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**Lack of iNKT cells in patients with combined immune deficiency due to hypomorphic *RAG* mutations**

**Running title:** iNKT cells in Omenn syndrome

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**Abstract**

Hypomorphic mutations of the *RAG* genes in humans are associated with a spectrum of clinical and immunological presentations that range from T⁻ B⁻ Severe Combined Immune Deficiency (SCID) to Omenn syndrome. In most cases, residual V(D)J recombination activity allows for development of few T cell clones, that expand in the periphery and infiltrate target organs, resulting in tissue damage.

Invariant Natural Killer T (iNKT) cells play an important immunoregulatory role and have been associated with protection against autoimmunity. We now report on five unrelated cases of combined immune deficiency due to hypomorphic *RAG* mutations, and demonstrate the absence of iNKT cells in all five patients. These findings suggest that lack of this important immunoregulatory cell population may contribute to the pathophysiology of Omenn syndrome.

**Introduction**

Omenn syndrome (OS) is a combined immunodeficiency characterized by early-onset erythrodermia, lymphadenopathy, hepatosplenomegaly, and severe infections. Patients with OS have a variable number of autologous, oligoclonal, and activated T cells that infiltrate and damage target tissues[1,2].

OS may be due to heterogeneous gene defects that impair, but do not abolish, thymic T cell development. In particular, hypomorphic mutations of the *RAG1* or *RAG2* genes, involved in V(D)J recombination, are a common cause for OS[3-5]. The same defects may lead to a spectrum of clinical and immunological phenotypes that also include typical T⁻ B⁻ SCID or leaky SCID with residual presence of activated T cells, in the absence of typical features of OS[4,6].

The clinical and pathological features of OS are suggestive of T-cell mediated autoimmunity. In keeping with this hypothesis, we have found that expression of AIRE and of AIRE-dependent tissue-specific transcripts is reduced in the thymus from patients with OS, possibly contributing to impaired central tolerance[7]. In addition, defects of regulatory T cells have been also hypothesized in patients with OS[8]. Both abnormalities have been confirmed in a newly generated murine model of OS[9]. However, the possible contributory role of other immunomodulatory components in determining the phenotype of OS has not been carefully evaluated.

NKT cells represent a population of cells with significant immunomodulatory properties[10]. In humans, most NKT cells recognize glycolipids presented in the context of CD1d molecules and express NK cell markers along with an invariant T-cell receptor

(TCR) with a Vα24-Jα18 rearrangement. NKT cells expressing the invariant TCR (iNKT) can be identified by using α-galactosylceramide (α-GalCer)-loaded CD1d tetramers[11]. iNKT cells are generated in the thymus from CD4^{+} CD8^{+} thymocytes that rearrange the invariant TCR and are directed to the NKT lineage following cognate interactions with CD1d-expressing cortical thymocytes[12,13]. Therefore, NKT cells development is absolutely dependent on V(D)J recombination, consistent with the observation that iNKT cells are severely reduced in a *Rag2* knock-in mouse model of OS[9].

In this study, we provide the first evidence that iNKT cells are absent in peripheral blood of patients with hypomorphic *RAG* mutations, a situation where substantial numbers of autoreactive T cells develop. We speculate that the absence of iNKT cells may contribute to the pathophysiology of OS.

**Materials and Methods**

*Patients*

Five unrelated patients with hypomorphic *RAG* defects were studied; four of them (Pt. #1, 2, 3 and 5) had typical OS, whereas Pt. #4 had the phenotype of T[+]B[-] leaky SCID (Table 1). Informed consent was obtained according to protocols approved by Children's Hospital, Boston; the Department of Pediatrics, University of Brescia, Italy; and the Department of Pediatrics, Tor Vergata University of Rome, Italy. Maternal T-cell engraftment was ruled out by molecular analysis using microsatellite markers.

*Mutation analysis*

Genomic DNA was extracted by standard procedures from blood samples. The single exons of *RAG* genes were amplified by PCR using thirteen pairs of overlapping primers, and the presence of mismatches was revealed by denaturing HPLC (DHPLC). When mismatches were found, direct sequencing was performed using the Big Dye Terminator kit (Applied Biosystems) on an ABI PRISM 3130 automatic sequencer (Applera).

