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Research Article

Aging

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A novel immunodeficiency associated with hypomorphic RAG1 mutations and CMV infection

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Amorphic mutations in the recombination activating genes *RAG1* and *RAG2* have been reported to cause T-B-SCID, whereas hypomorphic mutations led to the expansion of a few autoimmune T cell clones responsible for the Omenn syndrome phenotype. We report here a novel clinical and immunological phenotype associated with recessive *RAG1* hypomorphic mutations in 4 patients from 4 different families. The immunological phenotype consists of the oligoclonal expansion of TCR $\gamma\delta$ T cells combined with TCR $\alpha\beta$ T cell lymphopenia. The clinical phenotype consists of severe, disseminated CMV infection and autoimmune blood cell manifestations. Repertoire studies suggest that CMV infection, in the setting of this particular T cell immunodeficiency, may have driven the TCR $\gamma\delta$ T cell clonal expansion. This observation extends the range of clinical and immunological phenotypes associated with RAG mutations, emphasizing the role of the genetic background and microbial environment in determining disease phenotype.

Introduction

SCID defines a group of genetic disorders characterized by profound defects in T cell differentiation (1). Ten SCID conditions have been identified, based on immunological, enzymatic, and molecular genetic analysis. Adenosine deaminase deficiency results in a profound impairment of T, B, and NK lymphocyte development (2). Defects in IL-dependent T and NK cell differentiation caused by mutations in the γc gene (γc deficiency) or the γc -associated Jak-3 kinase gene (*JAK3* deficiency) result in the T-B⁺NK⁻ SCID phenotype (3, 4). Deficiency of the α chain of the IL-7 receptor (5) of CD45 (6, 7) and of either of the CD3 chains (CD3 δ and CD3 ϵ) accounts for the T-B⁺NK⁺ type of SCID (8, 9) phenotype. Finally, defective V(D)J recombination of the T and B cell receptors is responsible for the T-B⁺NK⁺ form of SCID (10–12). Defects in *recombination activating gene 1* (*RAG1*) and *RAG2* or in the *Artemis* gene can disrupt V(D)J recombination, thereby blocking the differentiation of T and B cells. However, hypomorphic mutation of the *RAG1/2* or *Artemis* genes may generate a different phenotype, characterized by residual T $\pm$ B cell differentiation. Various *RAG1* and *RAG2* mutations have been found to be associated with a spectrum of immunodeficiencies extending from T-B⁺NK⁺ SCID to Omenn syndrome (OS) (11, 13). OS is characterized by the massive expansion of oligoclonal T lymphocytes (11, 13–15).

TCR $\gamma\delta$ T cells account for 1–10% of peripheral T cells in the blood, while they are more prominent in mucosal sites (16). They display a restricted repertoire, using a limited set of V γ and V δ gene elements (16). There is now considerable evidence to suggest that TCR $\gamma\delta$ directly recognizes various microbiological products, such as nonpeptide phenyl pyrophosphates from *Mycobacterium tuberculosis* (17) and other microorganisms (18–21). Experimental data from mouse models strongly suggest that TCR $\gamma\delta$ T cells play a critical role in host defenses against a variety of infectious agents, including herpes simplex virus type 1 (22). In humans, the expansion of $\gamma\delta$ T cell subsets has been documented in *Mycobacterium* infection (17) and in immunocompromised transplanted patients infected with the CMV (23–25).

In this study, we analyzed a novel inherited immunodeficiency caused by hypomorphic *RAG1* mutations. This phenotype consists of TCR $\alpha\beta$ T cell lymphopenia combined with expansion of the TCR $\gamma\delta$ T cell population, severe CMV infection, and autoimmunity. We suggest that CMV infection triggers the expansion of TCR $\gamma\delta$ T cell clones in conditions of restricted T cell clonal expansion.

Results

Immunological characteristics. Four unrelated patients, each born to first-cousin parents, presented with severe, persistent CMV infection in the first year of life in the absence of evidence for prenatal CMV infection. In 3 cases, CMV infection was associated with autoimmune cytopenias (anemia in patient 2 [P2], P3, and P4 and neutropenia in P2). Autoimmunity was further demonstrated by the detection of autoantibodies against red blood cells in 3 patients (P2, P3, and P4) and anti-polymorphonuclear autoantibodies in P2. Anti-nuclear autoantibodies were also detected in P2 and P3.

Nonstandard abbreviations used: BMT, bone marrow transplantation; OS, Omenn syndrome; P2, patient 2; PHA, phytohemagglutinin; *RAG1*, *recombination activating gene 1*; TCRVB, T cell receptor variable β chain.

Conflict of interest: The authors have declared that no conflict of interest exists.

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**Table 1**

Patients' immunological characteristics: blood lymphocyte subsets and proliferation studies

	P1	P2	P3	P4	Age-matched control values
Age at investigation (mo)	3	12–19	13	19	
Lymphocytes/ μ l	1,100–1,400	3,000–5,000	4,000–5,000	3,500	2,900–5,400
Eosinophils/ μ l	200–800	60–100	0–100	NE	50–100
Lymphocyte subsets (%)					
CD3 ⁺	30	47–78	68–81	55	62–69
CD4 ⁺	5	5–21	6–9	4	30–40
CD8 ⁺	5	6–16	4–10	4	25–32
CD19 ⁺	0	3–51	4	12	21–28
CD56 ⁺ CD3 ⁺	34	13	13	38	4–15
HLA class II ⁺ /CD3 ⁺	ND	77	94	62	6–16
TCR$\alpha\beta$⁺ (%)					
TCR $\alpha\beta$ ⁺ /CD3 ⁺	20–23	20–33	14–19	8	86–97
TCR $\alpha\beta$ ⁺ /CD8 ⁺	ND	8	5	0.5	85–100
CD45RO ⁺ /CD4 ⁺	ND	1	1	1	66–88
CD45RA ⁺ CD31 ⁺ /CD4 ⁺	ND	ND	ND	1	60–80
TCR$\gamma\delta$⁺ (%)					
TCR $\gamma\delta$ ⁺ /CD3 ⁺	77–80	64–80	80–87	82	2–13
TCR $\gamma\delta$ ⁺ /CD8 ⁺	ND	82	86	72	0–15
CD45RO ⁺ /TCR $\gamma\delta$ ⁺	93	ND	78	ND	
CD28 ⁺ /TCR $\gamma\delta$ ⁺	2	ND	4	ND	
CD27 ⁺ /TCR $\gamma\delta$ ⁺	ND	ND	5	ND	
Proliferation ($\Delta \times 10^{-3}$ cpm)					
PHA	ND	151	44	ND	>50
Tetanus toxoid	ND	95 ^A	38 ^A	ND	>15
CMV	ND	12 ^A	4 ^A	ND	>15

