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MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

Identification and Characterization of Recombination-Activating Gene 1/2 Deficiency as Cause of Primary Antibody Deficiency with Progressive Lung Disease

verfasst von / submitted by

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Declaration

This master thesis is written in a cumulative form and includes two manuscripts in which the author of this thesis is first-author. Part of the research presented in this thesis was an international collaborative effort accomplished by several academic institutions.

Manuscript #1 (chapter 3.1) entitled "Leaky RAG Deficiency in Adult Patients with Impaired Antibody Production against Bacterial Polysaccharide Antigens" was published in PLOSOne by Geier, Christoph B. et al, 2015. The author of this thesis conceived, designed and performed the majority of experiments, analyzed clinical and experimental data and wrote the manuscript. Hermann M. Wolf and Martha M. Eibl co-designed and supervised experiments, contributed reagents, materials and analysis tools, cared for patients and co-wrote the manuscript. Alexander Piller assisted in next-generation sequencing analysis and performed site-specific PCR. Angela Linder contributed knowledge to optimize transfection experiments and westernblot analysis. Kai M.T. Sauerwein performed immunoglobulin ELISA.

Manuscript #2 (chapter 3.2) entitled "Prevalence and clinical challenges among adult primary immunodeficiency (PID) patients with RAG deficiency" was published in the Journal of Allergy and Clinical Immunology by Lawless, Dylan and Geier Christoph B. et al., 2018. Dylan Lawless and the author of this thesis contributed equally to this paper and share the first-authorship. The author of this master thesis contributed, collected and analyzed clinical and immunological data of patients with RAG deficiency, furthermore he conducted next generation sequencing of the Austrian PID cohort and analyzed bioinformatics data, and he co-wrote the manuscript. Dylan Lawless conducted next generation sequencing of the UK PID cohort and analyzed bioinformatics data, he characterized novel RAG variants and co-wrote the manuscript. Hana Lango Allen, Daniel Thwaites, Matthew Brown, Siobhan Burns O., Sri Deevi V.V., William Egner, Fernando Moreira, Alexander Piller, Paula Rayner-Matthews, Ravishankar Sargur, Ken G.C. Smith, Georgios Sogkas, Kathleen Stirrups, Adrian J. Thrasher, Rashida Anwar, and Joan Boyes performed functional, and immunogenetic analyses of patients. David Buchbinder, Jocelyn R. Farmer, Faranaz Atschekzei, Manish J. Butte, Krisztian Csomos, Stephan Ehl, Martha M. Eibl, Olajumoke Fadugba, Zsofia Foldvari, Deanna M. Green, Neil D. Romberg, Sarah E. Henrickson, Steven M. Holland, Tami John, Christian Klemann, Taco W. Kuijpers, Reinhold E. Schmidt, Claudia Schröder, Catharina Schuetz, Svetlana O. Sharapova, Carsten Speckmann, Hermann M. Wolf, Luigi D. Notarangelo, and James Thaventhiran cared for the patients and provided clinical and

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Abstract

Recombination-activating gene 1 (RAG1) and RAG2 encode lymphoid-specific proteins that are essential for V(D)J recombination, promoting receptor editing and diversification of the T and B cell repertoire (TCR and BCR). Complete mutations in RAG1 and RAG2 with low recombinase activity (<5%) present early in life with severe infections and/or clinical signs of systemic inflammation, such as severe dermatitis and/or colitis. Hypomorphic RAG1/2 mutations with more preserved residual V(D)J recombination activity (in average 5-30%) result in a distinct phenotype of combined immunodeficiency with granuloma and/or autoimmunity (CID-G/A).

Herein, we characterize RAG deficiency beyond the spectrum of combined immunodeficiency as a novel cause of selective polysaccharide antibody deficiency. Clinical manifestations included upper respiratory tract infections, cutaneous vasculitis and progressive lung disease. Patients harbored a combination of a null allele and a novel hypomorphic allele. One of these novel mutations affected the start codon of RAG1 and resulted in an aberrant gene and protein expression. The second novel RAG2 mutation leads to a truncated RAG2 protein, lacking the C-terminus with intact core RAG2 and reduced VDJ recombination capacity as previously described in a mouse model. Both patients presented with severely decreased numbers of naïve CD4+ T cells and defective T-independent IgG responses to bacterial polysaccharide antigens, while T cell-dependent IgG antibody formation against protein antigen was intact. Further, we sought to identify the prevalence of RAG deficiency amongst adult PID patients by analyzing the canonical regions of RAG1 and RAG2 in two separate PID cohorts. We estimate that the prevalence of RAG deficiency in adults with PID ranges from 1% to 1.9%. Finally, we recruited an international cohort of adult RAG deficient patients to provide detailed clinical features, complications and treatment outcomes. We identified progressive restrictive lung disease as the leading factor for mortality and morbidity among these patients.

Zusammenfassung

Recombination-activating gene 1 (RAG1) und RAG2 kodieren Lymphozyten-spezifische Proteine, welche essential für die V(D)J Rekombination, Rezeptoren Editierung und Diversifikation des T-Zell bzw. B-Zell Repertoire sind. Vollständige Mutationen in RAG1 und RAG2, mit niedriger Rekombination-Aktivität präsentierten sich früh im Leben mit schwerer Infektionsanfälligkeit die zu lebensbedrohlichen Infektionen und systemischer Entzündung führt. Unvollständige RAG1/2 Mutationen, mit Restaktivität zwischen 5-30% führen zu einem abgegrenzten Phänotyp mit kombinierten Immundefekt und dem Auftreten von Granulomen und/oder Autoimmunität.

In dieser Arbeit beschreiben wir RAG Defizienz als neue Ursache für Polysaccharid Antikörperbildungsstörung. Wir konnten in zwei nicht verwandten Patienten, mit wiederkehrenden Infektionen des oberen Respirationstrakts, kutaner Vasculitis und progressive restriktive Lungenerkrankung neue RAG1/2 Mutationen identifizieren. In diesen Patienten wurde eine Kombination aus einer bereits beschrieben kompletten Mutation und eine neuartige hypomorphe Mutation mit Restaktivität identifiziert. Ein dieser neuen Mutationen war eine RAG1 Mutation, welche das Startcodon alteriert und zu reduzierten Geneexpression bzw. Proteinexpression führt. Die zweite neuartige Mutation führt zu einem verkürzten RAG2 Protein, mit fehlenden C-Terminus aber intakter Kern-RAG2 Region und reduzierter VDJ Rekombination Aktivität. Beide Patienten präsentieren sich mit deutlich reduzierten naiven $CD4^+$ T-Zellen und einer defekten Antikörperbildung gegen bakterielle Polysaccharide. In beiden Patienten war jedoch die Antikörperbildung gegen Proteinantigen intakt. Weiter konnten wir zeigen, dass die Prävalenz von RAG Defizienz innerhalb von Patienten mit Antikörperbildungsstörung 1% bis 1,9% beträgt. Dafür sequenzierten wir die RAG1/2 Gene in zwei separaten Kohorten von Patienten mit Antikörperbildungsstörung. Zusätzlich haben die größte bisher beschriebene internationale Kohorte von Erwachsenen mit RAG Defizienz rekrutiert um die klinischen Manifestationen, Komplikationen und Therapiestrategien zu beschrieben. Wir konnten feststellen, dass die progressive restriktive Lungenerkrankung der Hauptfaktor der Mortalität und Morbidität dieser Patienten ist.

Table of Content

1 Introduction

1.1 Development of Adaptive Immunity

In 1909 it was first postulated that hematopoiesis is organized as a cellular hierarchy derived from self-renewing hematopoietic stem cell (HSC). 1 However, it took almost 50 years before experimental evidence could support this idea. Lethal irritated mice could be rescued from bone marrow failure after transplanting healthy donor bone marrow cells.2

All blood cells originate from HSC located in the bone marrow, that do have the capacity to self- renew, while contributing to the pool of differentiating cells. These stem cells differentiate into progenitor cell intermediates that undergo a gradual fate restriction to develop into mature blood cell. HSC are derived from mesoderm in the yolk-sac blood island and the aorta. During embryogenesis they populate different blood forming sites including fetal liver, spleen, thymus and bone marrow. 3

HSC are phenotypically distinct from other cell types located within the bone marrow, CD34 in combination with CD90 (Thy1) demarcates HSC from progenitor cells intermediates. 4 Several transcription factors have been found to play critical roles in HSC physiology, including SCL (stem cell leukemia hematopoietic transcription factor), GATA-2, cMyb, Lmo-2 and AML-1.5 6

Multipotent stem cells further differentiate into two fundamental branches the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). CMP yields a number of distinct, fully differentiated, short-lived cell types including erythrocytes, the megakaryocytes, which give rise to thrombocytes and the myeloid lineage, consisting of eosinophils, neutrophils, basophils, mast cells and monocytes. CLP produces lymphocytes that can be subdivided in adaptive immune system T- and B cells and innate lymphocytes like NK cells or innate lymphoid cells. 7

The bone marrow provides the appropriate support for the development of B, NK and dendritic cells, while T cells migrate as progenitor cell to the thymus to fully differentiate.8

1.1.1 Development of T lymphocytes

In contrast to other hematologic lineages, T cells do not fully mature in the bone marrow. They rather migrate as bone marrow-derived progenitor cells to the thymus.9 The thymus develops early in embryo genesis and is located in the anterior superior mediastinum, in front of the heart and behind the sternum. Each lobe of the thymus can be divided into a central medulla and a peripheral cortex, which is populated with hematopoietic cells, thymocytes, intrathymic dendritic cells and macrophages. 10 In the absence of a thymus T cells are reduction in numbers and impaired in function, leading to immunodeficiency. (e.g. DiGeorge Syndrome) 11

Within the thymus thymocytes undergo several differentiation steps which are defined based on their expression of CD4 and CD8. In the first differentiation stage thymocytes are CD4– CD8– double negative (DN) with non- rearranged T cell receptor genes, but express CD3. During the DN stage thymocyte differentiation can be further subdivided on the basis of CD25 and CD44 expression. 12 At stages DN1 and DN2 double-negative thymocytes express high levels of CD44. 13 Subsequently at DN3 stage they downregulate CD44 and its expression stays low throughout T cell development. 14 CD25 on the other hand is upregulated at the DN2 and DN3 stage and subsequently lost at DN4 stage. In contrast to CD44, CD25 is upregulated upon T cell activation and during development of regulatory T-cells. The full commitment to the T-cell lineage occurs during the DN2a-to-DN2b transition upon induction of several transcription factors including e.g. TCF1, Bcl11b, GATA3.15–17 Thymocytes lose the capacity to become non-T cells and undergo irreversible genome recombination of the T-cell receptor beta/gamma locus forming the pre-TCR complex.

During CD4+CD8+ double positive (DP) state thymocytes coexpress the complete antigen receptor, αβTCR following recombination of the TCR alpha gene locus. 18 Double positive thymocytes undergo two major selection steps, based on their TCR reactivity to fully mature into naïve T-cells. First they undergo positve selection. T cells that express TCRs with intermediate affinity /avidity for self-peptide–MHC complexes are induced to differentiate into mature single-positive thymocytes. 19 Depending on the initial positive-selection signals induced by MHC-I and MHC-II, thymocytes differnetiate in either CD8 or CD4-single positive thymocytes. CD4 expression is maintained in MHC-II-selected thymocytes that downregulate and shut off CD8 expression, whereas MHC-I-selected thymocytes upregulate CD8 expression and shute off CD4 expression. 20 After positiv selection double-positive or

single-positive thymocytes undergo negative selection. T cells that express TCR with high affinity for self antigens undergo apoptosis. 21

1.1.2 Development Lymphocyte Receptor Repertoires

The hallmark of the adaptive immune system is that cells can express a virtually unlimited variety of cell surface receptors that enable them to recognize millions of possible antigens. This plasticity of T and B lymphocytes is due to the combinatorial joining of variable (V), diversity (D) and joining (J) gene segments that encode the antigen binding regions of TCRs and BCRs. 22 V, D and J gene segments are flanked by recombination signal sequences (RSSs). 23 These RSSs contain specific consensus heptamer (CACAGTG) and nonamer (ACAAAAACC) elements that are separated by nonconsensus DNA elements called spacer with a specific length of 12 or 23 nucleotides. 24,25

The proteins that initiate the process of V(D)J recombination are RAG1 and RAG2 that are expressed during the early stages of T cell and B cell development. 26 RAG1 and RAG2 form a heterotetramer, that sequentially forms synapsis of one 12–RSS and one 23–RSS, that is regulated by DNA accessibility due to epigenetic modification of the VDJ loci. 27

Upon binding of the flanking RSSs RAG1 introduces a DNA double-strand break at the junction and forms a sealed hairpin with blunted ends. 28,29 The blunt DNA ends trigger the activation of DNA-PKCs and Artemis which open the hairpin. 30,31These signals subsequently trigger the activation of the non-homologous end joining (NHEJ) pathway that imprecisely repairs broken ends. P-nucleotide addition, N-nucleotide addition by TdT and exonucleolytic cleavage diversifies TCR and immunoglobulin repertoires. 32

1.2 Inherited Disorders of the Immunesystem

1.2.1 RAG 1/2 Deficiency as a Cause of Severe Combiend Immunodeficncies

Recombination-activating gene 1 (RAG1) and RAG2 encode lymphoid-specific proteins that play a fundamental role in the process of variable (V), diversity (D) and joining (J) recombination and diversify the T and B cell repertoire (TCR and BCR) to recognize millions of possible antigens.22,33 RAG 1 and RAG2 was first described in 1996 by Schwarz and colleagues as a cause of T-B-NK+ severe combined immunodeficiency. 34 RAG deficiency has an estimated disease incidence of 1:181,000 including severe combined immunodeficiency (SCID) at a rate of 1:330,000. 35,36

Complete or atypical variants of SCID secondary to low recombinase activity (<5%) present early with severe infections and/or clinical signs of deregulated inflammation, such as severe dermatitis and/or colitis.34,37,38 In addition to complete SCID, in recent years partial RAG mutations with higher residual V(D)J recombination activity have been described and these hypomorphic mutations result in a distinct phenotype of combined immunodeficiency with granuloma and/or autoimmunity (CID-G/A).39–41 Beyond the spectrum of combined immunodeficiency, RAG deficiency has been implicated among patients with the diagnosis of antibody deficiencies such as common variable immunodeficiency (CVID)42, agammaglobulinemia 43 and selective IgA deficiency. 44 However, in the majority of cases T cell studies eventually confirmed naïve CD4+ T cell lymphopenia. There are also individual case reports of idiopathic CD4+ T cell lymphopenia 45 and hyper-IgM syndrome. 46

Currently over 150 mutations in RAG1 and 60 mutations in RAG2 have been described. The majority of mutations are missense, whereas non-sense and frameshift mutations are less common. Disease-associated missense mutations in RAG1 have been predominantly detected in the zinc-binding region and core domain. In contrast, 38 mutations in RAG2 occur in the core domain.26

The standard treatment for patients with SCID or Omenn syndrome is hematopoietic stem cell transplant (HSTC). Overall survival is approximately 80% after HSTC from matched related donors. However, survival is poorer (60-70%) for patients after HSTC from unrelated haploidentical donors with or without myeloablative conditioning.47,48

1.2.2 Primary Antibody Defiencies

Primary antibody deficiencies (PADs) are the most abundant primary immunodeficiencies (PIDs) in humans. Since the first description of BTK as a cause of X-linked agammaglobulinaemia (XLA) in 1993 , a pleiotropic spectrum of B-cell intrinsic as well as extrinsic defects leading to agammaglobulinemia have been described. 49,50 The prototypical B-cell intrinsic agammaglobulinemia is X-linked agammaglobulinemia (XLA), caused by mutations in the Bruton's tyrosine kinase (BTK), which accounts for approximately 85% of all pediatric patients with agammaglobulinemia and loss of B lymphocytes. BTK encodes a tyrosine protein kinase of the Tec family, which is essential in Pre-B-cell activation and differentiation. Thus, mutations in BTK lead to a severe block in early B-cell differentiation and agammaglobulinemia due to a stop in B-cell differentiation in the bone marrow.51 Furthermore, several autosomal recessive genetic defects affecting either the expression of the B-cell receptor (λ5 chain, μ-chain, Igα and Igβ) or BCR signaling (PI3K and BLNK), result in the selective absence of circulating mature B-cells and all immunoglobulin isotyps. 52–57 In contrast, hypomorphic mutations usually result in disturbed B-cell homeostasis and impaired antibody response.58,59

In contrast to the absence of all immunoglobulins which results in severe susceptibility to recurrent infections partial absence of one or more immunoglobulin results in a diverse clinical spectrum.

