

Novel RIPK1 Mutations Causing Infantile-onset IBD with Inflammatory and Fistulizing Features

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Research Article

Keywords: RIPK1, monogenic IBD, VEOIBD, necroptosis, inflammasome, Crohn's

Posted Date: February 24th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1347173/v1>

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Abstract

Purpose: Receptor-interacting serine/threonine-protein kinase 1 (*RIPK1*) is an important regulator of necroptosis and inflammatory responses. We present the clinical features, genetic analysis and immune work-up of two patients with infantile-onset inflammatory bowel disease (IBD) resulting from novel *RIPK1* mutations.

Methods: Whole exome and Sanger sequencing was performed in two IBD patients. Mass cytometry time of flight (CyTOF) was conducted for in-depth immunophenotyping on one of the patient's peripheral blood mononuclear cells, and compared to control subjects and patients with Crohn's disease.

Results: The patients presented with severe colitis and perianal fistulas in the first months of life, without severe/atypical infections. Genetic studies identified novel and pathogenic genetic variants in *RIPK1*. Using CyTOF unbiased clustering analysis, we identified peripheral immune dysregulation in one of these patients, characterized by an increase in IFN γ CD8⁺ T cells along with a decrease in monocytes, dendritic cells and B cells. Moreover, *RIPK1*-deficient patient's immune cells exhibited decreased IL-6 production in response to LPS across multiple cell types including T cells, B cells and innate immune cells.

Summary: Mutations in *RIPK1* should be considered in very young patients presenting with colitis and perianal fistulas. Given *RIPK1*'s role in inflammasome activation, but also in epithelial cells, it is unclear whether IL1 blockade or allogeneic hematopoietic stem cell transplantation can suppress or cure the hyper-inflammatory response in these patients. Additional studies in humans are required to better define the role of *RIPK1* in regulating intestinal immune responses, and how treatment can be optimized for patients with *RIPK1* deficiency.

Introduction

Significant progress has been made in the last 15 years in understanding the role of genetics in the pathogenesis of inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC)¹. More than 240 single nucleotide polymorphisms, typically with a relatively high mean allelic frequency (MAF) in the general population, have been identified as conferring risk for CD, UC or both². On the other hand, rare deleterious variants directly causing intestinal inflammation were identified using advanced sequencing technologies^{3,4}. To date, nearly a 100 distinct monogenic disorders associated with IBD were characterized, resulting from deleterious and rare mutations in genes important for immune responses, epithelial cell function, or both^{5,6}. Consequently, in many of these diseases intestinal inflammation is accompanied by an immunodeficiency state, manifesting as recurrent or atypical infections. Identification of a monogenic disorder can have a marked impact on care provided for these patients through the possibility of providing targeted therapies, as we and others have shown before with anakinra for patients with IL10 receptor mutations⁷ and abatacept for LPS Responsive Beige-Like Anchor Protein (LRBA) deficiency⁸.

Mutations in Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) associated with monogenic IBD were recently reported^{9,10}. The RIPK1 pathway along with other kinases, including RIPK3 and mixed-lineage kinase domain-like pseudokinase (MLKL)¹¹ is important for necroptosis, a regulated necrotic cell death mechanism. We present the clinical course, genetic analysis and immune work-up of two patients from unrelated families who presented with severe infantile-onset IBD and were found to harbor novel deleterious mutations in *RIPK1*.

Methods

Whole exome sequencing

The study was approved by the IRB committee at Sheba Medical Center and informed consent was obtained from all individual participants included in the study or their parents. Whole exome sequencing (WES) was performed using an Agilent v5 Sureselect capture kit and Illumina 2500 sequencing technology. Paired end reads (2X100 bp) were obtained, processed and mapped to the genome. The average sequencing depth of the target region is 92X with 95.63% of bases reached at least 10X coverage. We used the BWA mem algorithm (version 0.7.12)¹² for alignment of the sequence reads to the human reference genome (hg19). The HaplotypeCaller algorithm of GATK version 3.4 was applied for variant calling, as recommended in the best practice pipeline¹³. KGG-seq v.08 was used for annotation of identified variants¹⁴ and in house scripts were applied for filtering based on family pedigree and local dataset of variants detected in previous sequencing projects. Likely pathogenicity was assessed if the variant was truncating (splicing or non-sense) or missense; in-frame indels were considered if they were predicted to be pathogenic by online prediction tools including PolyPhen-2, SIFT, CADD and MutationAssessor.

Immune work-up

Cell surface markers of peripheral blood mononuclear cells (PBMCs) were determined by immunofluorescent staining using flow cytometry (Navios, Beckman Coulter, Brea, CA, US) with antibodies purchased from Beckman Coulter. T cell receptor excision circles (TREC) analysis was performed using DNA extracted from the patient's PBMCs. The amount of signal joint TREC copies per DNA content was determined by real-time quantitative PCR as previously described¹⁵.

Sanger sequencing

Exons 4 and 11 of *RIPK1* gene were amplified and sequenced by the Sanger method. Briefly, polymerase chain reaction (PCR) amplification was performed using three sets of the following primers:

exon 4-Fw: CAGAATTTTCATGTGAACGTTTCCT

exon 4 -Rw: GGCTAAGTCCTCACAAGCAGAA

exon 11-Fw: CTGCCAGTGCATCAACAGCTA

exon 11 Rw: CCCATTCTCCAGCTATGAAGTACA

The PCR reaction took place in a 25- μ L volume containing 50 ng of DNA, 10 ng of each primer, 1.5 mM dNTPs, in 1.5 mM MgCl₂, PCR buffer, with 1.2 units of Taq polymerase (Bio-Line, London, UK). After an initial denaturation of 5 min at 95°C, 30 cycles were performed (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s), followed by a final extension of 10 min at 72°C. PCR amplicons were sequenced in both directions using a commercial sequencing service [Hy Laboratories, Ltd. (Hylabs) Park Tamar Rehovot, Israel].

Mass cytometry (CyTOF) studies

Peripheral blood mononuclear cells from the a RIPK1-deficient patient, both parents, three healthy controls and three patients with active CD were stained with a panel of metal-chylated surface antibodies targeting markers of major immune cell lineages (**Supplementary Table 1**) per previously published protocol¹⁶. The samples were run on Helios2 mass cytometer (Fluidigm, San Francisco, CA, USA). FCS files obtained were analyzed with premium Cytobank software and pre-gated on CD45⁺/viable/single/DNA⁺ events before initiating the analysis. Normalization beads were used and gated out of the analysis. The data was automatically clustered with Pheograph through cytofkit package in R and visualized with tSNE using all leukocytes (CD45⁺ cells) as input and manually labeled based on markers expressed in the individual clusters. Cluster abundance (% of CD45⁺ viable single events) and fold change over the RIPK1 abundance were computed and plotted for comparison between groups using Prism8 software. Additionally, clusters of similar cellular subtype were combined, and similarly, the ratio of CD4/CD8 cells was calculated.

To identify dysregulation in activation and cytokine production of the immune cells in RIPK1 deficiency, we stimulated PBMCs from Patient 1 and his parents with either lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) and Ionomycin. PBMC's from patient and parents were thawed and washed with T cell media, then centrifuged at 300g. The supernatant was discarded and pelleted PBMC's were resuspended in 1ml of T Cell media. GolgiStop and GolgiPlug were then added to each stimulation tube and each sample was stimulated with either LPS 1mg/ml or PMA 0.5ng/ml and Ionomycin 1mg/ml for 4 hours at 37⁰C. Samples were then centrifuged and washed again in T cell media then prepped for CyTOF where they were stained with heavy metal chelated antibodies (**Supplementary Table 2**) first for surface antigens, then fixed and permeabilized and finally stained with antibodies for intracellular antigens¹⁶. All samples were analyzed as above. In addition, mean expression of various cytokines (mean metal intensity) was calculated in the various populations indicated.

Results

Case description

Patient 1 was referred at the age of 16 months for evaluation of IBD. He was born to a consanguineous Muslim family (parents first degree cousins) and presented at the age of 9 months with fever and diarrhea that were initially attributed to amebiasis. Despite metronidazole treatment his condition deteriorated, and he developed perianal abscesses with multiple fistulas (**Figure 1A**). Blood tests were significant for anemia (hemoglobin 6.5 gram/dL), hypoalbuminemia (albumin 1.5 gr/dL) and elevated inflammatory markers (CRP 94 mg/L, normal <5 mg/L). Due to the severity of the perianal disease, the patient underwent double barrel protective ileostomy. Despite this intervention, his condition did not improve. At the age of 16 months the patient appeared cachectic. Weight was 7.4 kg (Z score -4), length 73 cm (Z score -2) and head circumference 46 cm (Z score -2). Abdominal exam showed double barrel ileostomy, with severe inflammation in the surrounding skin, and multiple perianal fistulas with deep ulcerations and perianal fissures. Colonoscopy demonstrated patchy areas of severe colonic ulcerations with pseudopolyps, with normal mucosa in between, while an upper endoscopy was unremarkable. Colonic biopsies revealed chronic active inflammation with focal cryptitis (**Figure 1B**).

Following a course of broad-spectrum antibiotics and nutritional supplementation, the patient was started on metronidazole, mesalamine and azathioprine, and later began adalimumab. Nevertheless, the patient failed to respond to this TNF α antagonist despite adequate drug levels and absence of anti-drug antibodies, thus experiencing primary pharmacodynamic failure, and at the age of 2.5 years developed colo-vesical fistula that required fistulectomy. Follow up colonoscopy showed severe ulcerations at the sigmoid and ascending colon. Anakinra, an IL1 receptor antagonist, was commenced with escalation of the dose up to 3mg/kg daily, though clinical or laboratory responses were not documented. Importantly, the patient did not develop severe or atypical inflammation until the age of 3 years.

Patient 2 was also born to a consanguineous Muslim family (parents first degree cousins) and presented at the age of 1 month with recurrent fevers, non-bloody diarrhea, oral ulcers, poor weight gain, abdominal distention and arthritis. During infancy he developed recurrent perianal abscesses. At the age of 20 months his weight was 9.7 kg (-2 SD), length 81 cm (-1 SD), head circumference 46 cm (-1 SD). Physical exam was noted for abdominal distention and hepatosplenomegaly, along with a draining perianal abscess. Blood tests demonstrated anemia (hemoglobin 8.8 g/dL) and mildly elevated inflammatory markers (ESR 29 mm/hour). Blood cultures were positive for *Providencia stuartii* and *Pseudomonas aeruginosa*, which were treated with Piperacillin-Tazobactam for two weeks. Colonoscopy showed patchy colitis, predominantly at the right colon, while an upper endoscopy was unremarkable. Pathologic assessment showed patchy chronic active colitis. The patient temporarily responded to mesalamine, metronidazole and azathioprine, with a 3 kg weight gain over 10 months and normalization of inflammatory markers. However, he continued to suffer from recurrent febrile episodes, oral ulcers, diarrhea, arthritis and perianal abscesses.

Basic immune studies

Immunoglobulin levels, including IgM, IgA and IgG were within normal limits for both patients (**Supplemental Table 3**). Moreover, immunoglobulin E was also normal for Patient 1. In addition,

lymphocyte subset analysis was within normal limits for both patients, beside slightly elevated CD8⁺ T cells (**Supplemental Table 3**). Finally, TREC levels for both patients were within normal limits, reflecting intact thymic function.

Identification of RIPK1 mutation

Following WES analysis, we identified 7,928 and 6,887 homozygous variants for Patients 1 and 2, respectively, that affect protein sequences. These numbers were reduced to 49 and 77 variants, respectively, after filtration for common variants (MAF \geq 0.01) in either our local in-house exomes database (n~3500) or external databases such as 1000 Genomes Project (1 KG; <https://www.internationalgenome.org/1000-genomes-browsers>) or dbSNP 135 database, the NHLBI Exome Sequencing Project (ESP) (<http://evs.gs.washington.edu/EVS/>) or gnomAD database (<https://gnomad.broadinstitute.org/>). A c.1934C>T missense mutation residing in Exon 11 was identified in Patient 1, and a c.580G>A *RIPK1* missense mutation residing in Exon 4 was identified in Patient 2 (**Figure 2**). Both mutations were verified using Sanger sequencing, were designated as deleterious to protein function and were not found in gnomAD Database, dbSNP, 1000G and ESP6500, nor in our in-house database.

Abnormal Cytokine Production in RIPK1-deficient Patient

To evaluate changes in the architecture and function of peripheral immune system in one of the RIPK1-deficient patients, we performed mass cytometry (CyTOF) using a panel of 36 antibody markers, and compared the results to in comparison to both maternal and paternal blood samples, non-IBD subjects (controls) and CD patients, An unbiased clustering algorithm (Phenograph) was performed on multiple immune populations. To begin, we clustered on all immune cells (CD45⁺) and were able to identify 28 unique populations (**Figure 3A-C**). When comparing abundances of major immune subsets, we identified an increased abundance of T cells, particularly CD8 effector population, in the RIPK1-deficient patient, compared to controls, along with a decrease in all other cell types including monocytes, dendritic cells and B cells (**Figure 3 C-E**). Interestingly, we also observed a reversal of the CD4 to CD8 ratio compared to all other samples (**Figure 3F**).

To gain further insight into the functional effects of RIPK1 deficiency, we examined cytokine production by PBMCs following stimulation with either LPS or PMA/Ionomycin, using CyTOF, and clustering on all leukocytes (CD45⁺ cells, **Supplemental Figure 1A-C**). We were able to identify 17 unique populations, again demonstrating a CD8 predominance in the patient (**Supplemental Figure 1A-B**). RIPK1-deficient patient's immune cells exhibited decreased IL-6 production in response to LPS, but not to PMA-I stimulation, across multiple cell types including T cells, B cells and innate immune cells (**Figure 4A**). In addition, we observed an increase in IL-22 production across the majority of immune cells, particularly under the LPS stimulation condition (**Figure 4B**).

Focusing on the individual cell populations, monocytes and dendritic cell's ability to produce inflammatory cytokines was drastically reduced in the patient, consistent with documented reduced NFkB

activity¹⁷ (**Figure 4C**). On the other hand, patient's T cells produced higher amounts of IFN γ than healthy controls upon PMA/I stimulation (**Figure 4E**). Interestingly, when analyzing immune populations with inhibitory potential, including myeloid derived suppressor cells (MDSC) and CD24⁺ B cells (containing the putative regulatory B cell populations), we demonstrate higher production of inflammatory cytokines (IL-23, IL1b, and IL-8), but also IL-10, compared with cells obtained from the parents (**Figure 4C**), suggesting a possible dysfunction of these cells.

Discussion

With the expanding use of next generation sequencing platforms in patients with unique IBD phenotypes, the list of monogenic disorders associated with intestinal inflammation is steadily growing, and many of them present with an increased susceptibility to severe and/or atypical infections. RIPK1 is a central regulator of apoptosis, inflammation, and necroptosis, and therefore, it is not surprising that patients that lack RIPK1-dependent signaling develop a severe multi-systemic disease with inflammatory phenotypes and infectious complications in some of them. To date, 14 patients with deleterious *RIPK1* mutations have been reported in the literature^{9,10,18,19}. All patients developed colitis, mostly in the first months of life, and in all but two patients perianal inflammation (abscesses or fistulas) was evident^{9,10}. Chronic upper gastrointestinal tract inflammation was also reported¹⁸. Additional clinical features included susceptibility to severe viral and bacterial infections, arthritis, skin lesions, recurrent fever and oral ulcers. Differences in clinical phenotype may be attributed to the functional consequences of the *RIPK1* mutation and whether it abrogates protein expression. This disorder should not be confused with a different autosomal dominant autoinflammatory disease caused by heterozygote mutations in *RIPK1*, leading to its abnormal cleavage by caspase-8, and manifesting as recurrent fever, lymphadenopathy and hepatosplenomegaly²⁰.

One of the pathways that has been shown to be upregulated in RIPK1-deficient macrophages is activation of the inflammasome, an intracellular complex that secretes the pro-inflammatory cytokines IL-1b and IL-18 in response to different stimuli, including lipopolysaccharide²¹. Impaired inflammasome activation has been reported in other monogenic disorders associated with intestinal inflammation, including Caspase-8 deficiency²¹, mutations in IL-10 receptor (IL10R)⁷, mevalonate kinase (MVK) deficiency²² and in gain-of-function mutations of *NLRP4*²³. We have previously shown that anakinra, an IL1 receptor antagonist, was effective in suppressing intestinal inflammation in two patients with deleterious *IL10RA* mutations and history of severe infantile-onset IBD⁷, and, similarly, blocking IL-1 is effective in patients with MVK deficiency²⁴. Over-activation of the inflammasome in patients with *RIPK1* mutations may suggest that IL-1 blocking agents can be effective in suppressing the IBD phenotype, though additional pro-inflammatory cytokines (e.g. IL6) and pathways may also be dysregulated and contribute to the hyper-inflammatory immune response observed in this disorder. A trial of anakinra (IL-1 receptor antagonist) was performed in Patient 1, but the dose provided was only 3mg/kg, much lower than the dose we successfully used for the IL10R-deficient patients (10mg/kg)⁷.

Our CyTOF data of PBMCs from a single RIPK1-deficient patient highlighted alterations in both innate and adaptive immune cells. The patient displayed increased frequency of naïve CD4⁺ and CD8⁺ T cells, compared to controls and also to patients with active CD, similar to observations by Li and colleagues⁹. In addition, we demonstrated decreased IL-6 production by various innate and adaptive immune subsets. This finding is in-line with reports from Cuchet-Lourenco and colleagues who found that RIPK-deficient fibroblasts have reduced ERK and NfκB phosphorylation and a reduction in pro-inflammatory cytokines such as IL-6, and that the TLR4 (LPS) signaling is affected more than TLR independent pathways¹⁰. The dysfunction of B cells and MDSC has been reported in IBD patients^{25,26}; however, this is the first reporting of our knowledge in RIPK1 deficient patients.

The question whether hematopoietic stem cell transplantation (HSCT) can cure RIPK1 deficiency is unclear at this point. Cuchet-Lourenço and colleagues reported 3 siblings (One was sequenced and found to harbor a *RIPK1* mutation and the others with the presumable same mutation) that underwent HSCT; two of them died within weeks of the procedure due to multi-organ failure or disseminated viral infections. The third patient underwent HSCT at the age of 30 months with resolution of IBD and arthritis (up to 5 years of follow-up), though is still on antibiotics due to chronic and probably irreversible lung disease¹⁰. Mice with a specific deletion of RIPK1 in epithelial cells develop spontaneous lethal intestinal inflammation due to enhanced apoptosis and necroptosis in epithelial cells^{27,28}. Moreover, RIPK1-deficient intestinal epithelial cells show altered cell death responses in response to TNFα stimulation⁹. Given RIPK1's roles in governing both immune and epithelial responses, clinicians should be cautious about the ability of HSCT to cure these patients, as it might ameliorate the immunodeficiency phenotype but not intestinal inflammation. This might be similar to Nuclear factor-kappa B essential modulator (NEMO) deficiency due to *IKBKG* mutations, in which HSCT eliminates the increased susceptibility to recurrent/atypical infections, but does not cure the IBD phenotype²⁹.

