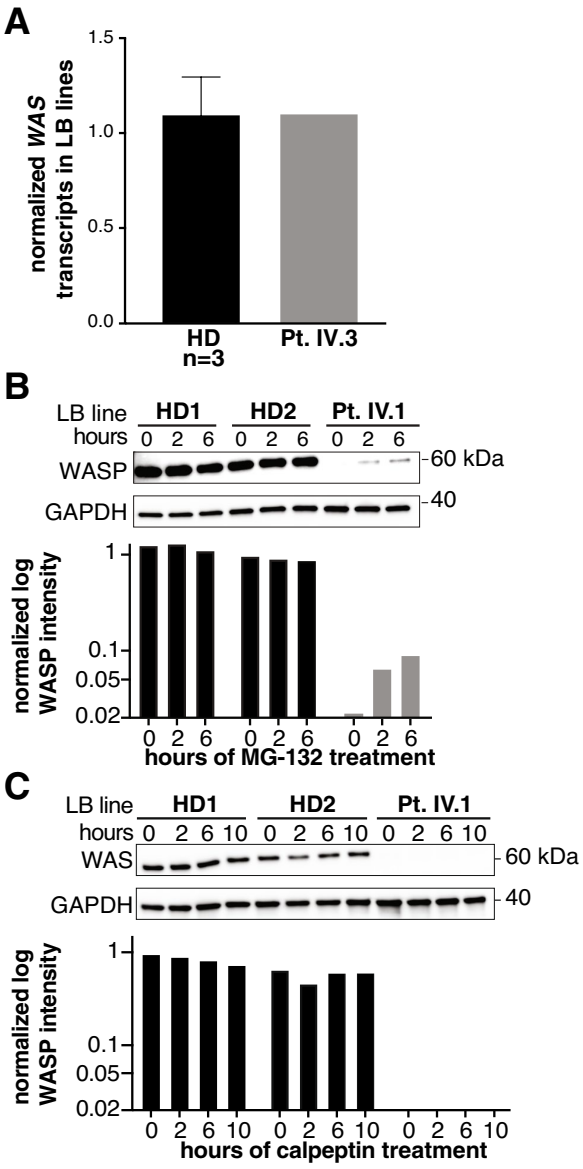

Fig. 4 WASPΔCA is preferentially degraded by the proteasome. **A** qPCR-quantified mean *WAS* cDNA transcripts of lymphoblasts derived from three healthy donors (HD) and patient IV.1. *WAS* transcript quantities are β-2 microglobulin normalized. The error bar reflects standard deviation. **B** WASP immunoblots of healthy donor (HD) or patient IV.1 lymphoblasts cultured with **A** proteasome inhibitor MG-132 (10 μM/L) for 0, 2, or 6 h or alternatively with **B** calpain inhibitor calpeptin (1 μM/L) for 0, 2, 6, or 10 h. Accompanying bar graphs depict GAPDH-normalized WASP immunoblot intensities on log scales

Discussion

Here we describe a patient with a novel, inherited *WAS* mutation encoding WASPΔCA. Although WASPΔCA is

stably expressed by HEK293 cells, patient PBMCs avoid WASPΔCA expression by employing one or more evasion strategies. Deep sequencing revealed that CTL#1 and CTL#3 cells employed strategic somatic mutations to either revert the germline frameshifting mutation in exon 11, or restore the native reading frame, or skip exon 11 entirely with post-transcriptional splicing. Other studied cell types possessed only the germline-inherited frameshifting mutation and associated mutated messenger RNA but did not express detectable WASPΔCA unless cultured with proteasomal inhibitor MG-132. Since both wild-type WASP (from gene reversion) and mutant WASP with intact C and A domains were clearly detectable in patient CTL#1 and CTL#3 cells, it appears WASPΔCA is specifically targeted by the proteosome for degradation.

Why do lymphocytes avoid WASPΔCA expression? One possibility is that WASPΔCA may exert negative selective pressure during lymphocyte development. Like murine thymocytes expressing WASPΔVCA, patient CTL#2 cells expressing WASPΔCA downregulated TCRαβ through an unclear mechanism [8]. Importantly, unlike developing mouse T cells, patient IV.1's cells may have avoided developmental arrest by suppressing WASPΔCA via targeted proteosome activity or other post-translational degradation processes. Such mechanisms appear absent or unutilized in HEK293 lines which are tolerant of WASPΔVCA expression. Additional effects from the patient's localized BCG infection in early life [13] may have favored expansion of CTL#1 and CTL#3 cells escaping WASPΔCA expression with strategically placed somatic mutations. Presumably if patient IV.1 was unable to post-translationally degrade and somatically evade WASPΔCA or his mother was unable to epigenetically silence it via XCI, either might have presented with the same severe T cell lymphopenia previously described in WASPΔVCA transgenic mice [8].

Although the germline *WAS* variant described here is novel, unrelated WAS patients with different germline mutations encoding WASPΔCA or WASPΔVCA have also been reported [14, 15]. In such cases, somatic *WAS* mutations that revert to wild-type WASP or alternatively abolish WASP expression were also identified using less comprehensive sequencing approaches. Together, these cases clearly demonstrate that like mouse counterparts, human lymphocytes expressing truncated WASP (WASPΔCA or WASPΔVCA) are under greater counter-selective pressure than lymphocytes expressing no WASP at all.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10875-022-01224-6.

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Author Contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Caroline Khanna, Carole Le Coz, Piyush Pillarisetti, Tori Knox, and Neil Romberg. Andrew Sy and David Buchbinder provided patient samples and clinical information. Bioinformatic analysis was performed by Courtney Vaccaro. Edward Behrens provided technical support. Neil Romberg supervised all aspects of the study. The first draft of the manuscript was written by Caroline Khanna and Neil Romberg and was critically reviewed by all authors. All authors read and approved the final manuscript.

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Data Availability The index patient's mutation has been deposited in the NCBI ClinVar database under accession number SCV001197985. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability No unique code or non-public electronic algorithms were used to generate this manuscript's dataset.

Declarations

Ethics Approval Human biospecimens were collected using research protocols approved by institutional review boards of Children's Hospital of Philadelphia and Children's Health Orange County.

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent to Publish No images, videos, or other material that could personally identify a research subject is contained in this manuscript.

Competing Interests The authors declare no competing interests.

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