For example, CVID is the most common clinically severe primary immunodeficiency in humans as 100% of patients require treatment. It comprises of multiple immunodeficiency phenotypes and affects various immune cell lineages. The main immunological phenotype observed in CVID in B cell abnormalities, resulting in impairment in humoral immunity with IgG, IgM and/or IgA reduction and a failure to produce pathogen-specific IgG antibodies. CVID B cells do not become fully activated or proliferate normally, nor do they terminally differentiate into plasma cells.60,61

Selective deficiency in antibody production against bacterial polysaccharide antigens (SPAD) leads to significant susceptibility to bacterial infections even in patients with normal levels of serum immunoglobulins. 62 First described in 1987 by Umetsu and colleagues in patients with IgG2-IgG4 subclass deficiency, impaired antibody production against bacterial polysaccharide antigens was later confirmed to occur in patients with normal IgG subclass levels, as well. 63

Genetic defects have only been characterized in a minority of patients. For example, hypomorphic mutations in BTK have been described as a cause of selective anti-polysaccharide antibody deficiency. 59 Furthermore impaired antibody production against bacterial polysaccharide antigens has been described in CD21 deficiency, CD20 deficiency, JAK3 deficiency, partial trisomy 19q13, nuclear factor-κB (NF-κB) essential modulator (NEMO) deficiency, and chromosome 18p deletion syndrome with IgA deficiency.64–68

2 Working Hypothesis & Aim of the thesis

Recent advances identified primary antibody deficiencies, such as CVID or selective IgA deficiency caused by leaky defects in RAG1/2. 44,69

Given the broad clinical spectrum among patients with RAG deficiency, we hypothesized that selective polysaccharide antibody deficiency is due to hypomorphic RAG mutations.

Furthermore, we were interested in the clinical features and complications of patients with late onset presentation of RAG deficiency. This highly vulnerable patient population with treatment-refractory lung disease and recurrent autoimmune manifestations has not been studied extensively. In addition, no studies have examined the prevalence of RAG deficiency in cohorts of adult primary immunodeficiency patients.

There is a great variability of diagnostic modalities for evaluation and treatment to control progression in cases reports of adult RAG deficient patients with no standardized guidelines in place. Overall, early recognition of adult patients with RAG deficiency and proper treatment of clinical complications may expedite timely curative treatment and improve outcome.

Here we aim to describe a cohort of 15 patients with the late presentation of RAG deficiency. In addition, we try to estimate the prevalence of RAG deficiency in adult PID patents following genetic analysis of RAG1 and RAG2 variants in two separate large cohorts of PID patients.

3 Results

3.1 Leaky RAG Deficiency in Adult Patients with Impaired Antibody Production against Bacterial Polysaccharide Antigens

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RESEARCH ARTICLE

Leaky RAG Deficiency in Adult Patients with Impaired Antibody Production against Bacterial Polysaccharide Antigens

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Abstract

Loss of function mutations in the recombination activating genes RAG1 and RAG2 have been reported to cause a $T^-B^-NK^+$ type of severe combined immunodeficiency. In addition identification of hypomorphic mutations in RAG1 and RAG2 has led to an expansion of the spectrum of disease to include Omenn syndrome, early onset autoimmunity, granuloma, chronic cytomegalovirus- or EBV-infection with expansion of gamma/delta T-cells, idiophatic CD4 lymphopenia and a phenotype resembling common variable immunodeficiency. Herein we describe a novel presentation of leaky RAG1 and RAG2 deficiency in two unrelated adult patients with impaired antibody production against bacterial polysaccharide antigens. Clinical manifestation included recurrent pneumonia, sinusitis, otitis media and in one patient recurrent cutaneous vasculitis. Both patients harbored a combination of a null mutation on one allele with a novel hypomorphic RAG1/2 mutation on the other allele. One of these novel mutations affected the start codon of RAG1 and resulted in an aberrant gene and protein expression. The second novel RAG2 mutation leads to a truncated RAG2 protein, lacking the C-terminus with intact core RAG2 and reduced VDJ recombination capacity as previously described in a mouse model. Both patients presented with severely decreased numbers of naïve $CD4^+$ T cells and defective T independent IgG responses to bacterial polysaccharide antigens, while T cell-dependent IgG antibody formation e.g. after tetanus or TBEV vaccination was intact. In conclusion, hypomorphic mutations in genes responsible for SCID should be considered in adults with predominantly antibody deficiency.

Introduction

The adaptive immune system is critically dependent on the diverse expression of B cell immunoglobulin receptor (BCR) and T cell receptor (TCR). [1] To obtain the necessary level of diversity, the recombination of the variable (V), diversity (D) and joining (J) segments that form these receptors is directed by recombination activating gene 1 (RAG1) and 2 (RAG2).

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RAG1 and RAG2 form the recombinase complex which binds and cleaves specific recombination signals that flank VDJ regions. [2,3] During lymphopoiesis, the levels of RAG1 and RAG2 are tightly regulated, with two major peaks of expression occurring during T cell development, first in the double negative stage during TCR β gene recombination, and subsequently in the double positive stage when the TCR α chain genes are rearranged. [4] During B-cell development, RAG1 and 2 are first expressed in pro-B cells during heavy chain loci rearrangement and once again in pre-B cells during light chain recombination. [5]

Defects in RAG1 and RAG2 are known to cause a $T^-B^-NK^+$ form of severe combined immunodeficiency. Since the first description of RAG1 and RAG2 deficiency in patients with severe combined immunodeficiency by Schwarz et al. in 1996 [6], a pleiotropic spectrum of phenotypes associated with RAG1/2 deficiency has been described. The spectrum of the disease has expanded to include Omenn syndrome, early onset autoimmunity, granuloma, chronic cytomegalovirus or EBV infection with expansion of gamma/delta T-cells, idiophatic CD4 lymphopenia and a phenotype resembling common variable immunodeficiency. [7–22]

While patients with pronounced hypogammaglobulinemia and agammaglobulinemia are well known to be susceptible to bacterial infections, [23] a clinically relevant selective deficiency in antibody production can also lead to significant susceptibility to bacterial infections even in patients with normal levels of serum immunoglobulins.[24] The most frequent selective antibody deficiency with normal immunoglobulin serum levels is a defect in IgG antibody production against bacterial polysaccharides in the presence of normal IgG antibody production to T-dependent protein antigens. First described by Umetsu et al. in patients with IgG2-IgG4 subclass deficiency, impaired antibody production against bacterial polysaccharide antigens was later confirmed to occur even in patients with normal IgG subclass levels. [25] Although such a specific antibody deficiency is a well-recognized predominantly antibody deficiency [26], the genetic defect has only been characterized in a minor subset of patients. Hypomorphic mutations in BTK have been described as a cause of selective anti-polysaccharide antibody deficiency [27]. Furthermore impaired antibody production against bacterial polysaccharide antigens was found in CD21 deficiency, CD20 deficiency, JAK3 deficiency, partial trisomy 19q13, nuclear factor-κB (NF-κB) essential modulator (NEMO) deficiency, and chromosome 18p deletion syndrome with IgA deficiency. [28–32]

In the present study we describe two unrelated adult patients with impaired antibody production against bacterial polysaccharide antigens and a leaky RAG1/2 deficiency harboring a combination of a null mutation on one allele and a novel hypomorphic mutation on the other allele. One novel mutation affected the RAG1 start codon and resulted in an aberrant gene and protein expression of RAG1. The other novel mutation affected RAG2. This RAG2 mutation had been shown in a mouse model to lead to a truncated protein lacking the C-terminal part while leaving the core region intact.[33] Both patients presented with a severe deficiency in naïve $CD4^+$ T-cells and a defective T-independent IgG response to bacterial polysaccharide antigens while production of T cell dependent IgG antibodies e.g. against tetanus toxoid or TBEV was intact.

Materials and Methods

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki. The patients gave their written informed consent that anonymized data collected as part of the routine medical attendance (immunological analysis, flow cytometry analysis and genetic mutation analysis) could be included in a scientific publication. All results presented in this study were obtained as part of the routine medical attendance the patients received and no extra intervention for

this study. The son of patient B was investigated for possible primary immunodeficiency on request of his mother, and written informed consent was given to present anonymized results of this investigation. All patient information contained in this study was anonymized and de-identified prior to analysis, and only anonymized and de-identified patient information is contained in this study. When we presented this non-interventional study to the appropriate ethics committee, the Ethics Committee of the City of Vienna, evaluation was refused, according to the legal regulations to be applied (§15a Abs. 3a Wiener Krankenanstaltengesetz) no ethics committee evaluation is required for this type of study. (S1 Supporting Information)

Patient and control blood samples

Peripheral venous blood was collected in either untreated (for antibody and immunoglobulin determinations) or lithium-heparin- (for genetic analysis and cell function tests) or EDTA- (for flow cytometry) containing tubes from patients, family members and healthy adult blood donors that served as controls. All patients were treated at the Immunology Outpatient Clinic in Vienna.

Flow cytometry

Peripheral blood was used to analyze lymphocyte subpopulations by multi-color flow cytometry using standard protocols. S1 Table shows the monoclonal fluorophore conjugated antibodies used. Data were analyzed on a FACSCalibur (Becton Dickinson; USA), using standard protocols and evaluated using CELLQuest software (Becton Dickinson; USA).

Determination of serum immunoglobulins and antibodies

Serum concentrations of immunoglobulins and IgG subclasses were determined by standard laser nephelometry on a Siemens nephelometric analyzer (Siemens Healthcare; Germany) using reagents purchased from Siemens-Behring Division. IgG and IgM antibodies against bacterial and viral antigens were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IgG antibodies against tetanus and diphtheria toxoid, tick borne encephalitis (TBE) virus, Haemophilus influenza type b (Hib)) or an in-house produced ELISA (IgG and IgM antibodies against 23-valent pneumococcal capsular polysaccharide) as previously described.[34]

DNA isolation and targeted resequencing

Genomic DNA was prepared from peripheral blood by spin column purification (QIAamp DNA Blood Mini Kit; QIAGEN, Germany). Targeted resequencing of 222 primary immunodeficiency genes listed in the 2011 IUIS expert committee report was performed for the two index patients. (S2 Table) Nextera Custom Enrichment kit was used according to standard protocols (Illumina, USA). Targeted DNA library was quantified and validated using Illumina Eco Real-time (Illumina; USA) and Agilent Bioanalyzer (Agilent Technologies; USA). The library was sequenced in a multiplex pool on a single (151 bp paired-end reads) Miseq flowcell. (Illumina, USA) Data analysis was performed using CLC Genomic Workbench (QIAGEN, Germany)

Allele-specific PCR

The coding sequence of RAG1 and RAG2 was amplified using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific; USA). Allele-specific PCR was used to characterize RAG1 M1V and R737H alleles. Each allele (wild type and mutant form) was amplified separately with an allele-specific primer in combination with a general primer using Maxima Hot Start Taq

DNA Polymerase (Thermo Fisher Scientific, USA). The resulting amplicons were purified and custom Sanger sequenced (Eurofins Genomic; Germany). Sequences were aligned to NM_000448.2 RAG1 and NM_001243785.1 RAG2, using CLC Genomic Workbench (QIAGEN, Germany)

T-cell CDR3 Vβ Spectratyping

Examination of the TCR Vβ repertoire of $CD3^+$ T-cells was performed by spectratyping analysis as described previously. [35] The primer sequences used for amplification are shown in S3 Table. Fragment length analysis sequences were acquired using an ABI 3130 XL Sequencer (ABI Applied Biosystems; USA) and analyzed using Peak Scanner2 software (ABI Applied Biosystems; USA).

Cloning of RAG1

Two constructs were generated: wild type RAG1 (NM_000448.2) and mutant M1V RAG1. The full-length RAG1 ORF was synthesized (Life Technologies; USA) and the constructs were subcloned into the pcDNA3.3 Vector System. (Life Technologies, USA) The plasmids were transfected into Freestyle HEK 293 cells (Life Technologies, USA) to express wild type RAG1 or mutant RAG1. Mock transfected cells were used as a control. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in complete FreeStyle 293 Expression medium (Life Technologies, USA). Samples were harvested at 96 hours post transfection.

RNA isolation, reverse transcription and quantitative Real-Time PCR (qRT-PCR)

RNA was extracted from frozen RAG1 transfected HEK 293 cells using RNeasy Mini Kit (Qiagen; USA). RNA quality was assessed using Agilent RNA 6000 Nano Kit (Agilent Technologies, USA) on a 2100 Bioanalyzer Instrument (Agilent Technologies, USA), and only RNA with an RNA Integrity Number (RIN) higher than 9 was used for subsequent analysis. Equal amounts of RNA were reversely transcribed into cDNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies, USA) following standard protocols. qRT-PCR was performed as previously described.[36] In short, 10ng of cDNA was amplified using an Eco Real-Time PCR System (Illumina, USA) with KAPA SYBR FAST SuperMix (PEQLAB). The ΔΔCT-method was used to calculate relative gene expression. Expression of the housekeeping gene HPRT served as an internal standard. RAG1 and HPRT primer sequences are shown in S3 Table.

In-vitro immunoglobulin secretion and proliferation

Peripheral blood mononuclear cells (PBMCs) of patient A and controls were stimulated with Epstein-Barr virus (EBV) using the supernatant from B 95–8marmoset cell line (ATCC, Rockville, MD) as previously described ([32]). 1^*10^6 cells per milliliter were incubated for 8 days in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 100 IU/ml penicillin, and 100μg/ml streptomycin (Gibco, Paisley, UK) at 37°C in the presence of 5% CO_2. After the incubation period supernatants were harvested and an in-house Enzyme-linked Immunosorbent Assay was performed to determine IgG and IgM level as described previously.[32] Results are expressed as mean ± standard deviation of triplicate measurements. PBMC proliferation was assessed by ^{3}H-Thymidin incorporation using standard protocols as described previously.[37]

Western Blot

Wild type and mutant RAG1-transfected HEK 293 cells were lysed for 30 min in ice-cold RIPA lysis buffer system (Santa Cruz Biotechnology, USA), and insoluble material was removed by centrifugation (16,000 x g, 10 min, 4°C). 20μg of protein were resolved in 8%SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and immunoblotted with anti-RAG1 antibody (H-300) (Santa Cruz Biotechnology Inc; USA). Detection was performed using the SuperSignal West Pico ECL detection system (Thermo Scientific; USA).

Results

Case Report

Patient A is a woman who was first investigated for primary immunodeficiency at the age of 27 years because of a history of undue susceptibility to infections and known hypogammaglobulinemia first diagnosed at the age of 13 years. She was born to unrelated parents of Austrian origin, her brother and mother are healthy, her father died due to coronary heart disease. Recurrent bacterial infections started at the age of seven with recurrent pneumonia and bronchitis, recurrent sinusitis and otitis media. At the age of eight she was hospitalized because of vasculitis of the skin of both legs, with recurrence at 13 and 36 years of age at which time a biopsy was performed and the histology revealed leukocytoclastic vasculitis. At the age of ten chronic interstitial pneumonia was diagnosed, an open biopsy of the lung revealed diffuse panbronchiolitis. Chronic obstructive airway disease with recurrent bacterial infections of the upper and lower airways and hospitalizations due to infection-associated exazerbation of the lung problems constituted the clinical picture since the age of 13 years. Streptococcus pneumoniae was repeatedly identified in sputum cultures and middle ear effusions during episodes of upper and lower respiratory tract infections. IVIG substitution therapy was started at the age of 20 years because of hypogammaglobulinemia with a pretreatment serum IgG level of 393mg/dl and levels of IgA and IgM within the normal range. A treatment-free interval of 6 months at the age of 28 revealed mild hypogammaglobulinemia (serum IgG, mg/dl [normal range]: 697 [790–1700]) IgG1-subclass deficiency (serum IgG1, mg/dl [normal range]: 303 [500–880], normal levels of IgA, IgM, IgG2-4 and low pneumococcal IgG-antibodies despite the fact that recurrent pneumococcal infections had led to very high IgM-antibody titers (Fig 1A). IgG-anti-polysaccharide antibody deficiency was diagnosed and the rapid reappearance of pulmonary infections required resumption of two-weekly IVIG substitution therapy. While regular IVIG substitution therapy led to a long-term improvement of the susceptibility to pulmonary and middle ear infections, the clinical picture was finally dominated by obstructive lung problems with decreasing lung function that made a 24-hour oxygen-therapy necessary and ultimately led to the demise of the 48 year old patient.

Patient B is a 41-year-old woman living in the rural province of upper Austria who was first investigated for primary immunodeficiency at the age of 35 years shortly after her first pneumonia with a history of recurrent bacterial bronchitis during the last four years in the absence of known risk factors such as allergies or smoking. She has two healthy children, a son of nine years and a daughter of six years. IgA-, IgM- and IgG2-IgG3-IgG4 subclass deficiency (serum IgG, mg/dl [normal range]: 940 [790–1700]; serum IgG2, mg/dl [normal range]: 51 [150–600]; serum IgG3, mg/dl [normal range]: 5 [20–100]; serum IgG4, mg/dl [normal range]: <7 [8–120]; serum IgA, mg/dl [normal range]: <6 [76–450]; serum IgM, mg/dl [normal range]:29 [90–350]) with normal levels of IgG1 (data not shown) and antibody deficiency against bacterial polysaccharides was diagnosed. Regular IVIG substitution therapy was initiated at the age

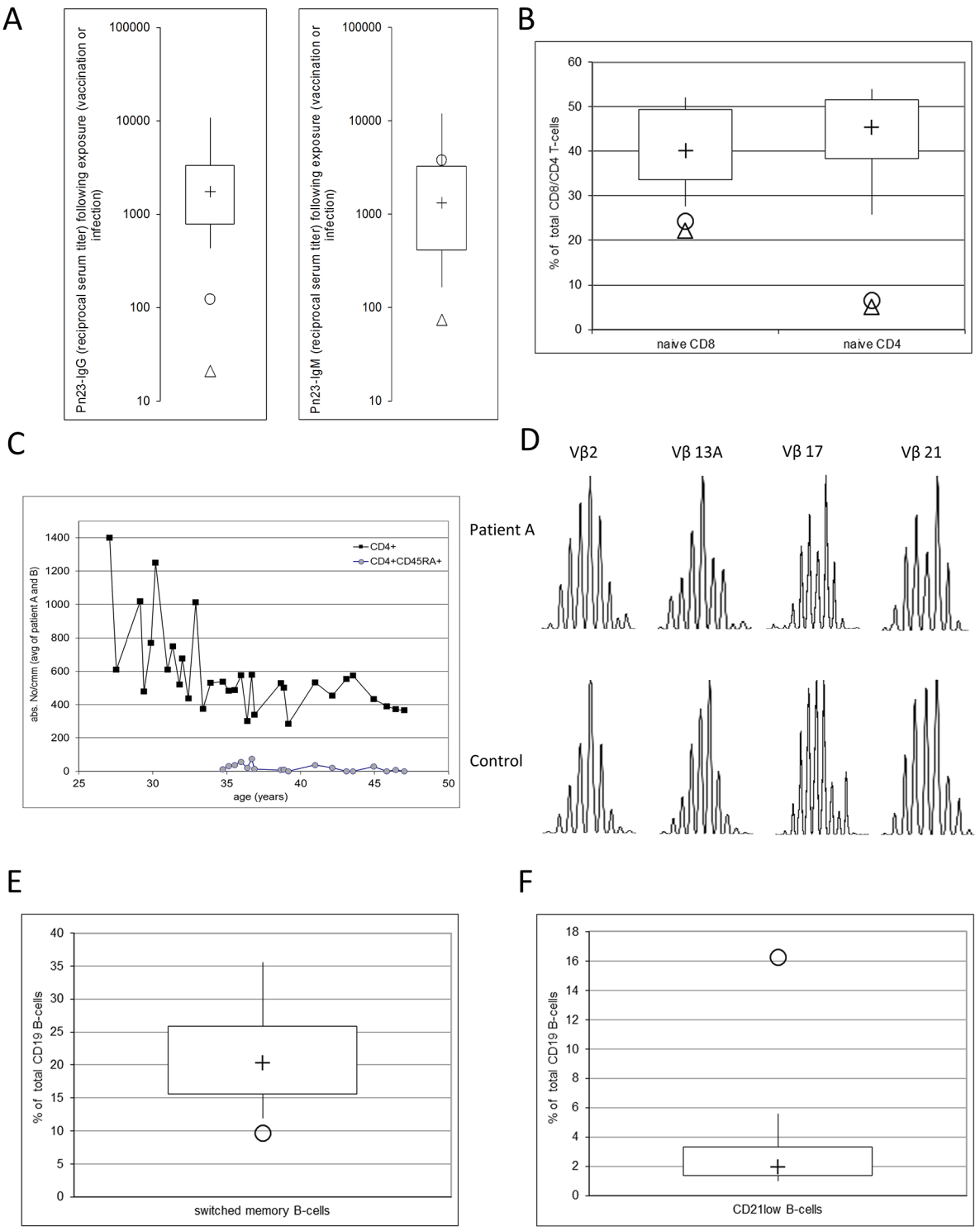

Fig 1. Immunological characteristics of patients with hypomorphic RAG deficiency and impaired antibody production against bacterial polysaccharide antigens. Patient A. . .circles; patient B. . .triangles 22 (A) Impaired antibody production against bacterial polysaccharide antigens in RAG-deficient patients. Box-plots represent pn23-antibodies in 41 healthy individuals (B) Numbers of $CD4^+CD45RA^+$ (naïve CD4) and $CD8^+CD45RA^+CD62L^+$ (naïve CD8) T cells given as percentage of total CD8- or CD4-positive T cells. Box-plots depict 50 healthy individuals (C) Kinetic of the total count of $CD4^+$ T cells and naïve $CD4^+CD45RA^+$ T cell depicted as average of both patients (D) Representative example of the distribution of TCR Vbeta genes 16, 17, 18, 22 (E) Numbers of switched memory B cells given as percentage of total CD19 B cells. Box-plots depict 50 healthy individuals (F) Numbers of $CD21^{low}$ B cells given as percentage of total CD19 B cells. Box-plots depict 50 healthy individuals (median,+; box, IQR; whiskers, q5-q95).

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of 35 years, which led to normalization of the susceptibility to infections. Following the birth of her third child and a substantial increase in the patient´s body weight another pneumonia occurred, followed by recurrent sinusitis and obstructive bronchitis with limited clinical improvement despite an increase in IVIG dosage and sinus surgery.

Hypomorphic mutations in RAG1 and RAG2

To determine the underlying disease-causing genetic defect we screened both patients for mutations in the primary immunodeficiency genes listed in the 2011 IUIS expert committee report. [26] Targeted resequencing of 222 primary immunodeficiency genes and potential candidate genes was performed for the two index patients, genes sequenced are listed in S2 Table. Data analysis and filtering strategy is depicted in S1 Fig. In both patients no disease-causing mutations were identified in genes known to be responsible for predominantly antibody deficiency syndromes. In contrast, genetic testing in patient A revealed a compound heterozygous mutation in RAG1 (c.125 A>G, p.M1V; c.2334 G>A, p.R737H) (Fig 2A and 2C). The c2334 G>A transition in exon 2 resulted in a missense mutation which led to an arginine to histidine substitution at position 737 in the active core of RAG1. This mutation has been previously reported by A. Villa et al. in a patient with Omenn syndrome. In this study the recombination efficiency of mutant RAG1 was examined and the R737H substitution led to a total lack in recombinase activity. [38]

The RAG1 mutation found in the second allele of patient A (c.125 A>G) was a novel mutation which affected the start codon of RAG1 (ATG>GTG). We hypothesized that this is a hypomorphic mutation which could result in an aberrant gene expression of RAG1. RAG1 expression was assessed by using HEK 293 cells transduced with either wild-type or M1V mutant RAG1. Mutant RAG1 mRNA expression was moderately, by approximately 35%,

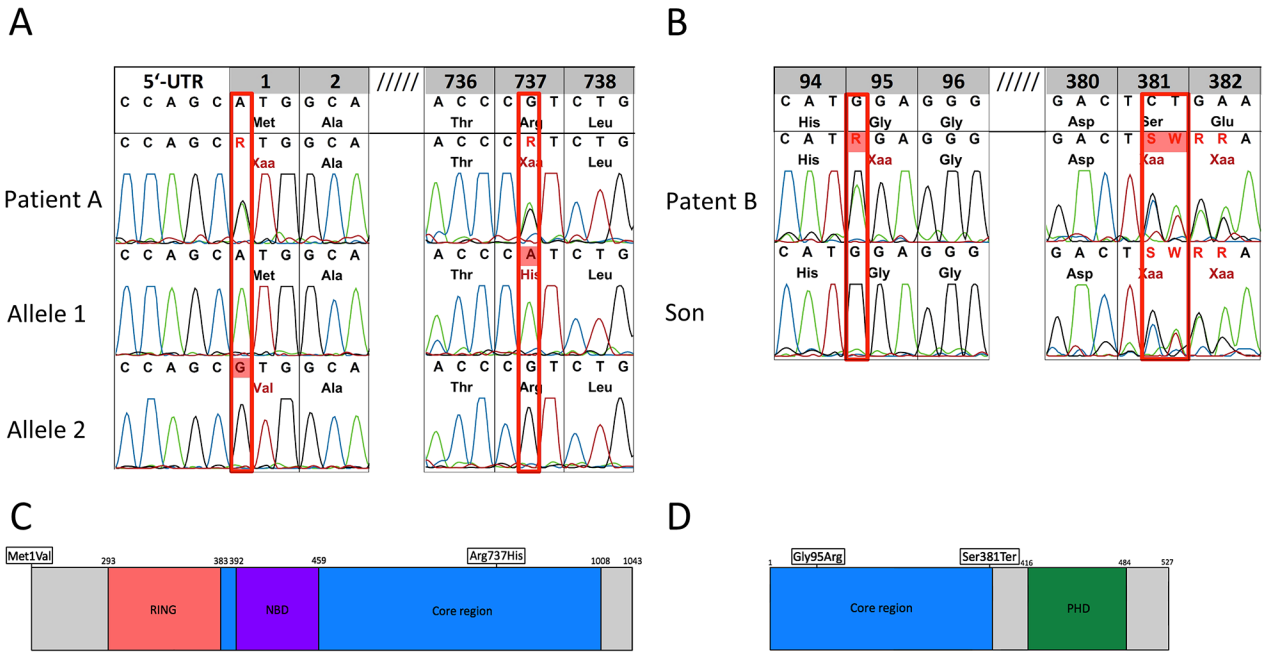

Fig 2. RAG1/2 Mutation analysis of genomic DNA from peripheral blood. (A) Patient A is compound heterozygous in RAG1, c.1 A>G, p.M1V; c.2322 G>A, p.R737H. Allele-specific PCR was used to characterize RAG1 M1V and R737H alleles. (B) Patient B is compound heterozygous in RAG2, c1347-8delCT, pSer381Terfs*1; c488G>A, pGlu95R. Mutation analysis of the son of patient B identified the compound heterozygosity of the RAG2 missense mutations. (C) Schematic depiction of RAG1 (D) Schematic depiction of RAG2. Mutations are indicated by rectangles.

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reduced as compared to wild type RAG1 gene expression. (Fig 3A). In contrast, western blot analysis revealed a profound defect in RAG1 protein expression in cells transfected with the mutant RAG1 gene (Fig 3B).

Patient B harbored two mutations in RAG2 (c1342-3delCT, pSer381Terfs*1; c683G>A, pGly95Arg) that were shown to be compound heterozygous by mutation analysis of patient´s son (Fig 2B and 2D). The c683G>A missense mutation changes glycine 95 into arginine and has previously been reported in a patient with Omenn syndrome. [39] G95 is conserved in the RAG2 gene of all known species and is located in a kelch motif in the enzymatically active core. Functional studies revealed an ablation of in vitro and in vivo recombination when RAG2 G95R mutant was tested.

The RAG2 mutation on the second allele of patient B (c.1342-3delCT) is a novel mutation never described before in men. We refrained from further investigating the molecular consequences of this mutation because a mouse model harboring a mutation, with similar consequences for Rag2 protein function, was previously described by Akamatsu and colleagues, [33] where it was shown that the mutation results in a frameshift and stop codon at position 381 (pSer381Terfs*1). The mouse model expressed a truncated RAG2 protein lacking the C-terminal 145 amino acids while leaving the catalytic core region of RAG2 intact. Mice expressing the mutant, truncated "core RAG2" (1–383) retained significant in vivo function of VDJ recombination but displayed a reduction in total numbers of B and T cells, presumably due to impaired lymphocyte development at the progenitor stage associated with reduced VDJ recombination, but cell numbers that were sufficient to prevent the development of a SCID phenotype.

Leaky RAG deficiency is associated with a severe reduction in naïve CD4 T cells

In both patients lymphocyte counts and numbers of $CD4^+$, $CD3^+$ and $CD56^+$ lymphocytes as well as numbers of Treg cells ($CD4^+CD25^+CD127^{low}$) were within the normal range, while iNKT cells ($CD3^+TCRV\alpha24^+TCRV\beta11^+$lymphocytes) were undetectable (data not shown)

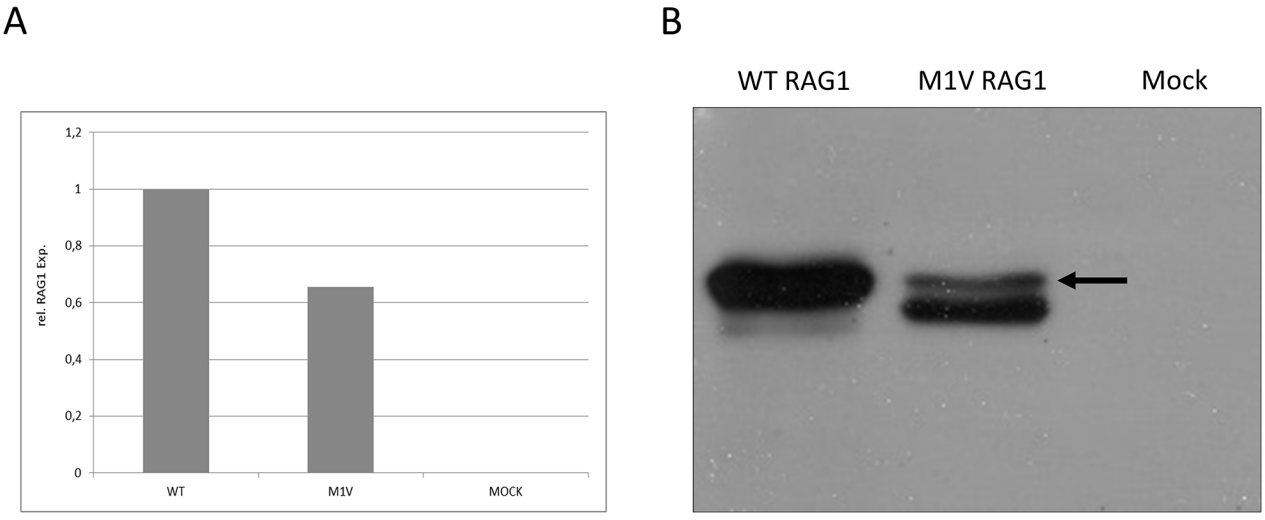

Fig 3. Molecular characterization of novel M1V RAG1 mutation. (A) Gene expression of RAG1 in HEK293 cells transfected with wild-type RAG1 and M1V RAG1 mutant by quantitative real time PCR. Cells transfected with mock vector served as a control. Wild type expression was normalized to 1 and percentage of RAG1 M1V gene expression was calculated. (B) Protein expression of RAG1 in HEK293 cells transfected with wild-type RAG1 and M1V RAG1 mutant by western blot. Cells transfected with mock vector served as a control. The arrow indicates RAG1 protein as identified through its molecular weight of 130kD.