NE, not evaluable; ND, not done. ^AAntigen-induced proliferation assays were positive in 1 test out of 4 in P2 and in 1 test out of 3 in P3.

Lymphocyte counts were normal in P2, P3, and P4 and low in P1 (Table 1). All lymphocyte subsets (T, B, NK) were detectable, and the counts for these subsets were close to normal values (except for P1). T cells were found to be of autologous origin, as shown by microsatellite analysis or by X/Y chromosome FISH. All patients displayed a marked decrease in T cells expressing the $\alpha\beta$ form of the TCR (Table 1), with counts of these cells well below 1,000/ μ l. This TCR $\alpha\beta$ ⁺ T cell deficiency persisted over several months (data not shown). The control of CMV infection by antiviral therapy had no effect on TCR $\alpha\beta$ T cell counts (data not shown). Both CD4⁺ and CD8⁺ TCR $\alpha\beta$ T cells were detected with a memory/activated phenotype (CD45RO/HLA class II⁺). The distribution of the TCR variable β chain (TCRVB) was assessed by immunofluorescence using specific mAb against CD4⁺ T lymphocytes or by PCR analysis. All members of the TCRVB family tested were present, but some were in excess (such as VB14 in P2 and P4; VB20 in P2; and VB3 in P3) (Figure 1). We were unable to evaluate the distribution of TCRVB on CD8⁺ T lymphocytes because most CD8 T cell subsets expressed the $\gamma\delta$ TCR (see below). A second striking feature of these 4 patients was the overrepresentation of TCR $\gamma\delta$ ⁺ T cells (Table 1). We showed, with antibodies specific for the V γ and V δ families and by PCR analysis, that these families were present in each patient but that their distribution differed between patients (Figure 2). We followed the distribution of these families over time in P2 and found it to be stable throughout a 4-month follow-up

period. $\gamma\delta$ T cells from P1 were mostly V δ 2 and V δ 3; $\gamma\delta$ T cells from P2 were mostly V δ 1 and V δ 5; $\gamma\delta$ T cells from P3 were mostly V δ 1; and $\gamma\delta$ T cells from P4 were mostly V δ 2. There was no obvious dominance of V γ families (Figure 2). Of note, the physiological overrepresentation of V γ 9 among blood $\gamma\delta$ T cells was not found in these 4 patients. Analysis of the distribution of CDR3 length within the various V γ and V δ T cells from P2, P3, and P4 showed fewer peaks than observed in control $\gamma\delta$ T cells (Figure 2). Sequencing of CDR3 from peaks showed limited diversity, as shown in Table 2 for V δ 3 and V γ 3 T cells clone for P4. This observation held true for all V δ and V γ families, except for V γ 9, which did not display a Gaussian distribution in controls (Figure 2). In both patients tested (P1 and P3), TCR $\gamma\delta$ ⁺ T cells were shown to be CD45RO⁺, CD45RA⁺, CD27⁺, CD28⁺, a profile characteristic of late differentiated memory T cells (26) (Table 1). The general expansion of an oligoclonal TCR $\gamma\delta$ ⁺ T cell population with a TCRV δ and -V γ usage different from that of the controls was therefore a feature common to these 4 patients. It was tested whether fresh or nonspecifically expanded $\gamma\delta$ T cells from P1 and P3 could be activated by CMV-infected cells (25) either to proliferate, produce TNF- α , or exert a cytolytic activity. Results were negative (data not shown). $\gamma\delta$ T cells could, however, proliferate in the presence of phytohemagglutinin (PHA) plus IL-2 (25).

In vitro, PHA-induced and antigen-induced proliferations in response to tetanus toxoid (P2, P3) and CMV antigen (P2), respectively, were detected. Following exposure to antigen, only TCR $\alpha\beta$ CD4⁺ cells were found to proliferate (data not shown). The T cells of the patients therefore displayed no gross signaling defect. Serum Igs were detected in 3 cases (P2, P3, and P4). In contrast, P1 had profound panhypogammaglobulinemia. Serum IgE levels, tested in 3 cases, were low. Remarkably, when P2–P4 were tested, antibodies against CMV of both the IgM (in P2 and P4) and IgG (in P2 and P3) isotypes were detected. However, serum concentrations of antibodies against tetanus toxoid and poliovirus were very low in P2 following immunization (Table 3), indicating a functional B cell immunodeficiency.

Detection of homozygous RAG1 mutations. The occurrence of severe opportunistic infections associated with profoundly abnormal T cell subset distributions in these patients born to consanguineous parents was suggestive of an autosomal recessive inherited form of severe T cell immunodeficiency. The NK[−] SCID phenotype was excluded by the presence of NK cells, and enzyme assays excluded adenosine deaminase and purine nucleoside phosphorylase deficiencies. The normal membrane expression of both the TCR/CD3 complex and CD45 ruled out the possibility of a deficiency of these proteins. A T/B cell combined immunodeficiency caused by a defective V(D)J TCR/BCR gene rearrangement was therefore considered, particularly as TCR heterogeneity was restricted in these patients, a characteristic of OS patients with leaky RAG1/2 mutations. Taking advantage of 2 informative consanguineous families (P2 and P3), a

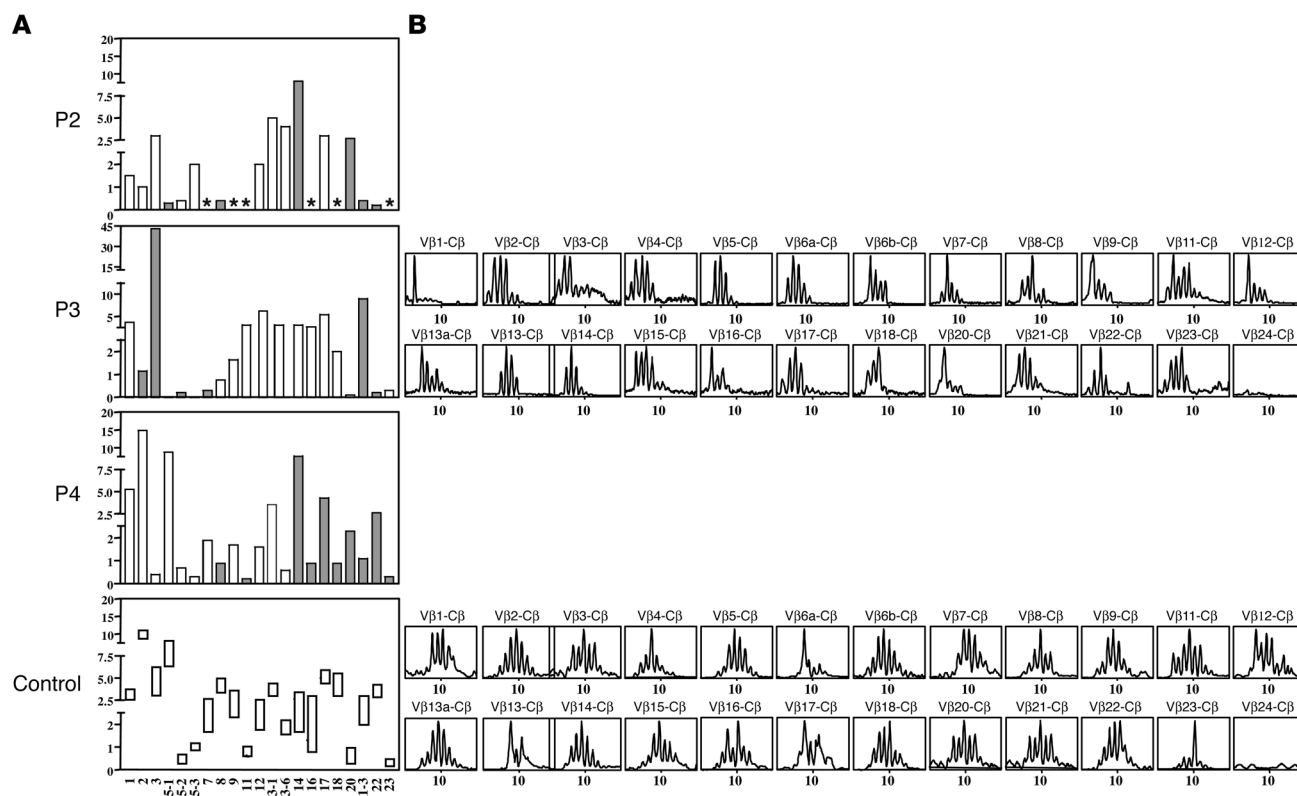


Figure 1

TCRVB usage by patients' T lymphocytes. **(A)** VB repertoire was determined either using available specific mAb in immunofluorescence assay in P2 and P4 or using real-time PCR analysis assay in P3. In this latter patient, similar results were obtained by PCR analysis and using available specific mAb in immunofluorescence assay (data not shown). The x axis indicates VB families; the y axis indicates relative frequency of usage (%) within the TCR $\alpha\beta$ T cells. Controls values shown in rectangles represent mean $\pm$ 2 SDs. Gray bars indicate abnormal VB usage. *Not done. **(B)** Profiles of the fluorescent V β -C β runoff products obtained with mononuclear cells. The x axis indicates CDR3 length.

genome-wide scan was conducted by homozygosity mapping (27) using GENEHUNTER software (28). A total of 18 subjects belonging to 2 families were genotyped for a panel of 381 microsatellite markers. The Linkage Mapping Set MD-10 (Applied Biosystems) formed the core marker set for the scan. Linkage was found (multipoint lod scores between 2.85 and 3.00) to a chromosome 11p region spanning from D11S1338 (5.9 Mb) to D11S905 (40.9 Mb). The *RAG1* gene is located in this chromosomal region (36.5 Mb).

Homozygous *RAG1* gene mutations were identified in all 4 patients (Figure 3). The heterozygous status of both parents was confirmed in all cases. A homozygous del T631 mutation was identified in P1. This mutation, which results in a frameshift at T173 followed by a premature stop codon, has been reported in several T-B- SCID and OS patients as well (11, 13, 29, 30). It does not result in a complete loss of function, as translation may be reinitiated by use of an alternative ATG (M202), 3' to the frame-shift deletion, resulting in a partially functional N-terminally truncated *RAG1* protein (31). The homozygous dinucleotide deletion del A368/A369 identified in P4, which has also been reported in previous studies, had similar consequences for *RAG1* expression, with an N-terminally truncated protein produced by translation initiation at M183 (13, 30, 31). P2 carried a homozygous missense mutation (A3054C) resulting in a Q981P amino acid substitution. This mutation, which to our knowledge has never before been described, affects the core domain of *RAG1*, in a region known to

contain other amino acid substitutions (R973, K992) found in OS patients (11, 13). Finally, P3 presented a homozygous C2633T missense mutation leading to a R841W substitution. This mutation has been observed in OS patients (13). Thus, the *RAG1* mutations identified in 3 patients (P1, P3, and P4) are known to result in residual *RAG1* activity and are therefore compatible with the presence of both B and T lymphocytes in these patients. *RAG1* Q981P is probably also hypomorphic.