*Immunophenotyping of peripheral blood mononuclear cells (PBMC)*

PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich). Immunophenotyping was performed by flow cytometry (BD FACSCanto) using the following anti-human antibodies: FITC-conjugated anti-CD4 (BD Pharmingen), FITC-conjugated anti-V$\alpha$24 (Immunotech), FITC-conjugated mouse IgG1 isotype control (eBioscience), PE-conjugated anti-V$\beta$11 (Immunotech), PE-conjugated anti-invariant NK T cells (clone 6B11, BD Pharmingen), PE-conjugated mouse IgG2a isotype control (BD Pharmingen), PE-conjugated mouse IgG1 (BD Pharmingen), PE-conjugated PBS57-loaded human CD1d tetramer or PE-conjugated unloaded human CD1d tetramer (National Institutes of Health Tetramer Facility), PE-Cy5 conjugated anti-CD45 (eBioscience), Alexa750-conjugated anti-CD3 (eBioscience). Data were analyzed using the FlowJo 8.3.3 software. Leukocytes were gated on CD45$^+$ cells. Lymphocytes were gated based on forward and side scatter. CD3$^+$ lymphocytes were then analyzed for expression of invariant T cell receptor (iTCR) of iNKT cells as determined by staining with PBS57-loaded CD1d tetramer (in comparison to unloaded CD1d tetramers). The percentage of iNKT cells in patients 3 and 5 was analyzed also by anti-V$\alpha$24 and anti-V$\beta$11 antibodies as well as by anti-invariant NKT cells (clone 6B11) antibody.

*V(D)J recombination analysis*

V(D)J recombination activity of the human RAG1 mutants G139R, G392E, and L732fs was assessed by a plasmid-based colony forming assay by co-transfecting into 293T cells the expression vectors of wild-type hRAG1 and wild-type hRAG2 (or of the mutants) with substrates pGG49 and pGG51 to allow to measure signal joints and coding joins formation, respectively[14].

**Results and discussion**

The five patients are representative of the spectrum of clinical and laboratory manifestations associated with hypomorphic *RAG* mutations. In all cases, the presence of at least one hypomorphic *RAG* allele allowed for residual T cell development, with accumulation of activated and anergic autologous T cells. The M435V mutation in the *RAG1* gene of patient 1 falls within the nonamer-binding domain, and has been previously reported in OS[6]. The G392E mutation of patient 3 involves the first residue of the GGRPR motif of the nonamer-binding domain (NBD) and had not been previously reported. While double mutations of murine GG 389-390 (corresponding to human residues 392-393) have a dramatic impact on nonamer binding[15], single aminoacid substitutions in the NBD allow for residual V(D)J recombination activity and have been identified in patients with OS[3,16]. The R561H mutation of patient 4 has been previously reported[3,6]. It affects the Rag-2 interacting domain of Rag-1 and reduces V(D)J recombination activity by 3 to 4-fold[3]. The *RAG1* L526R mutation of patient 5 is novel.

Finally, the *RAG2* G139R mutation of patient 2 falls in the first β strand of the third kelch motif within the β-propeller structure of the catalytic core of Rag-2, where several other disease-causing mutations have been identified[17,18]. The novel mutations detected in patients 2 and 3 were found to severely reduce, but not completely abrogate, the V(D)J recombination activity (Table 1).

The five patients examined had variable numbers of autologous peripheral blood CD4^{+} and CD8^{+} T cells (Table 1), indicating that at least limited V(D)J recombination occurred in these patients. Since CD4^{+} T cells in OS have been shown to produce Th2 cytokines[19], and CD4^{+} iNKT cells also produce Th2 cytokines[20], we asked whether any of the peripheral blood T cells in OS patients were iNKT cells. Using α-GalCer loaded CD1d tetramers, we were unable to detect iNKT cells (Fig. 1), while iNKT cells were present in healthy controls (Fig.1 and Supplementary Figure 1). Lack of iNKT cells in OS patients was confirmed by staining with the 6B11 antibody (which binds to the CDR3 region of human invariant TCR) and by analysis of Vα24[+]/Vβ11^{+} T cells (Supplementary Figure 2).

iNKT cells are important in protecting against autoimmune manifestations[21] and graft-versus-host disease (GvHD)[22]. Moreover, they can suppress pathogenic Th1 cells[23]. Numerical or functional defects of iNKT cells have been reported in numerous autoimmune conditions in humans and in mice[10,23]. Therefore, the absence of iNKT cells in OS may contribute to the development of the skin disease and colitis frequently observed in these patients.

In conclusion, our data demonstrate that hypomorphic mutations of the *RAG* genes in humans are not permissive for development of iNKT cells. The contribution of

this abnormality to the pathophysiology of OS could be addressed by adoptive transfer of purified iNKT cells into mice expressing hypomorphic mutations of the *Rag* genes[9,24].

## