Idiopathic CD4⁺ T lymphopenia without autoimmunity or granulomatous disease in the slipstream of RAG mutations

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Abstract:

A girl presented during childhood with a single course of extensive chickenpox and moderate albeit recurrent pneumonia in the presence of idiopathic CD4+ T lymphocytopenia (ICL). Her clinical condition remained stable over the last 10 years without infections, any granulomatous disease or autoimmunity. Immunophenotyping demonstrated strongly reduced naïve T and B cells with intact proliferative capacity. Antibody reactivity upon in vivo immunizations was normal. T-cell-receptor-Vβ repertoire was polyclonal with a very low content of T-cell-Receptor-Excision Circles (TRECs). Kappa-deleting-Recombination-Excision Circles (KRECs) were also abnormal in the B cells. Both reflect extensive in vivo proliferation. Patient-derived CD34+ hematopoietic stem cells could not repopulate RAG2−/− IL2Rγc−/− mice, indicating the lymphoid origin of the defect. We identified two novel missense mutations in RAG1 (p.Arg474Cys and p.Leu506Phe) resulting in reduced RAG activity. This report gives the first genetic clue for ICL and extends the clinical spectrum of RAG mutations from severe immune defects to an almost normal condition.
Introduction

Selective depletion of T lymphocytes is common in both primary and secondary immunodeficiencies. Idiopathic CD4⁺T lymphocytopenia (ICL) is defined by an unexplained persistent CD4⁺T lymphocyte count of <300 cells/μL or <20% of the total T-cell count[1].

Since the discovery of human retroviruses, sporadic ICL patients were recognized with a CD4⁺ lymphocytopenia not infected by HIV or HTLV-1[2-7]. Smith et al[2,3] reviewed 230179 cases from the CDC AIDS Reporting System and described 47 ICL patients. Of these cases, only 3 (6%) were asymptomatic. Screening of healthy blood donors confirmed a low prevalence of ICL of 0.2-0.6%[4,5].

The disease may have a transient nature over the years but mostly persists[3]. The immunological parameters of ICL consist of a prolonged decrease in CD4⁺T cell numbers, sometimes with a concomitant decrease in CD8⁺T cells and B cells as well. Immunoglobulin levels are normal[2-5], which helps to distinguish ICL from Common Variable Immunodeficiency (CVID)[1,6].

Although function declines with age, thymic output is well maintained into late adulthood[7-9]. Thymic size correlates with numbers of CD4⁺CD45RA⁺ naïve T cells. At very young age the T-lymphocytopenia is often caused by congenital defects resulting in severe combined immunodeficiency syndromes (SCID)[10].

Null mutations in RAG1 or RAG2 account for 70% of SCID cases with the classic T⁻B⁻SCID phenotype[10]. Hypomorphic mutations with residual RAG activity occur in typical Omenn syndrome[10], or rare cases with lymphocytopenia, hypogammaglobulinemia, granulomas in the skin, mucosa, internal organs and viral complications, including EBV-related lymphomas[11-12]. In the present paper, we describe hypomorphic RAG1 mutations that corresponds with mild CD4⁺T lymphocytopenia, normal in vitro lymphocyte function and in vivo vaccination responses.
Material and Methods:

Subjects and blood samples: Heparinized venous blood was collected from healthy (age-matched) donors, patient and family members. The study was approved by the institutional medical ethical committee and informed consent for the research purpose described was obtained from the parents of the child and age-matched controls in accordance with standards of the 1964 declaration of Helsinki.

Lymphocyte phenotyping: Absolute numbers of T cells, B cells and NK cells were determined with Multitest six-color (FACSCantoII; BD-Biosciences, Erembodegem, Belgium). For T- and B-cell subset-analysis directly conjugated monoclonal antibodies (MoAbs) were obtained from BD except for CD45RA-RD1 (Beckman-Coulter, Mijdrecht, The Netherlands) and CD27-FITC (Sanquin, Amsterdam, The Netherlands)[13,14].

Lymphocyte activation, determination of T-cell receptor CDR3 spectratype and TRECs: Lymphocyte proliferation was performed as described[13]. CD45RA+naive T cells were purified (>95%) to determine CDR3 patterns in CD4+ and CD8+T cells or to isolate DNA for Sj-TRECs analysis[14,15].

KREC assay and Vk3-20-specific restriction enzyme hot-spot mutation assay (IgκREHMA): Four B-cell subsets were purified from PBMCs using FACS-DiVa-cell-sorter[16,17]. Somatic hypermutation in these subsets was assessed by the Vk3-20-specific Igκ-REHMA assay[16].

RAG gene analysis and in vitro V(D)J recombination assay: The RAG1 and RAG2 genes were amplified by PCR and sequenced[18]. The level of recombination activity of the RAG1 mutant proteins were compared to the wt RAG1.
Case: The patient was the first child of non-consanguineous Dutch healthy parents, born in May 1992. At the age of 5, she contracted chickenpox with numerous large hemorrhagic skin lesions and VZV-associated pneumonia. Skin lesions healed slowly with cicatriciation. Until the age of about 8 years she has suffered from recurrent episodes of fever and (viral) pneumonia for which she regularly received antibiotics. High-resolution CT scanning of neck, thorax and abdomen around that age showed mild bronchiectasis without enlargement of perihilar lymph nodes or granulomatous lesions in lungs, liver or spleen; a thymus was not detectable (Suppl.Fig.1). Dysmorphic features were not observed by clinical geneticists. Chromosomal abnormalities (including 22q11 hemizygosity) were excluded. She remained disease-free for the last 10 years using oral co-trimoxazole as prophylaxis.
Results and Discussion:

Lymphocyte subpopulations and humoral immune responses

Immunophenotyping of the patient’s PBMC showed low numbers of CD4^T cells and to a lesser extent CD8^T cells. The CD4^T lymphocytopenia was persistent over time with some fluctuations (Fig.1A). The percentage of CD4^ and CD8^T cells with a CD45RO^ memory phenotype was strongly increased as compared with the naïve T cells (Fig.1B).

Although B-cell numbers were normal, the distribution of the B-cell subsets was severely disturbed (Fig.1B). The frequency of class-switched CD27^memory B cells was low (3.4-9.2%) whereas the frequency of non-switched sIgD^CD27^memory B cells showed a high and stable percentage between 58%-72% (controls between 5-15 years: 18.4±7.2%, n=40). Humoral immunity was intact (Suppl.Table1).

In vitro functionality of T cells, thymic function and TCR repertoire

T-cell proliferation to general stimuli (i.e. CD3/CD28, cytokines), and specific antigens was intact (Fig.1C; data not shown). Cytokine release, CTL or spontaneous NK-cell killing of target cells were normal (data not shown).

In the absence of a detectable thymus, peripheral in-vivo T-cell proliferation was expected to be increased to sustain normal T-cell numbers. This was indeed demonstrated in two ways. First, lymphocyte expression of the nuclear proliferation marker Ki67 was not different from age-matched control samples (Fig.1D; data not shown). Secondly, the content of TREC$s was reduced compared to controls (Fig.1D), indicative for increased T-cell proliferation. However, despite extensive peripheral T-cell expansion to sustain the number of T cells, no restriction in the TCR repertoire was demonstrated (Fig.1D).
**Cellular and molecular properties of the circulating B cell compartment**

B-cell proliferation was found to be normal upon BcR-dependent and BcR-independent stimulation (Suppl.Fig.2A). The number of cell divisions of the transitional, naïve-mature and non-switched B cells was strongly increased as compared to healthy controls (Suppl.Fig.2B)[16,17]. Naïve B cells were not somatically mutated and the mutation frequency in the expanded fraction of non-switched natural effector B cells was only 4.7% (healthy controls 15%; n=5)(Suppl.Fig.2C). These results indicate that the number of total peripheral B cells is normal because of extensive (antigen-independent) proliferation of the transitional, naïve and non-switched B-cells.

**Lymphoid origin of the defect and genetic immunodeficiency screening**

T-cell maturation data suggested an intrinsic T-cell developmental defect with thymus hypoplasia from early age onward. Using a humanized RAG2−/−IL2Rγc−/− mouse model[19], normal human T cell reconstitution upon intrahepatic injection of 4x10⁵ patient-derived CD34⁺CD38⁻HSCs from the bone marrow of the patient failed, in contrast to control HSCs. In the blood, no patient T cells could be detected at 6 and 9 week, nor in the thymus at 10 weeks (Suppl.Table2), confirming the lymphoid background of the thymus hypoplasia.

**RAG defect**

Sequence analysis demonstrated two novel heterozygous missense mutations in the RAG1 gene (c.1420C>T and c.1516C>T), affecting the evolutionary conserved amino acids Arg474 and Leu506 (Fig.2A). The RAG1 p.Arg474Cys recombinant protein had 25% recombinase activity compared to wild-type RAG1, whereas the p.Leu506Phe mutant did not have any residual activity (Figs.2B-2C).
Hypomorphic mutations may result in typical Omenn patients with T cells showing an oligoclonal TCR repertoire, and poor development of precursor-B cells[18,20], which may contribute to hyperinflammation/autoimmunity because of low-affinity autoantibody production[21,22]. These features were absent in our patient.

Epicrise

V(D)J recombination activity of the RAG1 mutant proteins was very low. Even this residual RAG1 activity provides a low level of T- and B-cell production for effective host immunity. The functional immune system in terms of the mild clinical course in the absence of a detectable thymus could be explained by cytokine-driven proliferation, as reflected by the maintenance of a polyclonal repertoire[23]. The persistent lymphocyte activation could be compatible with cytokine-driven homeostasis (Fig.2A; Suppl.Table3).

The thymus hypoplasia associated with an immune defect similar to ICL and few clinical symptoms was identified to be caused by novel heterozygous RAG1 mutations with very low RAG activity. Instead of SCID, Omenn syndrome or extensive granulomatous disease, our case study adds to the complexity of hypomorphic mutations in the RAG genes.
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Author’s contribution:

Taco W. Kuijpers: diagnosis and treatment, design of research, writing the paper

Hanna IJspeert: performed research, cloning and expression of RAG variants, analysis of data

Ester M.M. van Leeuwen: design of research, data analysis

Machiel H. Jansen: performed research, data analysis

Mette D. Hazenberg: performed research, data analysis

Kees C. Weijer: performed vital mouse studies

Rene A.W. van Lier: design of research, writing the manuscript

Mirjam van der Burg: design of study and writing the manuscript

The authors have no conflict of interest to disclose.
References:


Legends

Fig. 1. Absolute numbers of CD4+ and CD8+ T cells, CD19+ B cells and CD3−CD16/56+ NK cells over time. Numbers of the patient’s lymphocytes are indicated in closed circles; the age-matched control levels for different age categories (i.e. 4-10 years; 10-18 years) are indicated in a range of ± 2SD (dotted lines) (A). Immunophenotyping of CD4+ and CD8+ T cells and CD19+CD20+ B cells, according to CD45RA/CD27 and sIgD/CD27, respectively, as compared to a healthy, age-matched control (B). Proliferative capacity of the patient’s T cells. T cell proliferation of the patient and a healthy control is shown by CFSE dilution after 6 days of culture. Polyclonal proliferation was induced by a combination of CD3/CD28 or IL-15, whereas antigen-specific proliferation was assessed by stimulation with tetanus toxoid (Tet Tox), cytomegalovirus (CMV) or varicella zoster virus (VZV). As expected in the presence of negative serology, CMV antigen did not activate her T cells (C). Increased proliferation of the peripheral CD4+ T cell compartment as demonstrated by nuclear Ki67 staining in naive (CD45RA+CD27+) CD4+ and CD8+ T cells, compared with healthy age-matched naive control T cells, as described [13]. TREC s in patient’s T cells for the early, so-called signal joint (Sj) over a period of 5 years. For T-cell repertoire analysis CDR3 spectratyping in the patient’s T cells was analyzed, being representative for 2 separate experiments in triplicate, more than two years apart (D).
Fig. 2. *RAG1* gene mutations and residual recombination activity. Sequence alignment of RAG1 protein of different species. Both the arginine (R) at amino acid position 474 and the leucine (L) at 506 are conserved residues. The parents were heterozygous for each of the mutation (A). Transfection of pDVG93, RAG1 and RAG2 in 3T3 fibroblasts results in an inversion rearrangement of pDVG93, which can be detected by the primers DG89 and DG147 (B). Only the R474C mutation results in residual recombination activity, as assessed by the *in vitro* recombination assay using pDVG93 (C).
Figure 1A

CD4+ T cells

CD8+ T cells

B cells

NK cells
Figure 1B

Patient

Control

CD4+ T cells

CD8+ T cells

B cells

CD27

CD45RA

IgD
Figure 2

A

<table>
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<tr>
<th>Species</th>
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