Discussion

We describe here a new immunodeficiency syndrome in 4 patients with recessive hypomorphic *RAG1* mutations. The immunological phenotype consists of TCR $\alpha\beta$ ⁺ lymphopenia, contrasting with markedly high counts of TCR $\gamma\delta$ ⁺ T cells. All T cells were of autologous origin, thereby excluding the possibility of maternofetal engraftment. These unusual features were associated with the early onset of multivisceral and recurrent CMV infection in all patients and with autoimmune cytopenia in all but 1 patient. Occurrence of the multivisceral CMV infection in a nonimmunocompromised infant was considered unlikely because of the associated infectious and persistent autoimmune manifestations, although these findings were not regarded as a definitive exclusion criterion, since coinfection and autoimmunity have been reported in infants with CMV infection (32). However, the persistent detection of low TCR $\alpha\beta$ T cell counts in all 4 patients was considered to be a con-

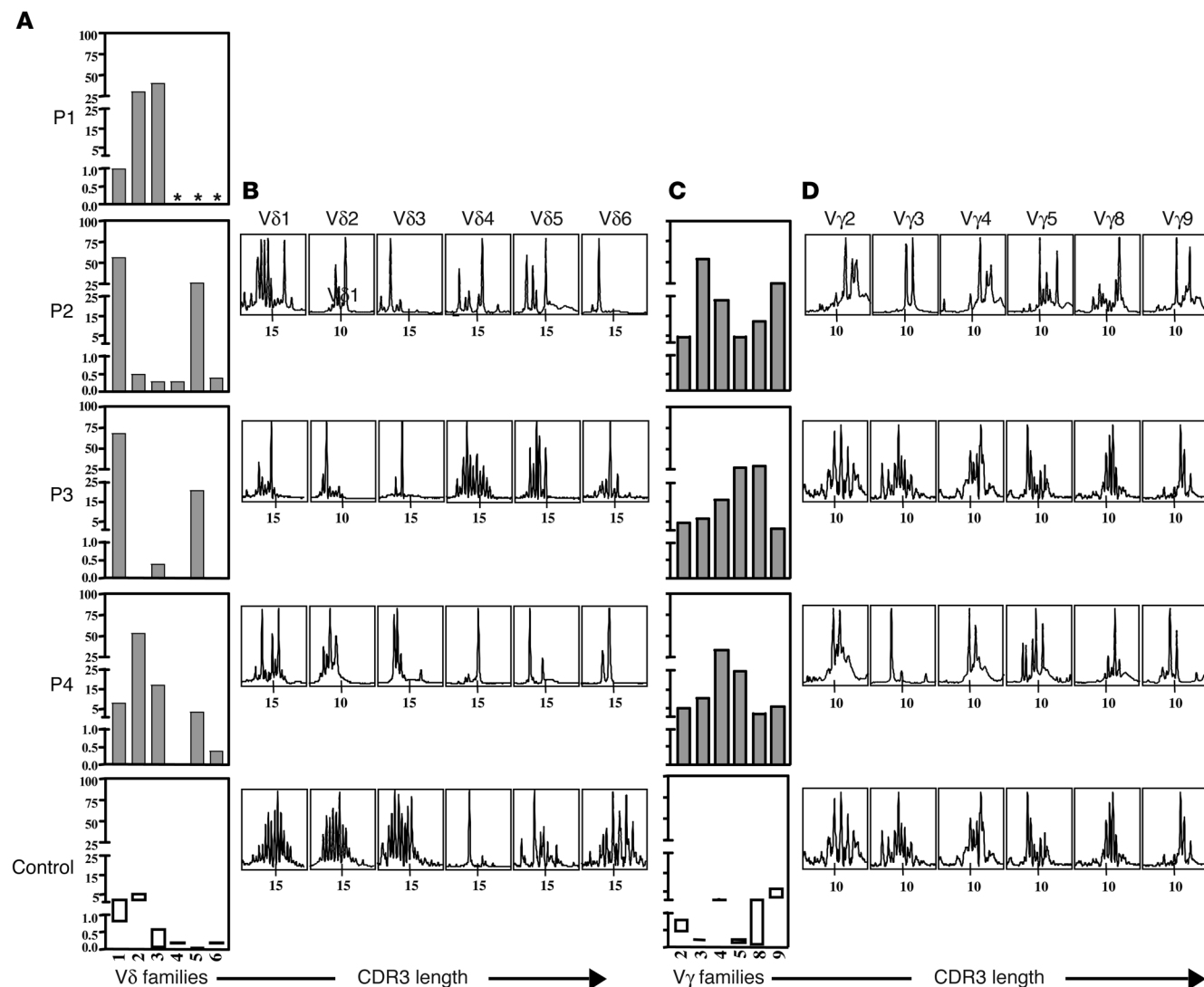


Figure 2

TCRV γ and -V δ usage by patients' T lymphocytes. V δ (A) and V γ (C) repertoire was determined either using available specific mAb in immunofluorescence assay in P1 or using real-time PCR analysis in P2, P3, and P4. The x axis indicates V δ family usage by TCRV δ cells; the y axis indicates relative frequency (%) within the TCRV δ T cells. Controls values shown in rectangles represent mean $\pm$ 2 SDs. Similar results were obtained using available specific mAb in immunofluorescence assay (data not shown). *Not done. Profiles of the fluorescent V δ -C δ (B) and V γ -C γ (D) runoff products obtained with mononuclear cells. The x axis indicates CDR3 length.