In conclusion, mutations in RIPK1 are a new monogenic form of IBD that should be suspected in patients with very early-onset IBD with inflammatory and fistulizing features, along with immunodeficiency. Given RIPK1's involvement in regulating inflammasome function it is plausible that targeting IL-1 and/or IL-18 may be effective, to some extent, in suppressing intestinal inflammation. Nevertheless, murine and human data, as well as in vitro studies, suggest that RIPK1 is important for epithelial cell function, and therefore, therapies targeting immune cells may be only partially effective in these patients. Moreover, the ability of HSCT to cure this disorder is still unclear. Additional human studies and observations are required to determine the role of RIPK1 in epithelial cells in sustaining mucosal homeostasis.

Declarations

Funding

This study was funded by the Leona M. and Harry B. Helmsley Charitable Trust.

Conflict of interest

DSS declares speaker's fees from Abbvie and research grant from Takeda. SBS declares the following interests: Scientific advisory board participation for Pfizer, Pandion, Celgene, Lilly, Takeda, Cosmo Pharmaceuticals, Merck, Sonoma Biotherapeutics, and EcoR1. Grant support Pfizer, Amgen Takeda, and Novartis. Consulting for Amgen, Kyverna, Bristol Myers Squibb, Third Rock, 89bio, GentiBio and Apple Tree Life Sciences. All other authors do not have any other conflicts to disclose.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

MZ, MA, IA – data collection, manuscript editing

NK, BM, LW, AL, OB, LK, RZ – data analysis, manuscript editing

SBS – conceptualizing of study, data analysis, manuscript editing

DSS - conceptualizing of study, coordination of study, data analysis, manuscript writing

The study was approved by the IRB committee at Sheba Medical Center and informed consent was obtained from all individual participants included in the study or their parents.

Ethical approval

The study was approved by the IRB committee at Sheba Medical Center.

Consent to participate

Informed consent was obtained from all individual participants included in the study or their parents.

Consent to publish

The authors affirm that human research participants provided informed consent for publication of the images in Figure 1.

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Figures

Figure 1

Presenting features of the patients. (A) Perianal fistula extending to the base of the scrotum in Patient 1. (B) Hematoxylin and eosin stain of rectal biopsy demonstrating chronic active colitis.

Figure 2

Identification of *RIPK1* mutations in index patients. Figure depicts chromatogram of *RIPK1* gene sequencing at the site of designated mutations for (A) Patient 1 and (B) Patient 2.

Figure 3

Alterations in circulating leukocytes in a patient with *RIPK1* deficiency. tSNE of Phenograph analysis of PBMCs as a (A) conglomerate of all samples with individual cellular population shown or as (B) density plots of the individual groups. (C) Heatmap of the markers expressed in each cluster on the left-hand-side, cluster abundance as a percent of all leukocytes in the middle and fold change of each cluster for each group shown as compared to the *RIPK1* patient. (D-E) Clusters combined by the immune populations indicated. (F) CD4 to Cd8 ratio. Blue arrow-relative reduction and red arrow-relative increase in the *RIPK1* patient compared to all other groups.

Figure 4

Changes in the cytokine profiles of PBMCs in a patient with *RIPK1* deficiency. (A) IL-6 and (B) IL-22 expression in the various populations indicated obtained from phenograph analysis of stimulated PBMCs with either LPS or PMA+. (C) Cytokine production by the immune populations indicated. (D) IFN γ production with stimulation by LPS or PMA/I in T cells.

Supplementary Files

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