Clinical, Immunological and Molecular Variability of RAG Deficiency: a Retrospective Analysis of 22 RAG Patients

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Clinical, immunological and molecular variability of RAG deficiency: a retrospective analysis of 22 RAG patients

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ABSTRACT

**Purpose:** RAG deficiency is associated with a variety of clinical phenotypes. We described clinical, immunological and molecular characterization within a cohort of 22 RAG patients focused on the possible correlation between clinical and genetic data.

**Methods:** Immunological and genetic features were investigated by Multiparametric Flow Cytometry and by Sanger or Next generation sequencing (NGS) respectively.

**Results:** Patients represented a broad spectrum of RAG deficiencies: SCID n=8, OS n=6, LS/AS n=4 and CID n=4. Four novel mutation in \textit{RAG1} gene and one in \textit{RAG2} were reported. The primary symptom at presentation were infections (81.8%). Infections and autoimmunity occurred together in the majority of cases (63.6%). Fifteen out of 22 (68.2%) patients presented autoimmune/hyperinflammatory manifestations. Four patients experienced severe autoimmune cytopenia refractory to different lines of therapy. Total lymphocytes count was reduced or almost lacking in SCID group. CD4 cells count was higher in OS patients. B lymphocytes were variably detected in AS and CID groups. Eighteen patients underwent HSCT permitting definitive control of autoimmune/hyperinflammatory manifestations in twelve of them (80%).

**Conclusion:** RAG deficiency still represents a challenge in the tracing of effective management and follow-up, notably considering the inability to predict the disease course in atypical cases. Immune dysregulation manifestations are common features often refractory to conventional medical management. Severe and early autoimmune refractory cytopenia is frequent and could be the first symptom of onset. Prompt recognition of RAG deficiency in patients with early onset of autoimmune/hyperinflammatory manifestations could contribute to the choice of a timely and specific treatment preventing the onset of other complications.

**Keywords:** RAG deficiency, RAG1/RAG2, hypomorphic mutation, CID phenotypes, cytopenia

**Abbreviation**

TCR, T-Cell Receptor
SCID, Severe Combined Immunodeficiency
OS, Omenn Syndrome
AS, Atypical SCID
LS, Leaky SCID
CID/G/A, Combined immunodeficiency with diffuse granulomatous disease and/or autoimmunity
EBV, Epstein-Barr virus
CVID, Common Variable Immunodeficiency
ICL, Idiopathic CD4+ T cell lymphopenia
NGS, Next Generation Sequencing
RTE, Recent Thymic Emigrants
IUIS, Union of Immunological Societies
PIDTC, Primary Immune Deficiency Treatment Consortium
CMV, Cytomegalovirus
IVIG, Intravenous Immunoglobulin
ITP, Immune thrombocytopenia
AIHA, Autoimmune Hemolytic Anemia
HSCT, Hematopoietic Stem Cell Transplantation
MAV, Myeloablative Conditioning
MMF, Mycophenolate Mofetil
INTRODUCTION

Systematic rearrangement of antigen receptor genes via V(D)J recombination is essential for maturation of progenitor lymphocytes, genesis of immunoglobulin and T cell receptor (TCR) and production of a broad repertoire of antigen-specific T and B cells [1-6]. Complete RAGs deficiency has been known to cause severe combined immunodeficiency (SCID) phenotype with lack of T and B cells (T-B-NK+ SCID) [7], life-threatening infections and failure to thrive in early infancy. Today, RAGs diseases are associated to an expanding broad spectrum of phenotypes ranging from SCID, Omenn syndrome (OS) [8-13], ‘leaky’ or ‘atypical’ SCID, (LS/AS) whose peculiarities reside in varying numbers of oligoclonal T and B cells, and in some cases a predominance of γδ+ T cells (γδ AS) and autoimmune cytopenias [14]. Hypomorphic mutations causing a residual RAG protein function leading to a delayed onset and diagnosis, characterized by diffuse granulomatous disease and/or autoimmunity (CID-G/A) and susceptibility to severe Herpesviridae infections (in particular EBV) [15-17]. Common variable immunodeficiency (CVID), idiopathic CD4+ T cell lymphopenia (ICL) [18], IgA deficiency and hyper-IgM syndrome have been also reported [19-21].

Although infections are the predominant presenting features in RAG deficiency patients, autoimmune manifestations including cytopenias, autoimmune hepatitis, myopathy, and nephrotic syndrome [17, 22-23] should be considered associated manifestations. This autoimmunity has been linked not only to checkpoint breaks in both T and B cell tolerance but also to intestinal microbiota which may play an additional role in sustaining autoimmune pathology [24].

Herein, we report the clinical, immunological phenotype and molecular characterization of 22 RAG patients referred to our Center presenting with a broad spectrum of symptoms including autoimmune and/or hyperinflammatory manifestations.

METHODS

Patients

A total of 22 patients from 18 different families were assessed between 2009 and 2020. Patients were classified into four groups (SCID, OS, LS/AS, CID) based on clinical presentation, immunological data, on the criteria published by the Primary Immune Deficiency Treatment Consortium (PIDTC) [25], ESID 2019 [26] and other groups [24,27,28].

- SCID: at least one of: invasive bacterial, viral or fungal/opportunistic infection; persistent diarrhoea and failure to thrive; affected family member AND manifestation in the first year of life AND two of 4 T cell criteria fulfilled: absence or very low number of T cells (CD3 T cells < 300/microliter), reduced naive CD4 and/or CD8 T cells; elevated γδ T cells; no or very low T cell function by response to mitogen or TCR stimulation; AND T cells maternal engraftment excluded AND HIV excluded

- OS: Desquamating erythroderma in the first year of life AND one of the following: lymphoproliferation, hepatomegaly splenomegaly; failure to thrive; chronic diarrhea; recurrent pneumonia AND eosinophilia or elevated IgE AND T-cell deficiency (detectable CD3 T cells, ≥ 300/microliter, low naive cells, reduced proliferation, oligoclonality) AND maternal engraftment excluded AND HIV excluded

- LS/AS: Reduced number of CD3 T cells (for age up to 2 years < 1000/microliter; for > 2 years up to 4 years < 800/microliter, for > 4 years < 600/microliter) AND Absence of maternal engraftment AND < 30% of lower limit of normal T cell function (as measured by response to PHA) AND Presence of activated, oligoclonal, and autologous T cells

- CID-G/A: Diffuse granulomatous disease and autoimmunity
not associated with typical features of OS. Expansion of γδ T cells upon cytomegalovirus (CMV) infection or Epstein-Barr virus (EBV)-driven lymphoproliferative disease and autoimmune cytopenias could be associated.

- CID: at least one of: severe infection (especially VZV, CMV, EBV, HPV, and molluscum); one manifestation of immune dysregulation (autoimmunity, IBD, severe eczema, lymphoproliferation, granuloma); malignancy; affected family member AND 2 of 4 T cell criteria fulfilled: reduced CD3 or CD4 or CD8 T cells (using age-related reference values); reduced naïve CD4 and/or CD8 T cells; elevated g/d T cells; reduced proliferation to mitogen or TCR stimulation.

The clinical characterization is summarized in Table 1. Two patients have been enrolled in the pCID study (DRKS00000497).

**Multiparametric Flow Cytometric Analysis**

All flow cytometric analysis were performed on ethylenediamine tetraacetic acid (EDTA) blood samples within 24 h of venipuncture. After red blood cells lysis with ammonium chloride the lymphocytes were incubated with the appropriate antibody cocktail for 30 min at 4°C, washed with PBS and suspended in PBS. At least 50,000 events in the lymphocyte live gate were acquired for each sample. Samples were acquired on FACSCANTO II (BD Biosciences, San Diego, CA, USA) and analyzed with FlowJo software (Tree Star Inc, version 8.8.6, Ashland, Ore).

**Genetic Analysis**

Ion Torrent Gene Target Library Preparation and NGS Sequencing were performed according to manufacturer’s instructions (Thermo Fisher Scientific). Next generation Sequencing (Ion Torrent) and Sequencing: genomic DNA isolated from peripheral blood of patients using standard protocols (QIAamp DNA Blood kit by QIAGEN GmbH, Hilden, Germany). PCR reactions were carried out using GoTaq DNA polymerase by standard methods (Promega, Madison, WI). Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI PRISM 3130 and 310 automated sequencers (Applied Biosystems). Sanger sequencing for all mutations and parents’ carrier status were performed.

**Ion Torrent bioinformatics analysis**

Mapping and variants calling were performed using the ion Torrent suite software v3.6. Sequencing reads were aligned against the USC hg19 reference genome using the program distributed within the Torrent mapping Alignment Program (TMAP) map4 algorithm (Thermo Fisher; https://github.com/iontorrent/TS). The aligned reads were processed for variant calling by using the Torrent Suite Variant Caller TVC program; variants found in Variant Calling Format (VCF) file were annotated using ANNOVAR. The called variants with minimum coverage of 20X, standard Mapping Quality, and Base Phred Quality were examined on Integrative Genome Viewer (IGV) and BIOMART. After applying filtering criteria, all nonsense, frameshift, and canonical splice site variants were evaluated to determine their potential pathogenicity.

**Statistical Analysis**

Data were analyzed with Graph-Pad Prism, version 6.2 (Graph Pad Software, la Jolla, CA). p < 0.05 was considered significant.

**RESULTS**

1.1 Clinical, Immunological, and Genetic Phenotypes of RAG-deficient cohort
We report clinical, immunological and molecular characterization within a cohort of 22 RAG patients diagnosed between 2009 and 2020.

The cohort characteristics are described in Table 1. The age at presentation ranged from birth to 6 years (72 months), with a median of 16.2 months whereas the median age of genetic diagnosis was 23.5 months (from birth to 11 years) with a diagnostic delay of 7.3 months.

In particular, the mean age of first symptom was 4.2 months (± 2.5 s.d.) and genetic diagnosis was 7.1 months (± 3.06 s.d) for SCID; 2.6 months (± 1.5 s.d.) and 3.4 months (± 1.8 s.d.) for OS; 13.2 months (± 7.9 s.d.) and 16.8 months (± 9.0 s.d.) for LS/AS; 4.7 years (± 4.2 s.d.) and 6.7 years (± 5.89 s.d.) for CID (figure 1B). Male patients were slightly predominant (54.2%).

The majority of patients (8 cases, 36.4%) were affected by SCID presenting with T-B-NK+ phenotype except for two patients with T+B-NK+ phenotype in which maternal T engraftment was observed, followed by OS (6 cases, 27.2%), LS/AS (4 cases, 18.2%) and CID (4 cases, 18.2%) (Fig. 1a). RAG1 and RAG2 mutations were present in 19 (86.4%) and 3 (13.6 %) patients respectively (Table 2). Clinical manifestations are summarized in Table 1.

Consanguinity was documented in 9 out of 20 families (45%). Noteworthy, there was no significant difference between patients with SCID and OS when comparing age at first clinical symptoms and at diagnosis (Fig. 1b). The median age of the first symptom as well as of the genetic diagnosis, was markedly lower in SCID and OS than in LS/AS and CID patients (p<0.05) (Fig. 1b). The symptom at presentation was infection (n=18; 81.8%) except for OS where dermatitis represents the onset manifestation in all cases. On the other hand, infections and autoimmunity occurred together in the majority of cases (n=14; 63.6%). Three SCID and one OS early diagnosed and prompt transplanted did not presented any severe manifestation (Fig. 1c). Chronic viremia was common among the cohort whereas respiratory and gastrointestinal infections were prevalent in SCID patients (Fig. 1d). Interestingly, 15 out of 22 (68.2%) patients presented autoimmune and/or hyperinflammatory manifestations (Fig .1c). The most frequent was dermatitis occurring in 11 patients (50%). Lymphoproliferation and hepatosplenomegaly were present in 5 patients (22.7%) (Suppl. Fig.1A). Nephropathy occurred in 2 patients whereas one patient developed Miller Fisher syndrome (Fig. 1d, and Table1).

Notably, four patients, three CID and one OS (PID-12, PID-13, PID-20, PID-10) with autoimmune/hyperinflammatory complications experienced a severe autoimmune cytopenia (AIC) (Fig. 2a, b) with a median age at onset of cytopenia of 3.2 years.

All patients received intravenous immunoglobulins (IVIG) and steroids as first-line therapy with limited response. No complete remission was observed in any patients despite second-line therapies with Rituximab (PID12 and PID20) (or Mycophenolate mofetil (MMF) (PID-20)).

Increased IgE levels were observed on the OS and CID groups and IgA and IgM resulted very low or undetectable in SCID and OS patients. AS and CID patients showed normal to hypergammaglobulinemia values. Serum IgG at onset resulted highly variable, for SCID and OS, probably reflecting maternal trans-placental transfer (Fig. 3a).

Total lymphocytes counts were particularly reduced or almost lacking in SCID group as expected, whereas CD4 cell count was higher in OS patients. Expansion of T-cell receptor γδ was documented in two patients (PID-3 and PID-11). Despite circulating T cells were present, the proportion of naïve T cells was reduced in CID patients (data not shown). Interestingly, B lymphocytes were variably detected in AS and CID groups (Fig. 3b). NK cells were present in all groups (Fig. 3b).

RAG1 mutations were detected in nineteen patients, while RAG2 mutations in three of them. Four novel mutations in RAG1 gene and two in RAG2 were reported. All mutations are summarized in Table 2. In total eighteen patients received HSCT (Fig. 3c).
Among fifteen patients with autoimmune/hyperinflammatory manifestations, HSCT was required for definitive management in twelve patients (80%). PID-13 and PID-18 died for multiorgan failure before HSCT, while PID-12 died of infectious complications after transplant (invasive aspergillosis already present at time of HSCT). Patients with immune dysregulation underwent HSCT at an older age compared to those without immune dysregulation (median 12.7 vs. 7.4 years) partially due to the diagnostic delay (Fig. 2d). Two patients resolved treatment-refractory AIC thanks to HSCT. Eighteen patients (81.8%) are alive at the time of this study. Thirteen patients experienced post-HSCT complications including herpetic infections, and low grade (i.e., grade I-II graft-versus-host disease).

1.2 Particular cases

Of note, three patients need further more in depth description because of the peculiarities of clinical phenotype. In details, PID-11 with an homozygous frameshift mutation was characterized by the persistent CMV viremia, _P. aeruginosa_ sepsis and mild BCGitis at the age of seven months. In addition, he developed Miller Fisher’s Syndrome (Fig. 4a, b). Immunological evaluation revealed marked CD4-penia, expansion of γδ+ T-cells and memory B cells with hypergammaglobulinemia and ITP. In order to control the expansion of B autoreactive clones, Rituximab therapy and plasmapheresis were used allowing a complete depletion of B cells and progressive improvement of neurological and respiratory function. Patient received HLA-haploidentical transplant from the father with full engraftment. Four months post-transplantation severe AIHA associated to a CMV reactivation was observed and BAFF plasma level resulted increased (12.000 pg/ml) as previously described in patients with RAG mutations and autoimmunity [29-31]. Antiviral drugs, high-dose IVIg, Rituximab, plasmapheresis and multiple blood transfusions were required to control autoimmunity and achieve a complete remission.

PID-3 carrying the homozygous p.R841W _RAG1_ mutation was characterized by long lasting diarrhea, erythroderma, candidiasis and tubulo-interstitial nephritis with infiltrate of lymphocytes, histiocytes, plasmacells and eosinophils. He showed T+B-NK+ phenotype due to maternal T engraftment. Immunological evaluation revealed CD4-, CD8-penia and γδ+ T-cells expansion.

Lastly, PID-14, previously reported [32], presented a disease onset at six years of age characterized by relapsing nasal polyposis, severe agammaglobulinemia and absence of B cell suggesting initially a humoral defect. His past history was notable for recurrent middle-ear infections, chickenpox and mild persistent EBV viremia. Bone marrow examination showed a marked decrease of B cell progenitors, with an incomplete arrest at pro-B cell stage and few pre-B and mature B cells. No sign of myelodysplasia was found. Later in the follow-up a marked reduction of CD4+/CD45RA+/CD31+ recent thymic emigrants (RTE) were detected over-time, along with a corresponding increase in the proportion of memory T cells. Since T lymphocytes pool deteriorate with age, we reassessed his clinical diagnosis that changed from agammaglobulinemia to CID. This patient, who did not receive HSCT, is currently alive and well on IVIGs at the age of 16 years.

DISCUSSION

We report 22 RAG deficient patients, documenting an increase in the number of RAG diagnosis following the application of NGS from a median of 1.5 up to 2.6 per year. In addition, we observed a median delay of 7.33 months between clinical onset and genetic diagnosis in the mild clinical presentation associated to hypomorphic RAG mutations. The increased frequency observed is consistent with recent reports including newborn screening that confirm _RAG1_ as second most prevalent genes associated with SCID and the most common genes associated with leaky SCID/CID [33]. _RAG1_ mutations have been reported more frequently than _RAG2_ [34] as occurred in our cohort.
Differently from other studies focused on populations with high rate of consanguinity [34,35], many patients enrolled in this work had non-consanguineous parents (59%), suggesting a highly mutation rate in these genes as well as a large occurrence of heterozygous carriers.

Intriguingly, although individuals with RAG heterozygous missense mutations do not seem associated to typical RAG phenotype, we observed three patients presenting as CID and autoimmune cytopenia carrying only a single missense heterozygous mutation in RAG genes (data not shown) suggesting that other factors, not yet fully understood, could influence this genetic background.

Two patients defined as AS and CID (PID-3 and PID-13), carrying the same homozygous mutations (p.R841W), showed distinct phenotypes, with severe autoimmune manifestations: the second one, despite having received a more timely diagnosis, died before being able to undergo HSCT; similarly, two siblings (PID-12 and PID-16) with compound heterozygosity (p.R405G and p.R624H) were diagnosed in different ages. The older sister diagnosed at 3 years of life died of invasive aspergillosis already present at time of HSCT. The younger sister thanks to a prenatal diagnosis underwent to HSCT at 3 months of life showing a different clinical outcome. This underlines the importance of other genetic or environmental factors on disease course and how a prompt HSCT may be crucial.

Furthermore, three compound heterozygous RAG1 deficient patients, classified as SCID (PID-16), CID (PID-12, PID-14) and OS (PID-7), carried the same p.R624H mutation in combination with the p.R405G, p.Y728H and p.R561H respectively. The p.Y728H showed a markedly decreased but detectable recombinase activity in line with his milder phenotype [33]. Nevertheless, other factors such as a chronic and uncontrolled infections or unknown genetic characteristics could influence genotype-phenotype correlation besides the level of recombinase activity. Time to diagnosis is also very different ranging from 2 months (PID7) to 3 and 6 years (PID12 and PID14) revealing a very different behavior under the same common p.R624H mutation. Of note the two sisters PID-12 and PID-16 received two different diagnoses at the birth (CID and SCID respectively).

In addition, chronic, uncontrolled Herpesviridae viremia was frequent among CID group. Usually, CID patients develop more severe manifestations (as autoimmunity) often triggered by viral infections that closely preceded the onset of autoimmunity as in our patients PID-11 [36].

Nonetheless, the presence of residual B cells in CID patients makes them prone to EBV infection with higher risk EBV-driven lymphoproliferation suggesting the need of a closer viral and radiological monitoring [36,37].

Considering the high frequency of hypomorphic mutations and milder phenotype, a longitudinal re-evaluation of patients lacking a molecular diagnosis is recommended, as in our PID-14 who was initially classified as humoral defect and only later reconsidered as CID.

Thus, RAG deficiency should be suspected also in patients where B cell defect is predominant and in those patients without typical sign of SCID or OS as immunodeficiency associated to immune dysregulation.

Our data, in line with other reports [22], showed that severe immune dysregulation manifestations are a common feature of RAG deficiency often refractory to conventional medical management.

In particular, severe and early autoimmune refractory cytopenia found in other cohort and others [22], seems to be very frequent in RAG deficiency also as the first symptom of onset. The early recognition of these patients in patients with early onset or severe autoimmune cytopenia could contribute to the choice of a prompt specific treatment and prevent the onset of others complications [38,39].

Literature reports show that HSCT using an HLA-identical donor demonstrates excellent overall survival greater than 75% [40,41]; moreover, innovative transplant approaches have showed encouraging results in case where a HLA-matched donor is not available [42,43]. Immunosuppressive or immunomodulatory drugs should be strongly considered to control
immunodysregulation in patients with a milder phenotype (CID or AS) and delayed presentation as well as patients affected by CVID or agammaglobulinemia in the absence of HLA-identical donor.

CONCLUSIONS

NGS has greatly accelerated the diagnosis of all RAGs cases preventing the worsening of disease thanks to a better and early treatment. On the other hand, it still presents a challenge in the tracing of effective management and follow-up, considering the inability to predict the disease course in atypical cases. This study describes the immunological, clinical and molecular characteristics of 22 patients with RAG deficiency highlighting the heterogeneity of manifestations associated with this condition in particular of patients with hypomorphic mutations and milder phenotypes. In this regard, extending RAG analysis to a cohort of older pediatric and adult patients affected by undefined CVID and/or immune dysregulation [44], may help to expand the knowledge of natural history of RAG deficiency.

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Code availability: not applicable

Author Contribution

CC, DA, BR, interpreted the results and wrote the manuscript
CC, GMU, SDC, MC, MGD, MD, GDM performed molecular and functional experiments and developed gene sequencing analysis.
CC, GMU, GDM created gene clusters to filter variants and integrated clinical and bioinformatics analysis of data retrieved by genetic platforms
DA, BR, LP, NC, MA, FG, GP, PM, FL, PP, PR, AF, and CaC provided or referred clinical samples and patient’s clinical data.
CC, DA, BR, GDM, CaC designed the research, participate to the study design and data interpretation.
FL, PP, PR, AF, GDM and CaC made substantial contributions to revising the manuscript.
All authors have critically revised and approved the manuscript

Ethics approval

This retrospective study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Ethical Committee of the Children’s Hospital Bambino Gesù in Rome approved this study. Informed consent was obtained from patient’s parents/legal guardians.

Consent to participate: Informed consent was obtained from patient’s parents or legal guardians.

Consent for publication: Informed consent for publication was obtained from patient’s parents or legal guardians.
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Table 1 Clinical features of RAG patients. SCID, Severe Combined Immunodeficiency; OS, Omenn Syndrome; AS, Atypical SCID; LS, Leaky/SCID; CID, Combined immunodeficiency, EBV, Epstein-Barr virus; CMV, Cytomegalovirus; HHV-6; Human herpesvirus 6; RSV, Respiratory syncytial virus; URI, Upper respiratory infections; LRI, Lower respiratory infections; AIHA, Autoimmune hemolytic anemia.

| ID   | AGE AT PRESENTATION | GENDER | ADMITTING CLINICAL DIAGNOSIS | FINAL CLINICAL DIAGNOSIS | GENETIC DIAGNOSIS | CHRONIC VIREMIA | OPPORTUNISTIC/RECURRENT INFECTIONS | IMMUNE DYSREGULATION AUTOIMMUNITY MALIGNANCIES | OTHER | HSCT |
|------|---------------------|--------|------------------------------|--------------------------|-------------------|----------------|---------------------------------|-----------------------------------------------|-------|------|
| PID-1| 5 MONTHS            | M      | AS SCID                      | RAG2                     | ADENOVIRUS        |                  |                                 | WIDESPREAD DERMATITIS, LYMPHOADENOPATY         | YES   |      |
| PID-2| 5 MONTHS            | F      | FEVER, VOMITING              | SCID                     | RAG2              | ADENOVIRUS      | FEVER                          | APHTHOUS GINGIVOSTOMATITIS                     | YES   |      |
| PID-3| 2 YEARS             | M      | AS SCID                      | RAG1                     | ADENOVIRUS,R HINOVIRUS | LONG-LASTING DIARRHOEA, CANDIDIASIS | TUBULOINTERSTITIAL NEPHRITIS WITH INFILTRATE OF LYMPHOCYTES, HISTIOCYTES, PLASMACELLS AND EOSINOPHILS | DERMATITIS | YES |
| PID-4| 2 MONTHS            | M      | FEVER                        | SCID (T-B-NK+)           | RAG1              | CMV ADENOVIRUS  | CMV RETINITIS, RSV, BRONCHIOLITIS, PNEUMONIA |                                                            | YES   |      |
| PID-5| 6 MONTHS            | F      | RESPIRATORY DISTRESS         | SCID (T-B-NK+)           | RAG1              |                  |                                 | YES                                           |       |      |
| PID-6| 4 MONTHS            | M      | DIARRHOEA, FEVER             | SCID                     | RAG1              |                  | LONG-LASTING ROTAVIRUS, ADENOVIRUS, DIARRHOEA, PNEUMONIA | YES                                           |       |      |
| PID  | Age   | Gender | Diagnoses                                                                 | Associated Conditions                                                                 | Genetic Syndrome | Outcome |
|------|-------|--------|---------------------------------------------------------------------------|----------------------------------------------------------------------------------------|------------------|---------|
| PID-7| 2 MONTHS | M      | HYPOGAMMAGLOBULINEMIA                                                    | OS RAG1 CMV                                                                            |                      | YES     |
| PID-8| 3 MONTHS | F      | DERMATITIS AND INFECTIONS                                                | SCID RAG1 OTITIS (PSEUDOMONAS AERUGINOSA), URINARY INFECTION, BRONCHIOLITIS           |                      | YES     |
| PID-9| 2 MONTHS | F      | OS OS RAG1                                                               | PNEUMONIA, STAPHYLOCOCCAL SEPSIS                                                     |                      | YES     |
| PID-10| BIRTH  | M      | OS OS RAG1                                                               | PNEUMOCYSTOSIS, LRI (FLAVOROBACTERIUM MENOGESEPTICUM)                                 |                      | YES     |
| PID-11| 11 MONTHS | M    | MILLER-FISHER (CMV-RELATED)                                              | AS RAG1 CMV P.AERUGINOSA PNEUMONIA, SEPSIS, BCGante                                   | MILLER-FISHER SYNDROME (CMV-RELATED), PLYRADICULONEURITIS WITH CRANIAL NERVE INVOLVEMENT | YES     |
| PID-12| 3 YEARS | F      | LRI CID RAG1                                                             | HHV-6, CMV, EBV URI, LRI AIHA, NEUTROPENIA                                           |                      | YES     |
| PID-13| 1 YEAR  | M      | RESPIRATORY INFECTION, THROMBOCYTOPenia                                  | CID RAG1 CMV, EBV HAEMOPHILUS INFLUENZAE, BOCAVIRUS RESPIRATORY INFECTION, LONG-LASTING ROTAVIRUS DIARRHOEA | THROMBOCYTOPENIA, AIHA | ACUTE RESPIRATORY FAILURE SEVERE HEPATOSPLENOMEGALY WITH LIVER FAILURE, DERMATITIS | NO      |
| PID-14| 6 YEARS | M      | AGAMMGLOBULINEMIA                                                        | CID RAG1 EBV URI, OTITIS, SEVERE CHICKEN POX                                          |                      | NO      |

14
| PID   | Age     | Gender | Diagnosis                          | Subtype | Cause                  | Infections                                      | Complications                  |
|-------|---------|--------|------------------------------------|---------|------------------------|-------------------------------------------------|-------------------------------|
| PID-15| 1.8 years | F      | URI, OTITIS, VOMITING, DIARRHOEA   | SCID    | RAG1                   | CMV, ADENOVIRUS RESPIRATORY INFECTION, OTITIS, LONG-LASTING DIARRHOEA | CHRONIC HEPATOPATHY YES |
| PID-16| Birth   | F      | PRENATAL DIAGNOSIS                | SCID (T-B-NK+) | RAG1                  | LRI, STAPHYLOCCUS SEPSIS, ENTEROBACTER CLOACAE URINARY TRACT INFECTIONS | ALOPECIA YES |
| PID-17| 5 months | M      | DERMATITIS                        | OS (T-B-NK+) | RAG2                  | PURULENT OTITIS, NECROTIZING FASCIITIS, CEREBRAL ABSCESS, SEPSIS | ALOPECIA EXUDATIVE DERMATITIS, EPATOSPLENOMEGALY, STAPHYLOCCUS SKIN INFECTION YES |
| PID-18| 3 months | M      | PURULENT OTITIS                   | OS      | RAG1                  | PURULENT OTITIS, NECROTIZING FASCIITIS, CEREBRAL ABSCESS, SEPSIS | DERMATITIS NO |
| PID-19| 7 months | F      | SCID                              |     | RAG1                  |                                                | YES |
| PID-20| 3 years  | F      | CID                               | RAG1         | HHV6                  | PNEUMONIA; LRI VITILIGO, AI THYROIDITIS AIHA | ECZEMA, EPATOSPLENOMEGALY NO |
| PID-21| 5 days   | F      | OS                                | RAG1         | HHV6                  |                                                | SEVERE DERMATITIS YES |
| PID-22| 5 months | M      | DIARRHOEA, BRONCHITIS             | SCID (with maternal engraftment) | RAG1                  | NOROVIRUS LONG-LASTING DIARRHOEA, PNEUMONIA, INFECTIOUS GASTROENTERITIS, HHV6 DNAemia | ECZEMA, EPATOSPLENOMEGALY, PAPULAR RASH YES |
Table 2: Molecular Characterization of RAG patients. SCID, Severe Combined Immunodeficiency; OS, Omenn Syndrome; AS, Atypical SCID; LS, Leaky/SCID; CID, Combined immunodeficiency; dbSNP, Single Nucleotide Polymorphism Database; In red novel not described mutations. * values from reference 45.