sequence of a primary immunodeficiency in the absence of HIV infection. Because of the restricted TCR repertoire, a partial T cell developmental defect was envisaged. A V(D)J recombination defect was thus considered. A genome-wide scan found a linkage in the 11p region where the *RAG1*/*RAG2*-encoding genes are located. The causative role of the *RAG1* mutations found in these patients was demonstrated in 2 ways: (a) no other known cause of primary immunodeficiency was found, despite thorough immunological investigations, and (b) 3 of the 4 mutations detected in these patients have been identified in patients with an immunodeficiency called Omenn syndrome (mutations found in P1 and P4) or atypical Omenn syndrome (mutation found in P3) (11, 13, 29–31). These mutations have been found to be hypomorphic: del T631 and del 368–369 lead to translation starting from an alternative initiation site, at a second methionine codon downstream from

the deletion (31). The missense mutations found in P2 (Q981P) and P3 (R841W) involve amino acids within the minimal core of *RAG1*, but the mutant proteins nonetheless display residual *RAG1* activity. The R841W mutation has been found in OS patients (13), and *RAG1* mutations leading to residue changes at positions close to 981 (R973, K992, R975) have also been reported to encode proteins with residual activities in vitro (13).

These findings broaden the spectrum of disease phenotypes observed in patients carrying recessive mutations of the *RAG1* and *RAG2* genes. Patients with null alleles display a typical T-B-SCID phenotype, as no productive rearrangement of the TCR or BCR can occur (13). In contrast, 2 other phenotypes have been described for patients carrying hypomorphic mutations. The most frequent — OS — involves the oligoclonal expansion of T cells with a Th2 phenotype, with eosinophilia and hyper-IgE syndrome. Th2

**Table 2**CDR3 sequences of V δ 3 and V γ 3 in patient 4

Nuclei sequences			F	L
Vδ3		Cδ		
TGTGCCTGT	AGCGGGGGATACGGCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	1	8
TGTGCCTTT	GGGGGGGTGCGTTGGACCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	1	9
TGTGCCTCA	AAGGCCCACTGGGGGGCGTCCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	2	
TGTGCCGTC	CGGGGGATACGTTATGACACCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	1	
TGTGCCTGT	TCGTGGGGGGGATAGGCCCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	6	10
TGTGCCTAC	TGGTACTGGGGGATACCGACGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	16	
TGTGCCTTT	TTTCCCTACTGGGGTTTACCGGGGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	78	11
TGTGCCTCC	TTAGGGGGACGTACTGGGGGATATCCATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	5	
TGTGCCTCC	CTGGAGTGGGGGAATCCCTCTACACCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	3	12
TGTGCCTTT	GGTTCTCGTACTGGGGGATACGCGAGTACCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	1	13
TGTGCCTTT	AAACGACGGAGACCCTTCCTAAAGTGGGATACTTACAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	1	14
TGTGCCTTT	AAGGCGCCTACCAGTCTACTGGGGGATCCTTCTGTGCCACCGATAAACTC	ATCTTTGGAAAAGGAACCCGTGTGACTGTGGAACCA	1	18
Vγ3		Cγ		
GCCACC	TGGCTCTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	46	
GCCACC	TGGGACATCCTGGGGAAA	CTCTTTGGCAGTGAACAACA	6	
GCCACC	TGGGACAGGCGTGGGAAA	CTCTTTGGCAGTGAACAACA	1	6
GCCACC	AGGATTTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	1	
GCCACC	CGGCTTTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	1	
GCCACC	CGGCTCTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	1	
GCCACC	TGGGACATTTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	1	7
GCCACC	TGGGATGGGTGGCGTTATAAGAAA	CTCTTTGGCAGTGAACAACA	1	
GCCACC	TGGGACAGGCGCGTTATAAGAAA	CTCTTTGGCAGTGAACAACA	2	8
GCCACC	TGGGACGAGCCGAATTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	7	
GCCACC	TGGGACCTACCAATTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	1	9
GCCACC	TGGGACCTACCACTTATTATAAGAAA	CTCTTTGGCAGTGAACAACA	2	

F, frequency of clones with identical sequences; L, CDR3 length (aa).

T cells infiltrate the skin, the gastrointestinal tract, and other organs, possibly in relation to autoimmune reactivity caused by defective central tolerance (11, 14, 33). Residual RAG1/2 activity enables a certain number of precursor cells to rearrange their TCR and BCR genes successfully (11, 29, 34), this rearrangement being followed by the massive expansion of a few autoaggressive T cell clones (14). Villa et al. described a third phenotype associated with recessive RAG1 or RAG2 mutations, which they named “atypical SCID/Omenn syndrome” (13). In patients with this syndrome, T cells are detectable in the periphery, albeit in small numbers, and there are only milder manifestations of immunopathological reactions in the skin or gastrointestinal tract, but most T cells express memory/activation markers. In half of these patients, B cells are also detectable (13).

The RAG1/2 gene mutations causing the OS and atypical SCID/OS phenotypes have yet to be clearly defined. There is clearly an overlap, because the same mutations have been found in patients with each of these phenotypes (A444V mutation, for example)

(11). A milder phenotype has also been described in a 6-year-old girl presenting a combination of maternal T cells and autologous functional B cells that produced anti-herpes simplex virus antibody upon infection. This phenotype was associated with 2 mutations of RAG1, one leading to the insertion of a premature stop codon in RAG1 (R897X) and the second being a missense mutation (R559S) in the core region of RAG1 (35). The missense mutant has been shown to display low but detectable levels of recombination activity in a cellular assay (35). Further overlap between a typical T-B⁻ SCID phenotype and OS, as observed in patients with the same mutations in several families (11), has suggested that the phenotypic consequences of RAG1 or RAG2 mutations depend on (a) the existence of residual V(D)J recombination activity (11, 31, 34) and (b) other yet-unknown genetic or environmentally determined factors. In this setting, it is tempting to conclude that the phenotype observed in the 4 patients described here results from such an environmental factor. The common feature of all 4 patients is early CMV infection. In all 4