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However, a more detailed characterization of the $CD4^+$ T cells showed a severe reduction in naïve $CD4^+$ cells ($CD4^+CD45RA^+$) in both patients (Fig 1B), while both $CD4^+$ memory subsets (central memory $CD4^+CD45RA^-CD62L^+$ and effector memory $CD4^+CD45RA^-CD62L^-$) were present in normal numbers (data not shown). The total count of $CD4^+$ T cells decreased over-time in both patients. (Fig 1C) In contrast to the severe reduction of naïve $CD4^+$ T cells, naïve $CD8^+$ T cells ($CD8^+CD45RA^+CD62L^+$) were only slightly reduced (Fig 1B). The number of $CD8^+$ T cell subsets ($CD8^+CD45RA^+CD62L^-$ effector cells, $CD8^+CD45RA^-CD62L^-$ effector memory cells, and $CD8^+CD45RA^-CD62L^+$ central memory cells) were comparable to healthy controls in patient A, while the number of $CD8^+$ effector memory cells was increased in patient B (% of $CD8^+$ lymphocytes [normal range] at age 41: 36.8 (7.4–24.8). Lymphoproliferative responses to mitogenic stimulation (PHA, ConA, PWM) were within the normal range in both patients (data not shown).

To further investigate a possible role of decreased RAG activity on T cell diversity, given that the number of naïve $CD4^+$ T-cells emigrating from the thymus was reduced, we analyzed T cell receptor of diversity $CD3^+$ T-cell in patient A. TCR spectratyping analysis revealed a diverse distribution of all 23 TCR Vbeta genes, thus giving no indication for a restricted TCR repertoire in this patient. Fig 1D shows the distribution of 4 frequently used TCR Vbeta genes. (Fig 1D) S2 Fig shows the distribution of TCR Vbeta genes 1–23.

Impaired antibody production against bacterial polysaccharide antigens in adult patients with leaky RAG1/2 deficiency

The antibody response to a range of T-cell dependent and T cell independent antigens was investigated and the results indicated a lack of IgG responsiveness to T-independent bacterial polysaccharide antigens in both patients. Patient B displayed a markedly reduced IgG and IgM response to vaccination with 23-valent unconjugated pneumococcal (Pneumo 23 "Merieux") and four-valent unconjugated meningococcal (Mencevax)-vaccine (reciprocal serum titer against $ACW_{135}Y$ meningococcal capsular polysaccharide: before vaccination, IgG <20, IgM <20; after vaccination, IgG <20, IgM <20; normal response in vaccinated healthy adults, IgG ≥ 100, IgM ≥ 100). Patient A displayed a poor Streptococcus pneumonia anti-capsular IgG response despite the fact that repeated culture proven pneumococcal infections led to high titers of IgM antibodies against these antigens (Fig 1A), resulting in a ratio of IgG- to IgM-Pn23-antibody serum titer of 0.03, well below the values observed in healthy adults either before or after Pn23 vaccination (ratio of IgG- to IgM-Pn23-antibody serum titer in 41 healthy adults [median, q5-q95]: before Pn23 vaccination, 2.00, 0.35–22.77; six-eight weeks after Pn23 vaccination¸ 0.95, 0.17–45.89). In contrast, IgG antibody responses to protein antigens such as tick-borne encephalitis virus (TBEV) vaccine were normal in both patients. (Serum TBEV IgG, ELISA, VIEU/ml: patient A, before vaccination 213, after vaccination 1609; patient B, before vaccination 51, after vaccination 1694; normal vaccination response in healthy adults ≥ 310). Furthermore, serum IgG antibodies against tetanus toxoid, diphtheria toxoid, polio I,II,III could be examined in patient B after vaccination and before IVIG replacement therapy and were found to be within the normal range (data not shown). In addition, serum IgG antibodies against measles, mumps, rubella and VZV were detectable in patient B before IVIG replacement therapy (data not shown).

B cell differentiation is impaired in leaky RAG1/2 deficiency

Patient A displayed normal levels of circulating B cells (CD19- and CD20-positive lymphocytes as determined by flow cytometry) but we observed a shift in peripheral blood B cell subsets. Naïve $CD27^-IgD^+$ and MZ-like $CD27^+IgD^+$ B cells were present in normal numbers, while

numbers of IgD$^-$CD27$^+$ switched memory B cells (9,7% of total B-cells) were decreased. (Fig 1E) Furthermore we observed an expansion of CD21^{low} B-cells, as numbers of this B cell subset were increased to 16,3% of total B-cells as compared to 1,95% in healthy adult donors (median of N = 50) (Fig 1F). CD21^{low} B cells are proposed to be an exhausted, anergic B-cell population with possible autoreactive capacities.

In patient B B cell numbers (identified as CD19-positive lymphocytes by flow cytometry) were significantly reduced but detectable upon first examination (CD19$^+$ lymphocytes at age 35 [normal range]: % of lymphocytes, 2 [7–23]; absolute number/μl, 21 [71–549]) and decreased continuously over time until they were completely undetectable at age 41.

Decreased in-vitro IgG production by EBV-transformed B cells with leaky RAG1/2 deficiency

To assess the capacity of in-vitro immunoglobulin production in patient A with hypomorphic RAG1 mutation, peripheral blood mononuclear cells (PBMCs) were simulated with Epstein-Barr virus (EBV). IgG in-vitro production was markedly reduced in patient A, compared to healthy controls. In contrast IgM in vitro levels were was comparable to the controls (Table 1) Further we analyzed proliferation response following EBV transformation, there was no significant reduction observed between patient A and healthy blood donors. (Table 1)

Discussion

We report herein a novel presentation of leaky RAG1/RAG2 deficiency which is associated with selective IgG antibody deficiency and massively decreased numbers of naïve CD4$^+$ T-cells. Both adult patients presented with intact production of T-cell dependent IgG antibodies but a defective T-independent IgG response to bacterial polysaccharide antigens. In patient B defective IgG antibody response to bacterial polysaccharide could be demonstrated by measurement of post-vaccination antibody response. In patient A however, it was not possible to examine IgG response after pneumococcal vaccination before the initiation of immunoglobulin replacement therapy providing substantial levels of IgG antibodies against 23-vqlent pneumococcal

Table 1. In vitro immunoglobulin production and ^{3}H-Thymidin incorporation of PBMCs and lymphoblastoid cells.

		immunoglobulin production in vitro (ng/ml)					
		control			patient		
		mean		SD	mean		SD
unstimulated	IgG	89	±	37	n.a.		
	IgM	103	±	53	n.a.		
EBV transformed	IgG	6388	±	4239	887	±	76
	IgM	24078	±	7946	38127	±	1805
		^{3}H Thymidin incorporation dpm					
unstimulated		1920	±	783	n.a.		
EBV transformed		75979	±	2795	90914	±	305

Unstimulated peripheral blood mononuclear cells and EBV-transformed lymphoblastoid cells were incubated for 8 days and the supernatant IgM/G concentration was determined by ELISA. Additionally the ^{3}H-thymidin incorporation was determined. Results are expressed as mean and standard deviation (control unstimulated IgM/G: n = 9; control EBV-transformed IgM/G: n = 3; patient EBV-transformed IgM/G: n = 1 (triplicate); control unstimulated ^{3}H-thymidin incorporation: n = 9; control EBV-transformed ^{3}H-thymidin incorporation: n = 1 (triplicate); patient EBV-transformed ^{3}H-thymidin incorporation: n = 1 (triplicate)).

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polysaccharides (Pn23). However, despite recurrent culture-proven pneumococcal infections such as pneumonia IgG-Pn23-antibodies were low (representing a mix of IVIG-derived and patient-derived antibodies), while IgM-Pn23-antibodies (entirely derived from the patient) were very high, resulting in a ratio of IgG- to IgM-Pn23-antibody serum titer that was well below the minimal value observed in our laboratory in healthy adults either before or after Pn23 vaccination. The patients described experienced recurrent pneumonia, sinusitis, otitis media and recurrent cutaneous vasculitis in one patient. While patient A´s clinical symptoms started in childhood, patient B presented in her fourth decade of life for the first time with a relative short history of disease. In both patients IVIG substitution corrected most of the susceptibility to infections.

Using a novel strategy of targeted resequencing a multitude of known primary immunodeficiencies genes in patients with predominantly antibody deficiency we identified compound heterozygous mutations in RAG1 or RAG2 in the two patients described. Delayed onset and relatively mild clinical manifestations were consistent with a residual function of the described novel hypomorphic RAG mutations. Patient A displayed a well described R737H substitution in one allele of RAG1. A. Villa and colleagues previously reported this mutation in a homozygous form in a patient with Omenn-syndrome and found that this is a null mutation associates with a complete lack of recombination activity.[38] Patient A however harbored a novel M1V mutation on the other RAG1 allele that affected the initiation codon of RAG1 (ATG>GTG). Subsequent characterization of the novel M1V mutation revealed a reduced but detectable Rag1 protein expression. Residual expression of Rag1 might explain the relatively mild clinical phenotype. Although the compound heterozygous combination of one null RAG1 allele and one hypomorphic allele results in a severe reduction of total RAG1 protein, this aberrant expression might be sufficient enough to prevent the development of a SCID phenotype.

In patient B we identified compound heterozygous mutations in RAG2. Analogous to patient A one mutation was a null RAG2 mutation as described in the literature. [39] The mutation on the second allele was a novel frameshift mutation that, resulting in a truncated RAG2 protein with an intact catalytic core region. Although not required for the basic biochemistry of V(D)J recombination, the non-catalytic region of RAG1/RAG2 is conserved throughout evolution. The C terminus of RAG2 is predicted to contain a plant homeodomain (PHD), a motif required for chromatin protein interaction. Akamatsu Y. et al. generated a transgenic mouse model with an analogous mutation resulting in a truncated RAG2 with intact core region. [33] These transgenic mice displayed a phenotype similar to patient B. The overall number of peripheral T-cells was unaffected however mice exhibit a partial block in T-cell development. These data are consistent with our finding that levels of circulating naïve $CD4^+$ T-cells were severely decreased in the RAG2-deficient patient as well. Although residual RAG1/2 expression/activity is sufficient to prevent the development of a SCID phenotype, impairment of lymphoid development may result from altered V(D)J recombination or decreased cell proliferation. As TCR spectratyping in patient A revealed a diverse repertoire one might speculate that the hypomorphic RAG1/2 mutations lead to a proliferation disadvantage. Cell cycle-regulated periodic destruction of RAG2 at the G1-to-S transition is induced through the phosphorylation of Thr-490 located in the C-Terminus that is deleted in patient B. [40] Such a dysregulation might result in aberrant expression of RAG2 during S phase and thus lead to decreased cell proliferation and/or increased cell death. In individuals younger than 20 years the bulk of naïve T cells is sustained primarily from thymic output, whereas proliferation within the naïve T cell population dominates in older individuals.[41] These findings are consistent with our findings that in leaky RAG deficiency a continuous decrease of absolute numbers of $CD4^+$ T-cells over time was observed. (Fig 1C) Patient B displayed a significant B-cell lymphopenia, and a comparable impairment in B cell differentiation was observed in core

RAG2-deficient mice, as in this animal model mature circulating B cell numbers were severely reduced. Patient A exhibited a moderate B cell differentiation defect with a pronounced expansion of circulating $CD21^{low}$ B-cells and decreased numbers of circulating switched memory B cells. This decrease in the percentage of switched memory B cells reflects the hypogammaglobulinemia and selective IgG antibody deficiency found in the patient and could also be responsible for the observed impairment in IgG production by patient A´s EBV-transformed B cells, although an additional effect of the patient´s RAG1 deficiency on immunoglobulin class switching cannot be ruled out.[42] Expansion of CD21low B cells is predominately found in HIV infected individuals and in a specific subgroup of CVID patients[43,44]. Recent findings suggest an anergic status of $CD21^{low}$ B cells in CVID associated with a defect in calcium-dependent BCR-activation. [45] Further reports have demonstrated an increased proportion of autoreactive B cells among $CD21^{low}$ B cells. [46] As patient A experienced episodes of leukocytoclastic vasculitis one might speculate that an increased expansion of autoreactive CD21low B cells might be implicated in the pathophysiology of her disease complications.

In both patients defects in B-cell function and differentiation were observed. A selective impairment in IgG antibody production against a certain type of antigen such as bacterial polysaccharides could be explained by a restricted B-cell repertoire, as has been shown in CD20 deficient patients. [30] It is feasible that a mutation in RAG leads to such a repertoire restriction, as V(D)J recombination is crucial to obtain BCR diversity. In contrast defects in cell-mediated immunity were limited to a decrease in naïve $CD4^+$ T-cells. No restriction in TCR diversity was observed. The results indicate that hypomorphic RAG mutations have more impact on humoral then on cell-mediated immune responses. Which is consistent with previous findings in mice overexpressing dominant negative RAG1 that led to impaired B-cell but intact T- cell development.[47]

Genetic defects in RAG1 and RAG2 are known to impair V(D)J recombination, thereby causing $T^-B^-NK^+$ severe combined immunodeficiency. However, in the last decade additional patients with RAG mutations were identified that present with a various degree of immune dysregulation and clinical manifestation. Most previous reports describing the different clinical phenotypes of RAG deficiency presented pediatric patients with late onset of combined immunodeficiency characterized by CD4 lymphopenia and/or impaired T cell function and hypo- or agammaglobulinemia with defective B cell differentiation [7,9,10,13–19] or early onset autoimmunity and granulomatous disease [8–11,15,16,20]. Few adult patients with leaky RAG deficiency have been reported, Abraham et al described 38-year-old man with skin rash and severe pan-T-cell lymphopenia rash but no susceptibility to infections or impaired antibody responses. A heterozygous frameshift mutation in RAG1 was found that was also present in his healthy father [21]. Recently, Buchbinder et al reported two unrelated adolescent patients with opportunistic infections, persistent lymphopenia and an early age of first clinical symptoms (recurrent bacterial sinopulmonary infections, viral infections, autoimmune disease) who exhibited compound heterozygous mutations in RAG1 and a prior clinical diagnosis of CVID made at the age of five years (patient 1) and 14 years (patient 2) of age [22]. The phenotype reported in our patients (adult RAG-deficient patients with a predominant clinical picture of antibody deficiency) confirms and extends the already reported spectrum of disease associated with RAG deficiency. In addition to a pronounced susceptibility to bacterial infections of the respiratory tract one of our patients showed episodes of cutaneous vasculitis reminiscent of the patient described by Sharapova et al.[16] In contrast to previous patients, in our adult patients the leaky RAG-deficient phenotype was dominated clinically and immunologically by an impairment in antibody production. Thus our findings contribute to the known complexity of divergent genotype-phenotype manifestations of RAG mutations and underline the importance of testing for hypomorphic SCID mutations even in adult patients with predominantly

antibody deficiencies. If predominantly antibody deficiency is associated with a hypomorphic SCID mutation the disorder is likely to be more complex as compared to patients with other defects leading to a deficiency of humoral immunity. Given that clinical manifestation and/or diagnosis can occur in adulthood, it will have to be decided whether alternative treatment such as HSCT will improve long-term prognosis as compared to immunoglobulin replacement alone. Further studies are needed to improve survival and long-term quality of life in other adult patients with a comparable clinical and immunological phenotype.

Supporting Information

S1 Fig. Next generation sequencing filtering strategy.
(TIF)

S2 Fig. Distribution of TCR Vbeta genes 1–23.
(TIF)

S1 Supporting Information. Ethics statement of the Ethics Committee of the City of Vienna.
(PDF)

S1 Table. Monoclonal fluorophore conjugated antibodies for flow cytometry.
(DOCX)

S2 Table. Primary immunodeficiency genes sequenced.
(DOCX)

S3 Table. Primer sequences for T-cell CDR3 Vβ Spectratyping and RAG1.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CBG MME HMW. Performed the experiments: CBG AP AL KMTS. Analyzed the data: CBG AP AL MME HMW KMTS. Contributed reagents/materials/analysis tools: MME HMW. Wrote the paper: CBG MME HMW.

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3.2 Prevalence and clinical challenges among adult primary immunodeficiency (PID) patients with RAG deficiency

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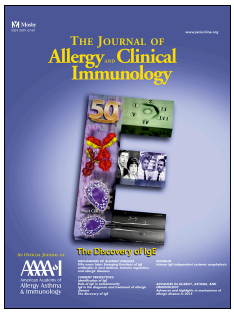

Prevalence and clinical challenges among adult primary immunodeficiency patients with RAG deficiency

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Prevalence and clinical challenges among adult primary immunodeficiency patients with RAG deficiency

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Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki. Patients gave their written informed consent that anonymized data could be included in a scientific publication. All results presented in this study were obtained as part of the routine medical attendance the patient received.

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Capsule Summary

Patients with RAG deficiency may survive in adulthood and our findings suggests that prevalence of such cases varies between 1%-1.9% in adult PID cohorts.

Key words

RAG1; *RAG2*; primary immunodeficiency; inflammatory lung disease; fibrosis

Abbreviations

AB (antibiotics), AIC (Autoimmune cytopenia), AIHA (Autoimmune hemolytic anemia), AN (Autoimmune neutropenia), BAL (bronchoalveolar lavage), BCR (B cell receptor), CID (Combined immune deficiency), COPD (Chronic obstructive pulmonary disease), COS-7 (CV-1 in Origin with SV40 genes), CT (computed tomography), CTD (carboxy-terminal domain), CVID (Common variable immunodeficiency), CXR (chest x-ray), DDBD (dimerization and DNA-binding domain), G/AI (granulomatous/autoimmune), HRCT (high-resolution computed tomography), HSCT (Hematopoietic stem cell transplantation), HYPOGAM (Hypogammaglobinemia), ICL (Idiopathic CD4+ T lymphopenia), ITP (Immune thrombocytopenia), IVIG (Intravenous immunoglobulin), MODS (Multiple organ dysfunction syndrome), NBD (nonamer-binding domain), NIHR (National Institute for Health Research), PFT (pulmonary function test), PHD (plant homeodomain), PID (Primary immunodeficiency), PreR (pre-RNase H), RAG (Recombination-activating gene), RNH (RNase H), RSS (Recombination signal sequence), SCIG (subcutaneous immunoglobulin), SPAD (Specific polysaccharide antibody deficiency), TCR (T cell receptor), V(D)J (Variable, diversity, and joining), ZnBD (zinc-binding domain).

To the Editor,

RAG deficiency has an estimated disease incidence of 1:181,000 including severe combined immunodeficiency (SCID) at a rate of 1:330,000.[1, 2] Complete or hypomorphic variants of SCID secondary to low recombinase activity (<5%) present early with severe infections and/or clinical signs of systemic inflammation, such as severe dermatitis and/or colitis.[3, 4] Hypomorphic *RAG1/2* mutations with more preserved residual V(D)J recombination activity (5-30%) result in a distinct phenotype of combined immunodeficiency with granuloma and/or autoimmunity (CID-G/A).[1, 2, 5] Beyond CID, RAG deficiency has been found in patients with predominantly primary antibody deficiencies[6, 7] and naïve $CD4^+$ T cell lymphopenia in most cases. Currently there is no published systematic evaluation for the presence of an underlying RAG deficiency in patients with primary antibody deficiencies. There is great variability among diagnostic modalities for evaluation and treatment for inflammatory lung disease in case reports of RAG deficiency with no standardized guidelines. Clinical features and lung disease for patients with late presentation of RAG deficiency have not been studied extensively. In addition, no studies have examined the prevalence of RAG deficiency in cohorts of adult primary immunodeficiency (PID) patients. Here, we describe a cohort of 15 patients with late presentation of RAG deficiency. We also estimate the prevalence of RAG deficiency in adult PID patents following genetic analysis in two separate large cohorts of PID patients.

We have analyzed the canonical regions of *RAG1* and *RAG2* in a total of 692 PID patients from two separate cohorts, one from the United Kingdom (UK) and one from Austria (Vienna). The UK cohort is part of the National Institute for Health Research BioResource – Rare Diseases (NIHRBR-RD) PID study, as previously described (Tuijnenburg et al, *in revision*). In the NIHRBR-RD PID cohort 558 patients (299 adults) and the Vienna cohort of 134 patients (106 adults) we report a total of five newly identified cases of RAG deficiency.

For details see repository text E1 and tables E1-3. Based on these findings we estimate that prevalence of RAG deficiency in adult PID ranges from 1%-1.9%. For all adult PID patients that are currently registered with the UK Primary immunodeficiency network database (3294 patients over age of 18 years) we expect to find an additional 32.9-62.6 cases of RAG deficiency. Gene variants are illustrated in Fig 1A. The cohort demographics are discussed in repository text E1.

Functional characterization of novel RAG variants is discussed in repository text E1. The activity of mutant RAG1 and RAG2 proteins normally required for catalyzing V(D)J recombination events are shown in Table E2. In addition to the method previously described,[8] we also employed a system to measure recombination activity in compound heterozygous cases by in vitro expression of murine RAG1 and RAG2. Both systems simulate the efficiency of protein expressed in patients in their ability to produce a diverse repertoire of TCR and BCR coding for immunoglobulins. Over half of the mutant proteins tested show almost complete loss of activity. All patients tested had an overall low combined RAG activity (6.4-28%).

The immune phenotypes and clinical diagnoses are shown in Fig 1B. Persistently low IgG and/or low IgA and IgM levels are seen in approximately 50% of cases (Table E3). The dominant laboratory features were naïve CD4+ T cell lymphopenia with low absolute number and fraction of naïve CD4 cells (CD4+CD45RA+), and B cell counts were variably low (Table E3). Enzyme-linked immunoassay was used to test for anti-cytokine antibodies (targeting IFNα, ω, and IL-12) on plasma from seven patients (not shown). Four patients were positive which is comparable to our previously reported cohort (56%).[5]

Most adult RAG deficient patients developed inflammatory autoimmune complications (87%) (Fig E1A, repository text E1). Organ-specific manifestations were most common (73%) and similar to previously described reports of 48-77%.[5, 9] Granulomatous

disease was seen in 40% of patients, with five out of six patients showing granuloma localization within interstitial lung tissue. Other complications were also seen (Fig E1E). Similar to recent reports (21-77%)[5, 9] cytopenias occurred in 40% of patients; autoimmune hemolytic anemia (27%), immune thrombocytopenic purpura (20%), and autoimmune neutropenia in one patient.

Progressive pulmonary disease was the leading causes of morbidity and mortality (93%) (Fig E1D); pneumonia being the most common, followed by bronchiectasis, chronic bronchitis, granuloma, fibrosis, chronic obstructive pulmonary disease, and bronchiolitis (Fig 1C). We observed a transition from acute infectious complications (pneumonia) (mean onset 14 years) to chronic inflammatory complications (mean age of 23 years) (Fig 2A). It was also the leading concern for successful HSCT. High-resolution computed tomography imaging of lung revealed bronchiectasis and granuloma. Histology of lung biopsies (patient 1 and 3) revealed atypical lymphoid hyperplasia with granulomatous features and giant cells formation (Fig 2B). Germinal center formations in patient 3 were comprised of CD3+ T-cells and CD20+ B-cells. Patient 1 had peribronchial fibrosis (Fig 2B). Pulmonary lung function data revealed median values of FVC 79.55%, DLCO of 75%, and FEV1/FVC ratio of 78%. We performed a retrospective analysis of PFTs over two or more years to assess the decline of respiratory function. Two of four patients had a significant decline indicating a variable degree of lung function in adult patients with RAG deficiency (Fig E1G)(repository text E1).

Thirteen out of fourteen patients (93%) received first line immunoglobulin replacement therapy (Fig E1B, Table E2). 57% received antibiotic prophylaxis, 21% antiviral drugs, and 14% disease-modifying anti-rheumatic drug. Five patients (36%) were considered for HSCT. Comparisons of therapeutics approaches revealed no statistically significant difference in survival. Three out of eight patients who only received Ig replacement therapy

were deceased. Among transplanted patients the major mortality cause was infections post HSCT (Fig E1C).

RAG1/2 are the most common defective genes associated with atypical SCID.[9] Patients may survive into adulthood and our findings suggests that prevalence of such cases varies between 1%-1.9% in adult PID cohorts. Total and naïve CD4+ T cell lymphopenia,[4, 9] autoimmunity, and progressive inflammatory lung disease should all prompt further investigations for RAG deficiency in adult PID patients. The relative absence of RAG deficiency in the pediatric cohort of 216 patients suggest that milder forms of RAG deficiency may not be diagnosed as readily as a PID in childhood. In the era of whole exome sequencing, the spectrum of RAG deficiency further broadens to include adults with autoimmune and inflammatory manifestations that may result in progressive decline. Systemic analysis of PID related genes[8] and functional in vitro assays that confirm decreased recombination activity are essential. Laboratory features of naïve CD4+ T cell lymphopenia and presence of anti-cytokine antibodies can further support the diagnosis of partial RAG deficiency. Where RAG deficiency is confirmed, therapy may be adjusted based on the mechanistic understanding and may ultimately provide targeted strategy for early intervention.

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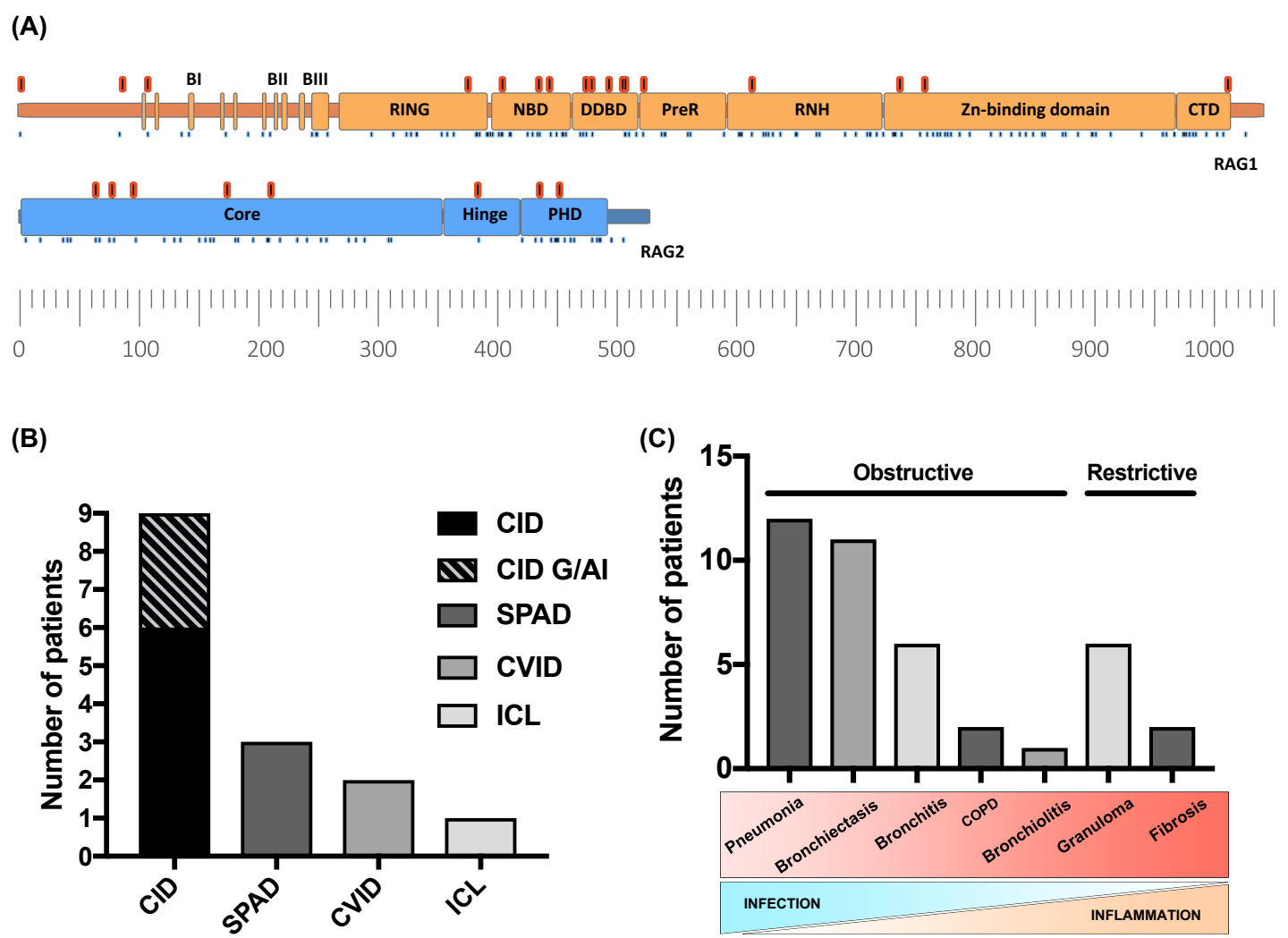
Figure 1. Gene variants and phenotypes of adults with RAG deficiency. (A) Schematic representation of *RAG1* and *RAG2* adapted from Notarangelo et al. 2016 (ref E19). Variants in this cohort (17 in *RAG1* and 8 in *RAG2)* are illustrated in red. Known pathogenic variants previously reviewed are shown as blue dots (ref E19). (B) The phenotypes of RAG deficiency in 15 patients including those from the NIHRBR-RD PID and Vienna cohort. (C) Presentation of lung disease. Bars represent age distribution, lines indicate median onset of lung disease, mean age of death, mean age (n=15).

Figure 2. Pulmonary disease. (A) Onset of pneumonia, bronchiectasis and granuloma/fibrosis/COPD (n=15). (B) High resolution computed tomography of patient 3

and 9. Histologic examination of lung biopsies from patient 1with atypical lymphoid hyperplasia with granuloma and fibrosis (H&E).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Obstructive
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Granuloma
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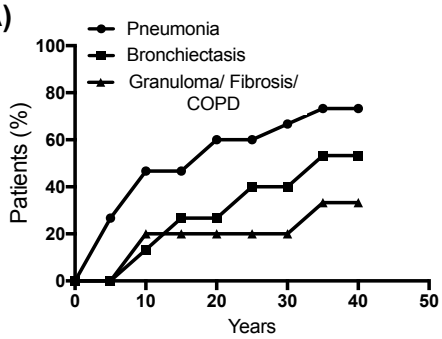
INFECTION
INFLAMMATION

(A)


Pneumonia
Bronchiectasis
Granuloma/ Fibrosis/
COPD
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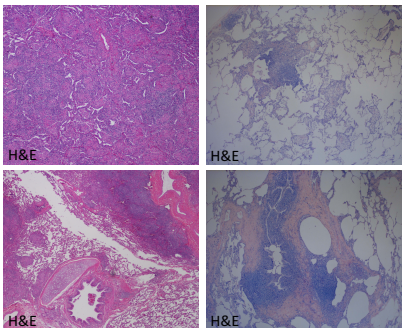
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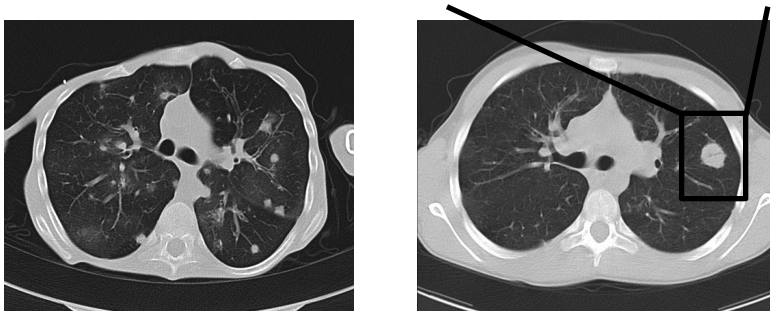

II. Methods

A. Whole genome sequencing

As part of the NIHR BioResource – Rare Disease study, 558 unrelated PID patients had their genomes sequenced, as described before (Tuijnenburg et al, *in revision*). Briefly, paired-end whole genome sequencing was performed by Illumina on their HiSeq X Ten system, and 95% of bases were covered by at least 15 reads. Substitutions and InDels up to 50bp were called by Isaac and then merged with AGG3 tool, while structural variants were called by Manta and Canvas (all software by Illumina). Only variants with an overall pass rate of >80% and were considered for further analysis. Structural variants were analysed for gene deletions, but none were identified.

B. Targeted sequencing

The canonical region of *RAG1* and *RAG2* in 134 patients was analyzed. Genomic DNA was prepared from peripheral blood by spin column purification (QIAamp DNA Blood Mini Kit; QIAGEN, Germany). Targeted resequencing of the canonical region of *RAG1* and *RAG2* was performed using Nextera Custom Enrichment kit according to standard protocols (Illumina, USA). DNA library was quantified and validated using Illumina Eco Realtime (Illumina; USA) and Agilent Bioanalyzer (Agilent Technologies; USA). The library was sequenced in a multiplex pool on a single (150 bp paired-end reads) flowcell on the Miseq System. (Illumina, USA)

C. Variant filtration

PID cohorts were assessed for the region of *RAG1* and *RAG2*; GRCh37 11:36,587,900-36,621,100. Filtering and prediction of functional consequences was performed using Variant Effect Predictor (http://www.ensembl.org/info/docs/tools/vep/index.html), Exome Variant Server (http://evs.gs.washington.edu/EVS/), The Single Nucleotide Polymorphism database (https://www.ncbi.nlm.nih.gov/projects/SNP/) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), The Exome Aggregation Consortium and The Genome Aggregation Database (http://gnomad.broadinstitute.org). Filtering of common variations and annotation was performed using vcfhacks (https://github.com/gantzgraf/vcfhacks) and in-house scripts. Candidate variants were required to pass the following filtering conditions: frequency (count/coverage) between 20-100%, according to VEP-annotation at least one canonical transcript is affected with one of the following consequence: variants of the coding sequence,

frameshift, missense, protein altering, splice acceptor, splice donor, or splice region; an inframe insertion or deletion; a start lost, stop gained, or stop retained, or according to VEP an ExAC frequency unkown, <=0.01, or with clinical significance 'path'.

D. Cell culture and transfection

COS-7 cells (fibroblast-like), from African Green Monkey kidney transformed with a mutant SV40 coding for the wild-type T-antigen, were used for recombination assays. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and Penicillin-Streptomycin (Gibco). Cells were seeded at $1.5x10^5$ per well (6 well plate) in 1.5ml culture medium 24 hours prior to transfection. Antibiotic free medium was substituted three hours prior to transfection. Cells were transfected with wild type or mutant form of murine *RAG1*, *RAG2* and recombination plasmids using 5uL Lipofectamine™ 2000 transfection reagent (Invitrogen) and 300 uL Opti-MEM® I reduced serum medium (Gibco). Experiments use a total concentration of 400 ng/μL *RAG1* construct, 200 ng/μL *RAG2* construct, and 1000 ng/μL of inversion recombination substrate. Experiments testing compound heterozygous mutations used half concentrations of both alleles for *RAG1* or *RAG2*.

E. RAG1, RAG2 and recombination substrate plasmids

Mammalian expression plasmids were constructed to contain either murine *RAG1* (3192bp in 4350bp pCS2-MT) or *RAG2* (1581bp in 5509bp pEF-XC). Site directed mutagenesis was used to produce mutant plasmids using Q5® Site-Directed Mutagenesis Kits (New England Biolabs, UK). Transfection assays used a combination of wild type or mutant *RAG1* and *RAG2* plasmids to reflect those of patient genotypes. A third plasmid was constructed and used in each transfection assay as an inversion recombination substrate (6009bp). The site targeted by the RAG1/RAG2 complex is a 557 nucleotide inversion sequence flanked with the 12 and 23 nucleotide RSS; CACAGTGCTACAGACTGGAACAAAAACC and CACAGTGGTAG TACTCCACTGTCTGGCTGTACAAAAACC, respectively (12 and 23 bp spacers with heptamer and nonamer flanking sequences).

F. Recombination assay

Triple-transfection of WT/mutant *RAG1*/*RAG2* and a recombination substrate was used to assess the functional activity matching that of patients with homozygous or compound

heterozygous genotypes. Transfection assays used a combination of RAG1 (400 ng/uL) co-transfected with RAG2 (200 ng/uL). Homozygous expression of a mutant gene was mimicked by co-transfecting a *RAG1* mutant with wild type *RAG2* and vice versa. To mimic compound heterozygous genotypes two equal half concentrations of mutant *RAG1* plasmids were co-transfected with wild type *RAG2* and vice versa. (i.e.200ng/uL WT *RAG1*, 200ng/uL mutant *RAG1*, and 200ng/uL WT *RAG2*). To measure the activity of these wild type or mutant proteins a third (or fourth in compound heterozygous instances) construct was used as an inversion recombination substrate (1000 ng/μL). The DNA sites targeted on this recombination substrate by RAG1/RAG2 complex are 12 and 23 nucleotide recombination signal sequences (RSS) flanking a 557 nucleotide inversion sequence. Successful recombination events, represented by a reversal of inversion sequence, were assessed by quantitative real-time PCR (qPCR) using comparative CT (ΔΔCT) (supplemental Fig E1H). qPCR primer sites were selected on the recombination substrate plasmid at 48 bp upstream of the 12RSS, prior to the inversion sequence, and a primer site 46 upstream of the of the 23RSS, laying internally on the inversion sequence, with both in the forward direction. A successful recombination event resulted in the reverse of inversion sequence and allows amplification of a 94 bp product. A second sequence of 94 bp on the recombination plasmid backbone, which is not affected by RAG activity, was used as a reference to calculate ΔΔCT values and assess relative recombination activity. The relative recombination activity is measured against wild type *RAG1/RAG2* to calculate the activity % of WT with mean ± SEM shown in Table 1.

G. Quantitative real-time PCR

Successful recombination events were assessed by quantitative real-time PCR (qPCR) using comparative CT (ΔΔCT). To recover recombination plasmid a modified Hirt's cell lysis extraction for low molecular weight DNA was performed before a phenol chloroform extraction (Thermo Fischer Scientific, 17909) and ethanol precipitation. DNA was diluted 50 times and Fast SYBR® Green Master Mix was used for qPCR. Experiments were performed on a QuantStudio® 5 Real-Time PCR System (Thermo Fischer Scientific, 4385610 and A28573).

H. Laboratory evaluation of immune phenotypes

Lymphocyte panel and immunoglobulin levels were determined by clinical laboratory testing. Anti-cytokine antibodies were detected by Enzyme-Linked Immunosorbent Assay (ELISA) as previously described.[1]

III. Discussion

Recombination-activating gene 1 (*RAG1*) and *RAG2* encode lymphoid-specific proteins that are essential for V(D)J recombination and diversification the T and B cell repertoire in the thymus and bone marrow, respectively.[2, 3] Hypomorphic *RAG1/2* mutations with residual V(D)J recombination activity (in average 5-30%) result in a distinct phenotype of combined immunodeficiency with granuloma and/or autoimmunity (CID-G/A).[1, 4-8] Currently there is no published systematic evaluation for the presence of an underlying RAG deficiency in patients with primary antibody deficiencies. Inflammatory complications are increasingly reported for RAG deficient patients with CID-G/AI phenotype and late diagnosis.[9-11] Allograft rejection and fatal post-transplant complications are more common among SCID patients with RAG variants than in other forms of SCID, especially if harboring infections.[12-14] In a recent multicenter study, hematopoietic stem cell transplantation (HSCT) was offered in 61% of cases, less frequently than in variants of SCID.[1] CID-G/AI patients may also fail to engraft stem cells or die from other post-transplant complications.[1, 9, 11, 15]

The UK cohort, part of the NIHRBR-RD PID study, included 558 cases with an antibody deficiency, 299 patients presented as adults; CVID were the largest group (n=305), followed by CID (n=101), and other antibody deficiencies (n=78). We report here only coding variants in *RAG1* and *RAG2*; variants identified in non-coding regions were not expected to affect protein expression or function. There were no instances where a patient had a damaging mutation in both *RAG1* and *RAG2*. Other known causes of PID were also excluded in these three patients. The Vienna cohort investigated 134 patients, including 106 adults; CVID (n=57), CID/late onset CID (LOCID) (n=36), primary antibody disorders (PAD) (including hypogammaglobinemia, selective PAD (SPAD) and agammaglobulinemia (n=41). In total five cases of RAG deficiency were identified and are reported in our study. Although these patients were primarily diagnosed with antibody deficiency, closer examination of the T cell compartment revealed low absolute number of naïve CD4 fraction (CD4+CD45RA+) suggestive of a CID phenotype (Table E3).

Complete data was collected for ten additional cases (>15 years of age) (Fig 1, Table E1-3). Of the fifteen patients described here the median age is 37 years (15-73 years), with five patients already deceased at ages 15, 22, 25, 37, and 43 (Table 1, 2). Clinical phenotype was predominantly CID (n=9, 60%), of whom three had clinical history of autoimmunity

and/or the presence of granulomas, followed by SPAD (3 patients, 20%), CVID (2 patients, 13%), and a single case of idiopathic CD4+ lymphopenia (ICL) (7%) (Table E1, Fig 1B). Most had late presentation. Although recurrent infections and lung disease were commonly seen in adolescence, severe disease and PID diagnosis generally occurred in adulthood.

Variants found in patients 1-10 were assessed as previously described.[16] The variants identified from patients 12-15 were assessed with in vitro expression of murine RAG1 and RAG2 in CV-1 in Origin with SV40 genes cells. The relative recombination activity was measured against wild type *RAG1/RAG2* to calculate the activity % of WT with mean ± SEM shown in Table 1. Both systems simulate the efficiency of protein expressed in patients in their ability to produce a diverse repertoire of TCR and BCR coding for immunoglobulins. As shown in Table E2 the mutations found in patients 12-14 have been tested by Lee *et al.*[26] and show very similar levels of recombination activity for individual mutations.[16] Over half of the mutant proteins tested show almost complete loss of activity. Patients 3, 5, 12, 13, and 15 all carry one allele with mutations that individually do not indicate any major loss of function. However, in our assay, we also had the ability to measure recombination activity in compound heterozygous cases and found a striking decrease that was less than the average activity of the two alleles in Patient 12, 13 and 15 (Table E1). All patients tested in our cohort had an overall low combined RAG activity (6.4-28%).

Inflammatory autoimmune complications developed in 87% of patients (Fig E1A). Organ-specific manifestations were the most common autoimmune complications affecting 73% of our adult cohort, similar to previously described reports with 48-77%.[1, 17] Gastrointestinal complications were the most prevalent organ specific manifestation followed by dermatological manifestations (Fig E1E). Granulomatous disease was seen in 40% of patients, with five out of six patients showing granuloma localization within interstitial lung tissue. Other complications included myopathies (14%), endocrine abnormalities, sarcoidosis, and polyarthritis was seen in one patient (Fig E1E). Cytopenias occurred in 40% of adult RAG deficient patients (Fig E1A), similar to recently reported cohorts (21-77%).[1, 17] Autoimmune hemolytic anemia was the most frequent (27%) followed by immune thrombocytopenic purpura (20%), and autoimmune neutropenia in one patient. Further studies may determine the underlining pathophysiology that drives autoimmunity in RAG deficient patients. 57% of the patients developed antibodies to cytokines, which may serve as a potential biomarker for adults with PID due to RAG1 and/or RAG2 mutations[1] (Table E3). It was recently demonstrated, that RAG deficient patients show a restriction of Treg repertoire diversity and a molecular signature of self-reactive conventional CD4+ T cells.[18]

Progressive pulmonary disease was prominent in our cohort of adults with RAG deficiency (Fig E1D). Of fifteen unrelated adult patients recruited, the median age at onset of lung disease was 11 years. The five patients (33%) that were deceased at the time of the study had a median age of 23.5 years (Fig E1D). We observed a diverse spectrum of pulmonary manifestations (Fig 1C). Clinical symptoms persisted for an average of 13 years. Lung disease was the leading cause of mortality. Of the five deceased patients two cases were due to progressive lung disease with pulmonary fibrosis. Two more patients died due to infections post-transplant. One patient died due to PML caused by John Cunningham virus infection. There was no significant difference in overall survival between patients presenting with pneumonia, bronchiectasis or granuloma, fibrosis, or chronic obstructive pulmonary disease. Computed tomography imaging revealed bronchiectasis and granuloma. Histology of lung biopsies are shown in Fig 2B. A retrospective analysis of PFTs indicated a variable degree of lung function (Fig E1G). Based on literature searches there are only four additional cases of similar adults.[1, 4, 5, 7] Of these four, two have died and two had a history of severe lung disease. To promote early intervention, we recommend high resolution chest CT every 1-3 years for signs of progressive pulmonary disease. Treatment of choice should be tailored to both infectious and inflammatory components.

Phenotype-genotype correlations are reported for regions of *RAG1* and *RAG2*.[19] Pathogenic missense variants in *RAG1* most frequently occur in the catalytic core (amino acids 387–1,011) (Fig 1A), predominantly in the zinc-binding domain. When normalized for domain length, a higher pathogenic variant rate is observed in the NBD and CTD.[19] Forty percent of *RAG1* patients reported here have disease-causing variants in NBD or CTD. These two domains constitute the highest reported pathogenic mutation rates.[19] A few *RAG1* missense mutations are associated with CID–G/AI. These variants are predominantly located in the domains DDBD, PreR and CTD.[19] Deviation from the phenotype-genotype correlation is illustrated by three patients found to have CID-G/AI; patient 3 (Table E1 and Fig E1F) was found to have compound heterozygous *RAG1* mutations in non-core and the catalytic RNase H (RNH) domain while presenting with CID-G/AI, and patients 6 and 7 (Table E1) reported as CID-G/AI due to compound heterozygous core/plant homeodomain (PHD) and homozygous core *RAG2* mutations, respectively. Fig 1A, E1F illustrates the distribution of mutations reported in this study amongst *RAG1* and *RAG2* functional domains.

Newborn screening for SCID and related conditions has identified RAG1/2 as the most common defective genes associated with atypical SCID[17, 20] Based on ExAC data analysis, we predicted the incidence of RAG deficiency as 1:181,000 individuals who are

homozygous or compound heterozygous for pathogenic RAG1/2 mutations.[7] Furthermore, based on two large cohort studies in Europe, we provide an estimate for the prevalence of RAG deficiency in adult PID between 1%-1.9%. With this estimate we expect to find an additional 32.9-62.6 cases of RAG deficiency in the UKPIN database. A robust systemic analysis of 79 individual mutations in RAG1 was conducted previously.[16] Our results were comparable to the published data.[16] Furthermore, we assessed activity levels in RAG1 and RAG2 compound heterozygous cases and found lower activity levels than the average of activity levels when each allele was measured separately. The relative absence of RAG deficiency in the pediatric cohort of 216 patients suggest that milder forms of RAG deficiency may not be diagnosed as readily as a PID in childhood. The low number of naïve CD4 cells may appear as idiopathic T cell lymphopenia subset when screened at birth.[21] Careful analysis of HSCT decision is needed and should be considered before onset of rapid or progressive decline in lung function.[15, 22] Interim analysis has been provided to guide difficult HSCT decisions for patients with SCID and profound CID.[17, 20] Patients with more severe phenotypes generally have a progressively pronounced restriction of their BCR and TCR repertoire diversity. Analysis of the TCR and BCR repertoire identifies skewed usage of V(D)J segment genes and abnormalities of CDR3 length distribution.[23] We also see, as recently published[24], that low IgA and IgM is associated with bronchiectasis in PID. Mutant RAG1 and RAG2 proteins with residual recombination activity in these patients likely provides antibody repertoire that may be sufficient during early childhood but immunodeficiency and progressive autoimmunity becomes apparent towards early adolescence. Phenotypic heterogeneity impedes prediction of the clinical phenotype, although at least 150 and 57 disease-causing variants which are likely to result in clinical intervention are reported for *RAG1* and *RAG2*, respectively.[19]

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Table E1. Patient details and *RAG1/2* gene variants causing mixed PID phenotypes. The general immunodeficiency phenotype as well as autoimmune or other autoinflammatory conditions are listed for 15 patients in this study. Recombination activity shown in brackets were previously reported.[1] Ages are listed as of May 2017 or age when deceased. Experimental recombination activity presented as % of WT with mean ± SEM. AIHA (Autoimmune hemolytic anemia), CID (Combined immune deficiency), CTD (carboxy-terminal domain), CVID (Common variable immunodeficiency), DDBD (dimerization and DNA-binding domain), G/AI (granulomatous/autoimmune), HYPOGAM (Hypogammaglobinemia), ITP (Immune thrombocytopenia), NBD (nonamer-binding domain) PHD (plant homeodomain), PreR (pre-RNase H), RNH (RNase H), SPAD (Specific polysaccharide antibody deficiency), ZnBD (zinc-binding domain).

Table E2. Pulmonary disease prominent feature in RAG deficiency. Details of pulmonary disease features from 15 RAG deficient patients.[25] AI (Other autoimmunity), AIC (Autoimmune cytopenia), AIHA (Autoimmune haemolytic anaemia), AN (Autoimmune neutropenia), BAL (bronchoalveolar lavage), BX (Biopsy), CID (Combined immunodeficiency), CID-G/AI (Combined immunodeficiency with granuloma autoimmunity), COPD (Chronic obstructive pulmonary disease), CT (Computerized Axial Tomography), CXR (Chest X-ray), HRCT (High Resolution computer tomography), HSCT (Hematopoietic stem cell transplantation), HYPOGAM (hypogammaglobulinemia), SPAD (Selective polysaccharide antibody), CVID (Common variable immunodeficiency), ICL (Idiopathic CD4 T cell lymphopenia), ITP (Immune thrombocytopenic purpura), IVIG (Intravenous Immunoglobulin), MODS (Multiple organ dysfunction syndrome), PFT (Pulmonary function testing).

Table E3. Lymphocyte cell counts and serum immunoglobulins. (A) Lymphocyte cell counts from multiple centers are shown. Local reference ranges differ between centers although progressive T cell lymphopenia is a notable feature for most patients. Reduced absolute lymphocyte counts or impaired T cell proliferation was found. Moderate to slightly low CD4+ and CD8+ T cells and severe B cell lymphopenia is also seen for several patients. MHC class I, MHC class II and CD18 appears normal for those tested but is not shown. (B) Most of this cohort (73%) demonstrates low serum immunoglobulins. 14-27% have normal or borderline low Ig measurements respectively. Low IgG subtypes (not shown) occur at roughly the same rate in this cohort where measured. Plasma from 7/15 patients was testing for anti-cytokine antibodies targeting IFNα, ω, and IL-12 as previously reported among patients with RAG deficiency.[1] Naive CD4+; (CD45RA+); Effector Memory CD4+ (CD45RA-CD62L-); Central Memory CD4+(CD45RA-CD62L+); Treg (CD4+CD25+CD127low); Switched Memory B cells (CD19+, CD27+, IgD-/IgM-).

Figure E1 Treatment, functional assay, and phenotype. (A) Autoimmune complications in adult patients with RAG deficiency. (B) Treatment strategies used in adult RAG deficient cohort. (C) Overall survival displayed as Kaplan–Meier survival curve. (D) Distribution of pulmonary disease (n=15). (E) Distribution of autoimmune diseases. Gastrointestinal includes duodenitits, collagenous colitis, coeliac disease, enteropathy, gastritis; dermatological includes alopecia, psoriasis, dyskeratosis, vitiligo; myopathy includes

myopathy, myoatrophy, myasthenia gravis, myofasciitis; endocrine include hypogonadism, hypothyroidism. (F) Phenotype-genotype distribution. RAG1 protein consists of 1008 amino acids. RAG2 protein is composed of 527 amino acids. (G) (Top) Pulmonary function data FEV1 (n=6), FVC (n=6), and DLCO (n=4). (Middle and bottom) Retrospective analysis of changes in pulmonary function tests over 2 or more years of patients with reduced lung function (n=2). (H) DNA recombination substrate containing 12 and 23 nucleotide recombination signal sequences flanking a 557 nucleotide inversion sequence. Recombination events are assessed by quantitative real-time PCR (qPCR).

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Further information is available from https://bioresource.nihr.ac.uk/rare-diseases/consortia-lists/

Study ID	Phenotype	Mutation	Recombination Activity (%)*	Gender	Current Age	Autoimmune cytopenia or other AI	Malignancy	Protein Domain	Reference
Patient 1	SPAD	*RAG1* : A c.125A>G p.M1V B c. 2334G>A p.R737H	na (0.2±0.0)	F	Deceased age 43	Leukocytoclastic vasculitis at age 8, 13, 36-38		Non-core ZnBD	Pt A (Geier, CB PLOS 2015) (E10)
Patient 2	SPAD	*RAG2:* A c.1342-3delCT p.S381fsTer1 B c.683G>A p.G95R	na (0.0)	F	50 years	AIHA		Hinge Core	Pt B (Geier, CB PLOS 2015) (E10)
Patient 3	CID-G/AI	*RAG1:* A c.256-257delAA p.K86VfsTer33 B c.1835A>G p.H612R	(2.7±0.3) (121.6±0.9)	F	20 years	AIHA, ITP, GLILD, vitiligo, duodenitis		Non-core RNH	PtA (Buchbinder, D J Clin Immunol 2015) (E9)
Patient 4	CID	*RAG1:* A c.322C>T p.R108Ter B c.1566G>T p.W522C	na (41.6±1.9)	M	41 years	Alopecia, hypogonadism, hypothyroidism		Non-core preR	Pt 55 (Dobbs, K Frontiers Immunol 2017) (E22)
Patient 5	ICL	*RAG1:* A c.1420C>T p.R474C B c.1516C>T p.L506F	(125.4±2.6) (1.0±0.1)	F	Deceased age 25	None		DDBD DDBD	(Kuijpers, TW Blood 2011) (E21)
Patient 6	CID-G/AI	*RAG2:* A c.230C>A p.T77N B c.629T>C p.G451A	(0.7)	F	22 years	Neutropenia, thrombocytopenia, Granulomas (spleen, lung) / bone marrow expansion, T-cellular infiltrates		Core PHD	Pt C (Schuetz, C N Engl J Med 2008) (E4)
Patient 7	CID-G/AI	*RAG2:* c.186C>A p.F62L homozygous	(20)	F	33 years	ITP		Core	Manish Butte (Walter, JE JCI 2015) (E1)
Patient 8	CID	*RAG2:* A c.518A>G p.N173S B c.1309 G>A p.E437K	na (1.0)	M	38 years	Psoriasis and progressive myopathy and muscle atrophy		Core PHD	Pt 59 (Dobbs, K Frontiers Immunol 2017) (E22)
Patient 9	CID	*RAG1:* A c.256-257delAA p.K86VfsTer33 B c.1331C>T p.A444V	(2.7±0.3) (1.4±0.2)	M	Deceased age 15	Granulomatous skin disease lymphohistiocytic infiltrates, dyskeratosis of epidermis.		Non-core NBD	Sharapova, SO (Human Immunology 2013) (E11)
Patient 10	CID	*RAG1:* c.2275C>T p.R759C homozygous		M	Deceased age 22	Collagenous colitis, coeliac disease	B cell lymphoma	ZnBD	Unpublished Christian Klemann
Patient 11	CID	*RAG1* : A c.1123C>G, p.H375D A c.1430delC, p.F478SfsTer14 B c.1420C>T, p.R474C	na na (125.4±2.6)	M	Deceased age 37	AIHA, vitiligo, lymphomatoid granulomatosis, leukocytoclastic vasculitis		Non-core DDBD DDBD	Schröder, C (Clin Immunol 2017) (E25)
Patient 12	HYPOGAM/SPAD	*RAG1:* A c.1303A>G p.M435V B c.3016A>G p.M1006V	16 (23.6) 97.2 (105.6)	F	64 years	Pernicious anaemia		NBD CTD	Unpublished NIHRBR-RD PID
		Compound	28.8±8.9						
Patient 13	CVID	*RAG1:* A c.1303A>G p.M435V B c.3016A>G p.M1006V	16 (23.6) 97.2 (105.6)	F	73 years	Sarcoidosis. Small bowel lymphangiectasia with protein-losing enteropathy. Antral gastritis. Low grade dysplasia.	Tubular adenoma	NBD CTD	Unpublished NIHRBR-RD PID
		Compound	28.8±8.9						
Patient 14	CID	*RAG1:* A c.1210C>T p.R404W B c.1520G>A p.R507Q	1.7 (Q1.2) 19.4 (W15.9)	M	36 years	None		NBD DDBD	Unpublished NIHRBR-RD PID
		Compound	15.7						
Patient 15	CVID	*RAG2:* A c.1352G>C p.I210T B c.629T>C p.G451A	74.3±5.2 56.6±6.1	F	39 years	Myasthenia gravis, alopecia, vitiligo. Chronic diarrhoea. Severe polyarthritis. Macrophagic myofasciitis.		Core PHD	Unpublished NIHRBR-RD PID
		Compound	6.4±2						

*Recombination activity shown in brackets represent values previously reported.
Activity % of WT with mean ± SEM
Age as of May 2017 or age when deceased

ID	Age*	RAG phenotype	Autoimmunity	Type of pulmonary disease, pathogens	Severity of lung disease	Age at onset of lung disease	Length	Diagnostic studies	Treatment			HSCT	Respose to therapy	Overall outcome	Reference
									First-Line	Second-Line	Third-Line				
Patient 1	43 yrs	SPAD	leukocytoclastic vasculitis	Diffuse panbronchiolitis with *Streptococcus pneumoniae* and Pseudomonas, COPD, bronchitis, granulomas, fibrosis	Progressive, severe, leading cause of death	13 yrs	30 yrs (until death)	PFT, CXR, HRCT, BX	IVIG, AB prophylaxis	considered for lung transplantation and HSCT but deceased	-	No	Poor, COPD with progressive decline in lung function	deceased, 43 yrs	Pt A (Geier, CB PLOS 2015) (E10)
Patient 2	50 yrs	SPAD	AIHA	Bacterial pneumonia (pathogen unknown), recurrent bacterial bronchitis	Progressive	35 yrs	15 yrs (ongoing)	PFT, CXR	IVIG, AB prophylaxis	considered for HSCT	-	No	Limited improvement	alive	Pt B (Geier, CB PLOS 2015) (E10)
Patient 3	20 yrs	CID-G/AI	AIHA, ITP GILD, vitiligo, duodenitis	Recurrent granulomatous interstitial lung disease, no history of bacterial infections, penicillium, corynebacterium, pseudomonas isolated in BAL	Progressive, severe, leading indication for HSCT	6 yrs	12 yrs (until HSCT)	PFT, CXR, BAL, HRCT	IVIG, infliximab	Abatacept	1. Steroid with sirolimus 2. Adalimumab	Yes, 18 yrs	Alive and well, on regular IVIG	alive	PtA (Buchbinder, D J Clin Immunol 2015) (E9)
Patient 4	41 yrs	CID	alopecia, hypogonadism, hypothyroidism	Bronchiectasis, pneumatocele	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	No	n.a.	alive	Pt 55 (Dobbs, K Frontiers Immunol 2017) (E22)
Patient 5	25 yrs	ICL	none	Varicella pneumonia	Progressive	7 yrs	18 yrs (until death)	HRCT	IVIG, AB prophylaxis	tobramycin, rhDNAse		Yes, 24 yrs	Poor, septicemia (6 mnth post-HSCT)	deceased, 25 yrs	(Kuijpers, TW Blood 2011) (E21)
Patient 6	22 yrs	CID-G/AI	AN, ITP granulomas (spleen, lung), bone marrow expansion, T-cellular infiltrates	Recurrent pneumonia, granulomas	n.a.	9 yrs	5 yrs (until HSCT)	HRCT	IVIG	-	-	Yes, 14 yrs	n.a.	alive	Pt C (Schuetz, C N Engl J Med 2008) (E4)
Patient 7	31 yrs	CID-G/AI	ITP	Recurrent sinopulmonary infections, progressive restrictive lung disease	Mild	22 yrs	10 y (ongoing)	HRCT	inhaled steroid, bronchodilator	tobramycin, rhDNAse	-	No	No improvement	alive	Manish Butte (Walter, JE JCI 2015) (E1)
Patient 8	37 yrs	CID	psoriasis and progressive myopathy and muscle atrophy	Recurrent pneumonia, transient bronchiectasis	Stable. Bronchiectasis was transient.	30 yrs	Uncertain duration of bronchiectasis, perhaps 2 years	HRCT, PFT	IVIG, AB prophylaxis	inhaled steroid, bronchodilator	-	Yes, 37 yrs	Pneumonia episodes respond well to antibiotics. Baseline respiratory status is good. Bronchiectasis was not progressive.	alive	Pt 59 (Dobbs, K Frontiers Immunol 2017) (E22)
Patient 9	15 yrs	CID	granulomatous skin disease lymphohistiocytic infiltrates, dyskeratosis of epidermis	Bronchiectasis, granulomas, pulmonary fibrosis	Progressive, leading cause of death	5 yrs	10 yrs (until death)	HRCT, BX	IVIG, AB prophylaxis	acyclovir, steroids (prednisolon),antifungal, bronchodilator (salbutamol)	-	No	No improvement	deceased, 15 yrs	Sharapova, SO (Human Immunology 2013) (E11)
Patient 10	22 yrs	CID	vitiligo, coeliac disease (HLADR1&DQ8 negative)	clubbing, FEV1 49 % Vcmax 73 %. DLCO 61 %, recurrent pneumonia (HiB+), bronchiectasis, constant peribronchial infiltrates	Progressive, severely damaged, leading concern as limiting factor for HSCT	7 yrs	15 yrs (until death)	PFT, CXR, BAL, HRCT	physiotherapy, salbutamol, IVIG, AB prophylaxis	inhal steroid, Colistin inhal, hypertonic saline inhalation, recurrent courses of oral Augmentin treatment for HiB (acyclovir for recurrent, severe HSV stomatitis)	constant therapy with augmentin p.o.	Yes, 22 yrs	poor, death due to GI mucositis Grade 4, leading to sepsis and MODS	deceased, 22 yrs	Unpublished Christian Klemann
Patient 11	37 yrs	CID	AIHA, vitiligo,lymphomatoid granulomatosis, leukocytoclastic vasculitis	Recurrent bronchopulmonal infections, COPD with GOLD 3, bronchiectasis in both lungs with chronic colonization of Pseudomonas aeruginosa.	Progressive, severe	32 yrs	5 yrs (until death)	n.a.	SCIG, AB	steroid, cidofovir, mianserine, mefloquine	-	No	death due to PML	deceased,37 yrs	Schröder, C (Clin Immunol 2017) (E25)
Patient 12	64 yrs	HYPOGAM WITH SPAD	Pernicious anaemia	No lung infections or inflammation	n.a.	n.a.	n.a.	CT	IVIG	-	-	No	n.a.	alive	Unpublished NIHRBR-RD PID
Patient 13	73 yrs	CVID	Sardcoidosis, small bowel lymphangiectasia, protein-losing enteropathy, antral gastritis	Severe bronchiectasis, pulmonary sarcoidosis	Progressive, moderate	50 years	23 yrs (ongoing)	PFT, CT	IVIG, AB prophylaxis	-	-	No	Ongoing infections , continues with intermittent hypoxia and poor PFTs	alive	Unpublished NIHRBR-RD PID
Patient 14	34 yrs	CID		Recurrent pneumonia, bronchiectasis	Progressive, moderate	Childhood	n.a.	n.a.	IVIG	-	-	No		alive	Unpublished NIHRBR-RD PID
Patient 15	39 yrs	CVID	AIHA Myasthenia gravis, alopecia, vitiligo. Chronic diarrhoea. Severe polyarthritis. Macrophagic myofasciitis.	Recurrent pneumonia (since 5 yrs), bronchiectasis (since 25 yrs) with persistent right lower lobe collapse	Progressive, severe	5 yrs	n.a.	n.a.	IVIG, steroid	DMARDS (TNF and IL1 antagonists)	Tocilizumab	No	Multiple relapse	alive	Unpublished NIHRBR-RD PID

* age as of May 2017 or age when deceased

Lymphocyte count		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13	Patient 14	Patient 15
CD3+	**%Ly**	57-63	79-82	73.4	83.8	-	-	62	79	50.9-94	82	49	80	71	79	56-66.8
CD3+	**cell/µl**	454-2280	818-1279	807	628	170	592-606	422	449	210-1635	400-700	125	1761	1560	304-926	101-667
CD4+	**%Ly**	35-51	24-52	50	54	-	-	30	63	8.1-33.3	27	25	66	27	24-39	43-53.8
CD4+	**cell/µl**	367-1400	362-374	550	405	336	108-184	200	360	92-385	-	64	1447	592	166-280	54-290
Naive CD4+$^{CD45RA+}$	**%Ly**	0	2-3	2.18	15.4	15.5	-	7	6	-	0.5	-	-	-	26	5
Naive CD4+$^{CD45RA+}$	**cell/µl**	0	31	12	116	-	-	12	34	-	-	-	-	-	-	-
Effector Memory CD4+	**%CD4+**	-	-	69.64	6.2	-	-	5.8	-	40.7	-	-	-	-	27	-
Central Memory CD4+	**%CD4+**	-	-	28	32.3	-	-	46	-	40	-	-	-	-	42	-
CD4+CD45RO+	**%Ly**	-	-	-	-	38.5	75-96	-	57	-	99	-	-	-	-	-
CD8+	**%Ly**	27-43	52-68	17.5	25.7	-	-	28	15	22.6-86.8	45	32	16	44	-	11.3-17
CD8+	**cell/µl**	194-1720	538-1061	192	193	-	360-420	191	86	83-1510	-	82	352	950	-	30-180
HLA-DR+	**%Ly**	21-31	35-61	-	6.4	-	-	-	5	-	$90^{monocyte}$	-	-	-	-	-
HLA-DR+	**cell/µl**	151-1240	362-952	-	48	-	-	-	29	-	-	-	-	-	-	-
CD3+HLA-DR+	**%Ly**	11-13	28-51	-	14.7	-	18	$20^{CD4}12.1^{CD8}$	-	5.9-60.9	$21^{CD4}7.3^{CD8}$	-	-	-	-	-
CD3+HLA-DR+	**cell/µl**	79-520	290-796	-	110	-	-	$34^{CD4}17^{CD8}$	-	22-1060	-	-	-	-	-	-
Treg $^{CD4+CD25+CD127low}$	**%CD4+**	4.77	4.91	-	-	-	-	-	-	2.1	-	-	-	-	-	-
CD56+	**%Ly**	18-30	9-10	13	15.1	-	-	23	18	5.1-38.9	10	43	9	25	16-19	28.1-36
CD56+	**cell/µl**	130-1200	104-140	143	113	-	131-355	159	103	89-385	-	110	191	555	87-210	150-244
CD19+	**%Ly**	8-10	0-2	15.6	1.6	-	-	13	2	0.4-4.3	0	1	10	3	2-5	3.6-7
CD19+	**cell/µl**	58-400	0-21	172	6	14	58-132	89	11	2-20	-	3	216	59	24-31	10-50
IgG		697	940	703	673	673	146	614*	393	0.04	n/a***	281	310	250	730	467
IgA		127	<6	<7	139	106	<6	<8	22	0.018	<5	21	60	50	258	930 *
IgM		60	< 7	<21	59	69	<5	16.9	19	0.02	7	17	50	40	2.5	234 **
IgE		256	256	<1,0	<1	<1	<5	<2	<2	<0,1	-	-	-	-	<2	
anti-IFNα		Positive	Negative	Negative	Positive	Positive	N/A	Positive	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A
anti-IFNω		Low-positive	Negative	Negative	Positive	Positive	N/A	Positive	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A
anti-IL-12		Negative	Negative	Negative	Positive	Positive	N/A	Positive	Negative	N/A	N/A	N/A	N/A	N/A	N/A	N/A

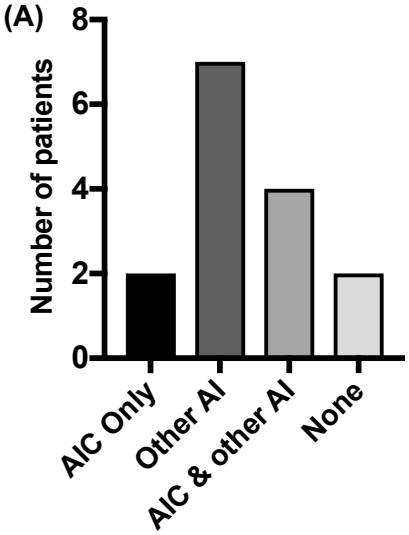
Naive CD4+; (CD45RA+), Effector Memory CD4+; (CD45RA-CD62L-),Central Memory CD4+; (CD45RA-CD62L+), Treg; (CD4+CD25+CD127low)

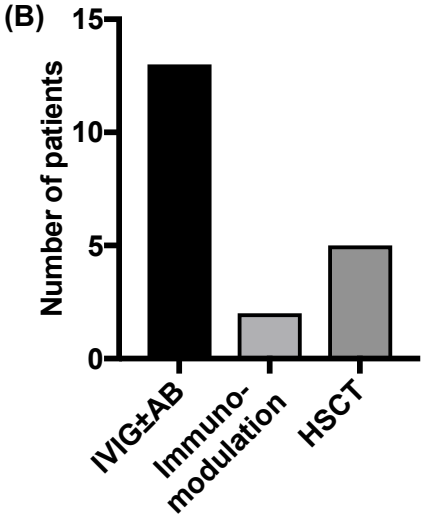
* variable low to high

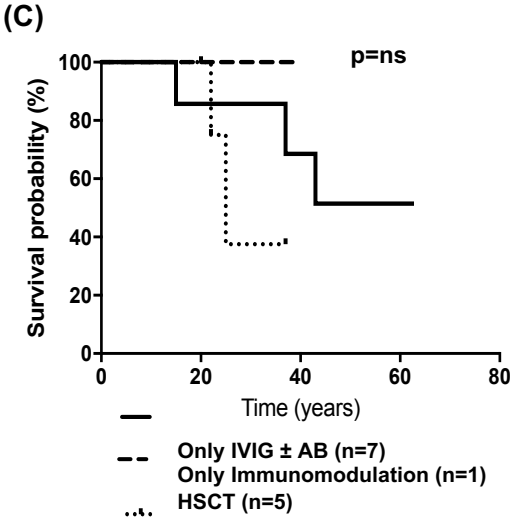
**Raised when unwell

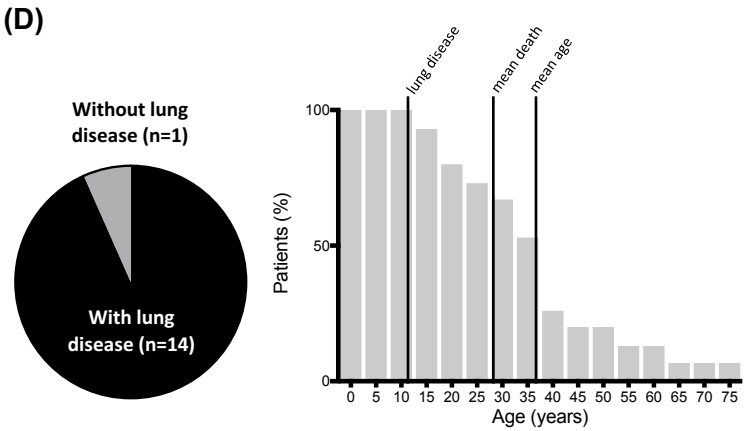
***CVID criteria fullfilled at age 7yrs. IVIG since

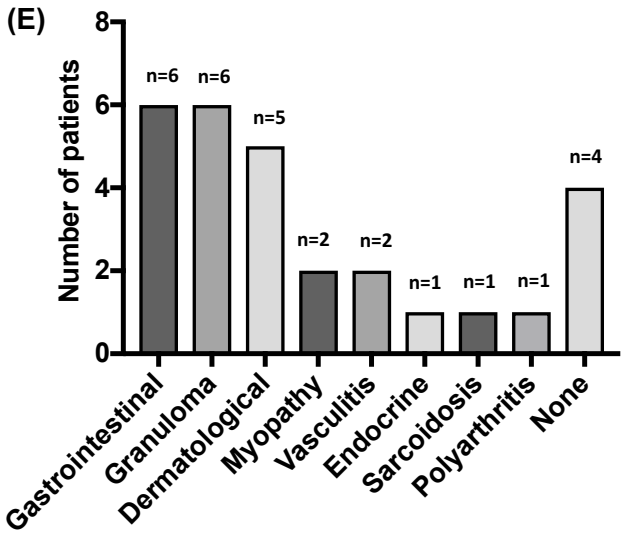
∆ Low borderline normal











(F)

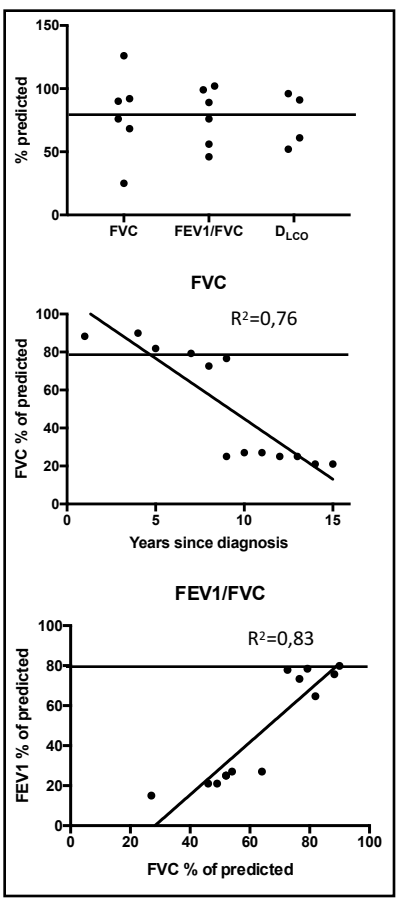
RAG1

	NBD	DDBD	PreR	RNH	ZnBD
NBD					
DDBD		ICL			
PreR					
RNH					
ZnBD					CID
CTD	CVID HYPO/SPAD				
Non-core	CID	CID	CID	CID-G/AI	SPAD

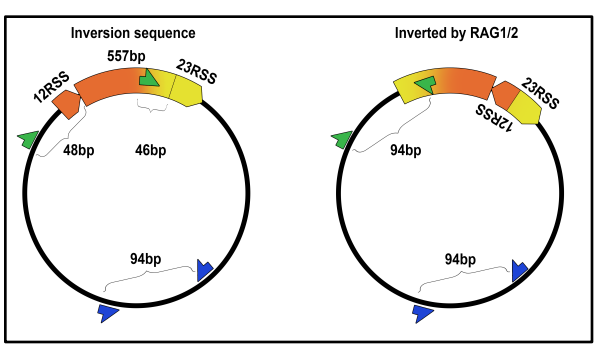
RAG2

Domain	Core	PHD
Core	CID-G/AI	CID CVID CID-G/AI
Hinge	SPAD	

(G)



(H)



4 Discussion

Genetic defects in RAG1 and RAG2 are known to impair V(D)J recombination, thereby causing T-B-NK+ severe combined immunodeficiency. However, in recent years the phenotypic spectrum was extended by describing additional patients with hypomorphic RAG mutations that present with a various degree of immune dysregulation and clinical manifestations. Most previous reports describe pediatric patients with late onset combined immunodeficiency characterized by CD4 lymphopenia and/or impaired T cell function and hypo- or agammaglobulinemia with defective B cell differentiation 38,39,42,43,45 or early onset autoimmunity and granulomatous disease 70–72. Only a few adult patients with leaky RAG deficiency have been reported. Abraham et al described a 38-year-old man with a heterozygous frameshift mutation in RAG1 presenting with skin rash and severe pan-T-cell lymphopenia but no susceptibility to infections or impaired antibody responses. 73 Buchbinder and colleagues report two unrelated adolescent patients with early onset opportunistic infections, persistent lymphopenia and compound heterozygous mutations in RAG1. 74

We herein report a novel presentation of hypomorphic RAG1/RAG2 deficiency which is associated with selective IgG antibody deficiency and decreased numbers of naïve CD4+ T-cells. Both adult patients presented with intact production of T-cell dependent IgG antibodies but a defective T-independent IgG response to bacterial polysaccharide antigens. In addition, the clinical picture was dominated in both patients by progressive obstructive lung disease, which was the major cause of mortality and morbidity. The phenotype reported in our patients confirms and extends the already reported spectrum of disease associated with RAG deficiency. In addition to a pronounced susceptibility to bacterial infections of the respiratory tract one of our patients showed episodes of cutaneous vasculitis reminiscent of the patient described by Sharapova et al. 72 In contrast to previous patients, in our adult patients the leaky RAG-deficient phenotype was dominated clinically and immunologically by an impairment in antibody production and progressive lung function. Thus, our findings contribute to the known complexity of divergent genotype-phenotype manifestations of RAG mutations and underline the importance of testing for hypomorphic SCID mutations even in adult patients with predominantly antibody deficiencies. If predominantly antibody deficiency is associated with a hypomorphic SCID mutation the disorder is likely to be more complex as compared to patients with other defects leading to a predominant deficiency of humoral immunity.

It is unclear if these adults could have been identified at birth with newborn screening for SCID or naïve CD4+ T cell lymphopenia that is a hallmark of adult RAG deficiency. The late presentation of RAG deficiencies presented here indicate that screening for RAG1 and RAG2 defects in early childhood among milder PID phenotypes may provide an opportunity for intervention before clinical manifestations including the onset of the pulmonary disease.

A possible biomarker for finding these patients is progressive CD4+ T cell lymphopenia (total and naïve T cell). RAG dependent immunodeficiency is known to produce immune dysregulation; granulomas and autoimmunity occurs with mutations retaining residual recombination activity.74–77 The majority of patients investigated have developed inflammatory and/or autoimmune complications in similar frequencies to previously described studies.26 A fraction of the patients also developed antibodies to cytokines. It needs to be determined when autoantibodies develop and whether antibodies against self-antigens, especially cytokines, may serve as additional biomarkers for adults with PID due to RAG1 and/or RAG2 mutations.74,75

Lung disease was the most prominent feature in our cohort of fifteen cases of adolescent or adult patients with RAG deficiency. Based on a literature search there are only four additional cases of adults, of these four patients two have deceased and two had a history of severe lung disease.

Clinical spectrum of lung disease may range from infections to immune dysregulation and is a key component of morbidity and mortality among patients with hypomorphic RAG1 or RAG2 variants. Severe non-infections complications including fibrosis are likely underestimated in the absence of close monitoring of lung disease. Although large cohort-based whole exome studies among patients with pulmonary fibrosis have not revealed RAG genes, our data suggests that RAG deficiency should be considered, especially if immune phenotype is supportive. To promote early intervention, serial lung evaluation with pulmonary function testing (PFT) including DLCO and lung volumes should be considered in these patients.

If PFT is abnormal, we recommend yearly high-resolution chest CT for signs of progressive pulmonary disease. Treatment of choice should be tailored to both infectious and inflammatory components. Timing of HSCT is critical and should be considered before the onset of rapid or progressive decline in lung function.48

Given that clinical manifestation and/or diagnosis can occur in adulthood, it will have to be decided whether alternative treatment such as HSCT will improve long-term prognosis as

compared to immunoglobulin replacement alone. Further studies are needed to improve survival and long-term quality of life in other adult patients with a comparable clinical and immunological phenotype.

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