| ID   | Disease | Gene | Mutation                                                                 | dbSNP and references       | Zygosity       | Inheritance | OMIM         | Protein Domain                                                                 | Recombinase activity allele a (approximately) | Recombinase activity allele b (approximately) |
|------|---------|------|--------------------------------------------------------------------------|----------------------------|----------------|-------------|--------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| PID-1| AS SCID | RAG2 | a) c.685C>T; p.R229W                                                      | rs765298019                | Homozygous     | Unknown     | OMIM *179616 | a) core region                                  | 10.5 ± 0.5*                                   |                                               |
| PID-2| SCID    | RAG2 | a) c.1A>G; p.M1V,b) c.1403_1406del AFCT                                  | Cifaldi C. et al.; rs786205616 | Compound heterozygous | Familial | OMIM *179616 | a) core region                                  | n.d.                                         | 0% deduced                                   |
| PID-3| AS SCID | RAG1 | a) c.2521C>T; p.R841W                                                    | rs104894287                | Homozygous     | Familial    | OMIM *179615 | a) Zn binding domain (catalytic core)           | 10.0 ± 0.5*                                  |                                               |
| PID-4| SCID    | RAG1 | a1) c.1681C>T; p.R561C,a2) c.1815G>C; p.M605I                             | rs104894285; Dobbs et al.   | Compound heterozygous | Familial | OMIM *179615 | a) pre RNAseH (catalytic core); b) catalytic RNAseH (catalytic core) | n.d.; n.d.                                   |                                               |
| PID-5| SCID    | RAG1 | a) c.1221G>A; p.R410Q                                                    | rs199474684; Cifaldi et al. | Compound heterozygous | Familial    | OMIM *179615 | a) NBD (catalytic core)                         | 0%                                            | 0% deduced                                   |
| PID-6| SCID    | RAG1 | a. c.595delIC; p.329fs                                                  | n.d.                       | Homozygous     | n.d.        | OMIM *179615 |                                               | 0% deduced                                   |                                               |
| PID-7| OS      | RAG1 | a) c.1682G>A; p.R561H                                                    | rs104894284; rs199474680   | Compound heterozygous | Familial | OMIM *179615 | a) pre RNAseH (catalytic core); b) catalytic RNAseH (catalytic core) | 2.0 ± 0.6*                                   | 0.0 ± 0.4*                                   |
| PID-8| SCID    | RAG1 | a) c.1228C>T; p.R410W                                                    | rs199474684; Cifaldi et al. | Compound heterozygous | Familial    | OMIM *179615 | a) NBD (catalytic core)                         | 0.0 ± 0.0*                                   | n.d.                                         |
| PID-9| OS      | RAG1 | a) c.351delT,b) c.1577T>G; p.L526A                                       | Cifaldi et al.              | Compound heterozygous | n.d.        | OMIM *179615 | a) pre RNAseH (catalytic core)                  | 0% deduced                                   | n.d.                                         |
| PID-10| OS      | RAG1 | a) c.1870C>T; p.R624C                                                    | rs199474688; rs104894287   | Compound heterozygous | Familial    | OMIM *179615 | a) catalytic RNAseH (catalytic core)            | n.d.                                         | 10.0 ± 0.5*                                  |
| PID-11| AS      | RAG1 | a) c.256,257del; p.K80VfsTer33                                          | rs772962160                | Homozygous     | Familial    | OMIM *179615 |                                               | 2.7 ± 0.3*                                   |                                               |
| PID  | CID   | RAG1          | Allele Details | Functional Details | OMIM | Recombinase activity |
|------|-------|---------------|----------------|-------------------|------|----------------------|
| PID-12 | CID   | RAG1          | a) c.1870C>T; p.R624H | rs199474680; Cifaldi et al. | Compound heterozygous | OMIM *179615 | a) catalytic RNAaseH (catalytic core) b) NBD (catalytic core) 0.0 ± 0.4* |
| PID-13 | CID   | RAG1          | a) c.2521C>T; p.R841W | rs104894287 | Homozygous | OMIM *179615 | a) Zn binding domain (catalytic core) 10.0 ± 0.5* |
| PID-14 | CID   | RAG1          | a) c.1871G>A; p.R405G | rs199474680; Cifaldi et al. | Compound heterozygous | OMIM *179615 | a) catalytic RNAaseH (catalytic core) b) Zn binding domain (catalytic core) 0.0 ± 0.4* 25.7% (REF) |
| PID-15 | SCID  | RAG1          | a) c.1767C>G; p.Y589X | rs991089005 | Homozygous | OMIM *179615 | 0% deduced |
| PID-16 | SCID  | RAG1          | a) c.1870C>T; p.R624H | rs199474680; Cifaldi et al. | Compound heterozygous | OMIM *179615 | a) catalytic RNAaseH (catalytic core) b) NBD (catalytic core) 0.0 ± 0.4* n.d. |
| PID-17 | OS    | RAG2          | a) c.281A>G; p.H94R | rs104894287 | Homozygous | OMIM *179616 | a) N-term nd n.d. |
| PID-18 | OS    | RAG1          | a) c.519delT; p.E174Sfs*26 | rs1241698978 | Homozygous | OMIM *179615 | 0.5 ± 0.2* |
| PID-19 | SCID  | RAG1          | a)c.1361 T>A; p.L454Q | rs199474677 | Homozygous | OMIM *179615 | a) NBD (catalytic core) 5.4 ± 0.7* |
| PID-20 | CID   | RAG1          | a)c.2095C>T; p.R699W | rs1241698978 | Homozygous | OMIM *179615 | 19.3 ± 1.8* n.d. |
| PID-21 | OS    | RAG1          | a) c.1870C>T; p.R624H | rs199474688; rs104894287 | Compound heterozygous | OMIM *179615 | a) catalytic RNAaseH (catalytic core) b) Zn binding domain (catalytic core) n.d. 10.0 ± 0.5* |
| PID-22 | SCID  | RAG1          | a) c.1210 C>T; p.R404W | rs199474688 | Homozygous | OMIM *179615 | a) NBD (catalytic core) n.d. |

* values from [45] Predicting the Occurrence of Variants in RAG1 and RAG2. Dylan Lawless, Journal of Clinical Immunology (2019) 39:688–701. Bold: novel not described mutations. SCID = Severe Combined Immunodeficiency; OS = Omenn syndrome; AS= Atypical SCID CID-G/A= combined immunodeficiency with granuloma/autoimmunity; CVID= Common Variable Immunodeficiency;
**FIGURES LEGEND**

**Fig. 1** Panel a RAG cohort. Clinical diagnosis of RAG patients for the four main categories (n=22). Panel b Difference in timing for first symptom of immunodeficiency and genetic diagnosis of four RAG groups. Symbols represent individual patients and red bars representing median. Panel c Infections and autoimmunity in RAG cohort (number of total cases). Panel d Infections and autoimmune manifestations in RAG cohort distributed among the four groups (frequency as % total cases).

**Fig. 2** Panel a Occurrence of autoimmune cytopenia (AIC) in RAG cohort (n=4/22). Panel b Severity of autoimmune cytopenias by cell nadir during disease flare (symbols representing individual patients, mean with SD. Dashed line normal range from Bambino Gesù Children’s Hospital. Autoimmune manifestation (AI); Autoimmune cytopenia (AIC); Absolute neutrophil count (ANC); Immune thrombocytopenia (ITP); autoimmune hemolytic anemia (AIHA); platelet (PLT).

**Fig. 2 Panel a** Immunoglobulin titers of each group. Panel b Immunophenotype of RAG cohort. CD3+, CD4+, CD8+ T cell, CD19+ B cells and NK cells count among groups Panel c Occurrence of HSCT in our RAG cohort. For each category, the total number of patients is indicated. Panel d Occurrence of HSCT in patients with (AI+ n=12) or without (AI- n=6) autoimmunity (frequency as % annotated total cases, n=18) and age of HSCT.

**Fig. 4** Panel a Abdominal CT scan and ultrasound showed hepatosplenomegaly in two CID patients. Panel b Magnetic resonance images showed marked thickening, impregnation of the roots of the cauda (left) and cranial nerves (right) reflecting a clinical picture of Miller Fisher’s Syndrome characterized by severe axial hypotonus, ophthalmoparesis, ataxia, generalized areflexia, progressive paralysis of cranial nerves.
Figures

Figure 1

Panel a RAG cohort. Clinical diagnosis of RAG patients for the four main categories (n=22). Panel b Difference in timing for first symptom of immunodeficiency and genetic diagnosis of four RAG groups. Symbols represent individual patients and red bars representing median. Panel c Infections and autoimmunity in RAG cohort (number of total cases). Panel d Infections and autoimmune manifestations in RAG cohort distributed among the four groups (frequency as % total cases).
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