Table 3
Serum Ig and antibody determinations

	P1	P2	P3	P4	Age-matched normal values
Age at investigation (mo)	3	12–19	13	19	
Ig					
IgG, mg/dl	<200	700–1240	650–1220	743 ^A	420–800
IgA, mg/dl	7	30–120	38–48	0	16–68
IgM, mg/dl	26	240–426	7–85	270	50–98
IgE, IU/ml	ND	11	3.6	<4	10–25
Antibodies against					
Tetanus toxoid (IU/ml)	ND	<0.06	NE	NE	>0.125
Polio virus (titer × 10 ⁻¹)	I	ND	NE	NE	>40
	II	ND	NE	NE	>40
	III	ND	NE	NE	>40
CMV IgG	ND	11	31	32	<0.9
CMV IgM	ND	Positive	Negative	Positive	

^AUnder intravenous Ig treatment.

patients, dissemination and recurrence of infection was observed each time antiviral therapy was decreased or stopped, probably reflecting a limited appropriate anti-CMV T cell immunity. The severity of the immunodeficiency was also demonstrated by the severe coinfections observed in 2 patients (*Pseudomonas aeruginosa* sepsis, *Pneumocystis jirovecii* pneumonitis). Thus, prolonged CMV infection in patients with profound (but not complete) inherited T cell immunodeficiency may have led to the activation and expansion of $\gamma\delta^+$ T cell clones. This assumption is based on the known ability of $\gamma\delta^+$ T cells to recognize products from microorganisms directly. The expansion of various $\gamma\delta$ T cells has been reported to occur in recipients of kidney allografts developing CMV infection (23). In this case, most of the $V\delta 1^+$ and $V\delta 3^+$ T cells detected were activated in vitro by CMV, demonstrating their specificity (23, 25). An analysis of junctional diversity in these $\gamma\delta^+$ T cells showed that the size distribution of CDR3 cells was restricted, indicating the probable selective expansion of antigen-specific clones (36). In the 4 patients carrying hypomorphic *RAG1* mutations, the diversity of TCR γ and δ CDR3 chain length was found to be fairly restricted. In vitro assay of CMV-infected stromal cell-induced $\gamma\delta$ T cell proliferation, cytotoxic activity, and TNF- α release (25) did not provide evidence to suggest that $\gamma\delta$ T cells were CMV specific. Nevertheless, the striking similarity between these patients' phenotype and the one of CMV-infected kidney transplant recipients with acquired immunodeficiency strongly suggests that expansion of the population of $\gamma\delta$ TCR T cells is directly related to the persistence of CMV infection (23, 25). The predominant location of CMV infection in mucosae (lung and gastrointestinal tract, where mostly Vdelta2- $\gamma\delta$ T cells are located; ref. 37) could also favor local $\gamma\delta$ T cell population expansion. Of note, in mice, $V\delta 1$ cells were also found to exert a protective effect against CMV infection in the liver (38). The lack of in vitro-detectable reactivity to CMV of patients' TCR $\gamma\delta$ T cells can either reflect usage of inadequate assays or the fact that these $\gamma\delta$ T cells do not directly react to CMV molecules. It has not been proven that expanded $\gamma\delta$ T cells found in chronically infected patients after organ transplanta-

tion directly recognize CMV molecules, although the $\gamma\delta$ TCR is required for activation (25). $V\delta 1$ T cells have been shown to be activable by self lipids associated to CD1c (39, 40) or through stress-induced MICA/B MHC class I-related molecules (41, 42). Presence of IL-12 produced by dendritic cells has also been shown to be necessary to induce in vitro $V\delta 1$ T cell activation (43). It is thus possible that chronic CMV infection, in patients with partial RAG1 deficiency, triggered the expression of stress proteins and/or self lipids leading to the activation of these $\gamma\delta$ T cells. CMV products might also trigger a superantigen effect, although the diversity of the TCRV δ and TCRV γ families (with restricted CDR3 length distribution) in the patients is consistent with the antigen-induced activation of a particular set of available clones. The CD45RO⁺CD27⁻CD28⁻ phenotype of the $\gamma\delta$ T cells is itself evidence for in vivo activation.

Another striking feature in 3 of these 4 patients was autoimmunity to blood cells (anemia in 3 patients and neutropenia in 1). No such autoimmunity has previously been reported in patients with *RAG1/2* mutations, with the exception of 1 patient with mild immunodeficiency who presented both autoimmune thrombocytopenia and neutropenia (35). This may reflect a trend toward the development of antibody-mediated immunity, provided that the disease is not fatal in the first few months of life and that B cells with sufficiently diverse BCRs are present, which is not the case in most patients with *RAG1/2* mutations. Nevertheless, it is also possible that the expanded $\gamma\delta$ T cell clones do also promote autoimmunity, as previously suggested (44). Chronic CMV infections are also known to be frequently associated with autoimmune hemolytic anemia, possibly due to the production of cross-reactive antibodies (45). An antibody production deficiency was also found in these patients. It may be the consequence of defective T cell help and/or reduced B cell repertoire diversity in the setting of a defective V(D)J rearrangement of B cell receptor genes.

In conclusion, this report extends the spectrum of disease phenotypes associated with *RAG1/2* mutations (Table 4). It strongly suggests that further phenotypic variability will be identified due to the effects of *RAG1/2* mutations, genetic background, and infectious environment. This finding may well apply to many other primary immunodeficiency diseases, such as X-linked BTK deficiency, the phenotype of which extends from agammaglobulinemia to a limited deficiency of antibody to polysaccharidic antigens (46, 47).

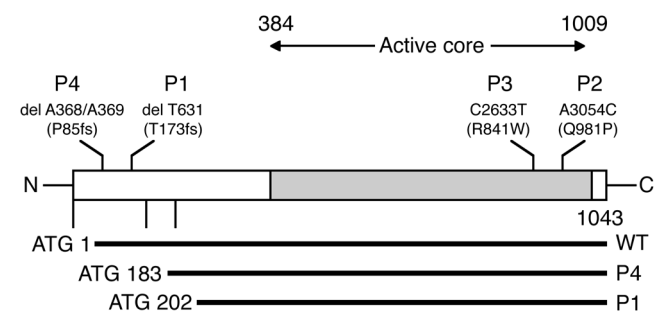


Figure 3
Schematic representation of the *RAG1* gene mutations. Mutations are represented and placed on the overall schematic primary structure of the *RAG1* protein. Horizontal bars represent the expected truncated products as compared with the wild-type *RAG1* protein. fs, frameshift.



Table 4
Spectrum of primary immunodeficiencies associated with RAG1 mutations

	T-B- SCID	OS	Atypical SCID/OS-mild ID	ID with $\gamma\delta$ T cell expansion and autoimmunity
T cells	0	N, $\uparrow$ (oligoclonal)	$\downarrow$	N ($\gamma\delta$ oligoclonal)
B cells	0	0, $\downarrow\downarrow$	$\downarrow\downarrow$ to N	$\downarrow$ to N
RAG1 mutation consequences	Null	Hypomorphic	Hypomorphic	Hypomorphic
Associated factors	–	?	?	CMV infection

ID, immunodeficiency. N, normal; $\uparrow$, increased; $\downarrow$, moderately decreased; $\downarrow\downarrow$, profoundly decreased.

Methods

Patients

P1. P1 was the first child born to first-cousin parents of Algerian origin. The child was healthy at birth. At 3 months of age, he was referred to Necker-Enfants Malades Hospital for *Pseudomonas aeruginosa* sepsis and CMV pneumonitis. CMV was detected in blood (viral load determined by PCR: 39,800 copies/ml of blood), in bronchial aspirates, and in urine. The immunodeficiency of this patient was treated by haploidentical T cell-depleted bone marrow transplantation (BMT), with the mother as a donor, following a conditioning regimen. Acute GVHD, together with reactivation of CMV disease, resulted in the patient's death 150 days after BMT.

P2. P2, a girl born to first-cousin parents of Moroccan origin after an uneventful pregnancy, was referred to Necker-Enfants Malades Hospital at 5 months of age. She had interstitial pneumonitis caused by *Pneumocystis carini* and CMV. CMV was also found in blood and urine specimens. At 1 year of age, she presented fever, protracted diarrhea, severe autoimmune neutropenia, and hemolytic anemia associated with CMV disease reactivation. The patient was treated with ganciclovir together with G-CSF and steroids. CMV infection was reactivated at 14 months and 18 months (pneumonitis) and treated with a combination of ganciclovir and Foscavir. At 19 months of age, the patient received a haploidentical, T cell-depleted bone marrow transplant, with her father as the donor, after a conditioning regimen. Liver and gastrointestinal GVHD, with the reactivation of CMV disease and autoimmune anemia, resulted in the patient's death 6 months after BMT.

P3. P3 was a boy born to first-cousins parents of Lebanese origin after an uneventful pregnancy. At the age of 6 months, he presented autoimmune hemolytic anemia, which was treated with steroids. At 10 months of age, he presented pneumonitis associated with hepatosplenomegaly and recurrence of hemolytic anemia. Lung biopsy showed severe bronchiolitis lesions. *Pseudomonas aeruginosa*, *Candida albicans*, and CMV grew from bronchoalveolar fluid. CMV was also detected in urine and blood. The child received appropriate anti-infection treatment, including ganciclovir and Foscavir. The patient was referred to Necker-Enfants Malades Hospital at 14 months of age, where he underwent BMT with HLA-identical bone marrow from his CMV⁺ mother, after a conditioning regimen. BMT was complicated by a severe skin, liver, and pulmonary GVHD, associated with CMV disease reactivation and autoimmune anemia, eventually leading to death 14 months after BMT.

P4. P4 was born to related parents of Turkish origin. At 5 months of age, she was referred to St Justine Hospital for severe autoimmune hemolytic anemia requiring treatment with a combination of steroids and rituximab. CMV was detected in urine, saliva, and blood. Appropriate treatment with ganciclovir and Foscavir was initiated. Further reactivation of CMV infection occurred at the ages of 16, 18, and 23 months. The patient underwent haploidentical BMT at 26 months of age, after a

conditioning regimen. The donor was her father. Six months after BMT, the patient was doing well, with no significant complications.

The results of complement studies (C3, C4, CH50) were normal, and results of HIV tests were negative for all patients.

Informed consent for the study was obtained from the families in accordance with the Helsinki Declaration.

Immunological investigations

We used the following mAbs for immunofluorescence analysis: anti-CD3: Leu 4 (IgG2a; BD); anti-CD4: Leu 3a (IgG1; BD); anti-CD8: Leu 2a (IgG1; BD); anti-CD19: J4 119 (IgG1; Immunotech, Beckman Coulter); anti-CD56: MY31 (IgG1; BD); anti-TCR $\alpha\beta$: BMA031 (IgG1; Immunotech, Beckman Coulter); anti-TCR $\gamma\delta$: IMMU 515 (IgG1; Immunotech, Beckman Coulter); anti-CD45RO: UCHL1 (IgG2a; Immunotech, Beckman Coulter); anti-CD45RA: ALB11 (IgG1; Immunotech, Beckman Coulter); anti-CD31 (IgG2; Immunotech, Beckman Coulter); anti-CD27 (IgG1; Immunotech, Beckman Coulter); anti-CD28 (IgG1; Immunotech, Beckman Coulter). We obtained anti-TCRBV1S1 and -TCRBV1S2 (IgG1), anti-TCRBV2S1 (IgG1), anti-TCRBV3S1 (IgM), anti-TCRV β 4 (IgM), anti-TCRBV5S1 (IgG2a), anti-TCRBV5S2 (IgG1), anti-TCRBV5S3 (IgG1), anti-TCRBV7S1 (IgG2a), anti-TCRBV8S1 and -TCRBV8S2 (IgG2a), anti-TCRBV9S1 (IgG2a), anti-TCRBV11S1 (IgG2a), anti-TCRBV12S2 (IgG2a), anti-TCRBV13S1 (IgG2b), anti-TCRBV13S6 (IgG1), anti-TCRBV14S1 (IgG1), anti-TCRBV16S1 (IgG1), anti-TCRBV17S1 (IgG1), anti-TCRBV18S1 (IgG1), anti-TCRBV20S1 (IgG) and anti-TCRBV21S3 (IgG2a), anti-TCRBV22S1 (IgG1), anti-TCRBV23S1 (IgG1), anti-TCRV δ 1 (IgG1), anti-TCRV δ 2 (IgG1), anti-TCRV δ 3 (IgG1), anti-TCRV γ 2/3/4 (IgG1), anti-TCRV γ 3/5 (IgG1), anti-TCRV γ 8 (IgG1), and anti-TCRV γ 9 (IgG1) antibodies from Immunotech, Beckman Coulter. Fluorescence staining was carried out with PE- or FITC-conjugated mAbs, on whole blood. Cells were analyzed in a FACScalibur flow cytometer (BD), using Cell Quest software.

Lymphocytes were isolated from patients by Ficoll density gradient centrifugation (Laboratoires Eurobio), and their [³H]thymidine uptake was measured in the presence of mitogens (PHA, OKT3) and antigens (tetanus toxoid, final dilution 1:250 [Aventis Merieux]; CMV antigen, final dilution 1:800 [Advanced Biotechnologies Inc.]) (48).

Serum Ig concentrations were determined by nephelometry, and serum antibody concentrations were determined by ELISA (tetanus toxoid, poliovirus, and CMV) in patients not receiving intravenous Igs.

RAG1 and RAG2 gene analysis

DNA was extracted from whole blood by conventional methods, and the RAG1 and RAG2 genes were amplified by PCR and sequenced as previously described (49). The RAG1 gene coding sequence was amplified by PCR from genomic DNA obtained from whole blood using 1F7b: CTG-GATCCTTATAAGATACATCAGTGGG; 1092R: CGGCAAGAGGGA-



CAAT; 1052F: CGGGTCTGCATTCTCAG; 2089R: CTCATCTGC-CAGCATAAGG; 2013F: TTGCCACAGCTCTCAGAAT; 2594R: CCTGCCACCTTTTCCTTTC; 2401F: GCGTTCCAACCTTACC; 3324R: GCCCCATACAGCAGTA primers and the Expand High Fidelity PCR System (Roche Diagnostics Corp.) according to the manufacturer's recommendations. Nucleotide primers were designed based on the HuRAG1 sequence obtained by Schatz and colleagues (50).

PCR analysis of V δ -C δ and V β -C β junctional diversity

TCRVB quantitative immunoscope analysis. As previously described (51), cDNA prepared from the P3 PBMCs was amplified with each of the 24 TCRVB family-specific primers together with a TCRBC primer and MGB-Taq-Man probe (Applied Biosystems) for TCRBC. Real-time quantitative PCR was carried out on an ABI PRISM 7300 system (Applied Biosystems). PCR products were then subjected to run-off reactions using a nested fluorescent primer specific for the C β segment. The fluorescent products were separated and analyzed on a 373A sequencer (Applied Biosystems). The size and intensity of each band were analyzed with Immunoscope software (52). The Gaussian distribution of the different CDR3 lengths was characteristic of normal V β repertoire.

TCRV δ quantitative immunoscope analysis. As previously described (36), cDNA produced from 0.2 mg of RNA was amplified in a total volume of 25 μ l with each of the 6 TCRV γ and the 6 TCRV δ primers specific for the TCRV families, in combination with a C δ or C γ primer. Real-time quantitative PCR was carried out on an ABI PRISM 5700 system (Applied Biosystems) and optimized for the SYBR green dye assay. The PCR cycling schedule was as follows: heating for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. During PCR, the SYBR green dye binds to double-stranded DNA; the increase in fluorescence is therefore proportional to DNA concentration. PCR products were then subjected to run-off reactions using nested fluorescent primers specific for the C δ or C γ segment. The fluorescent products were separated and analyzed on a 373A sequencer. The size and intensity of each band were analyzed with Immunoscope software. Fluorescence intensity was plotted in arbitrary units on the y axis, whereas the x axis corresponds to CDR3 length in amino acids.

The V gene family-specific and constant primers used, oriented 5' to 3', were as follows: V δ 2: TACCGAGAAAAGGACATCTATGGC; C δ : TGGGAGAGATGACAATAGCAGGATC; C δ -fam: ACGGATGGTTTG-GTATGAGGCTGA; V γ 4: CGGAAGCACAAGGAAGAACTTGAGAAT; C γ : GAATCGTGTGCTCTTCTTTTCTTGCC; and C γ -fam: ATAGT-GGGCTTGGGGGAAACATC.

Sequence analysis of TCR transcripts. PCR of V δ 3-C δ or V γ 3-C γ was performed on P4 cDNA with 5 U of Pfu polymerase (Stratagene) for 25 cycles. PCR products were then cloned into pCR-blunt II-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen Corp.). For sequencing purposes, PCR was conducted directly on *E. coli* colonies with Taq polymerase. Sequencing reactions were performed on these products using C δ or C γ primers and the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). These sequencing reactions were run on an ABI PRISM 3730 DNA analyzer. Sequences corresponding to the CDR3 region were extracted and analyzed.

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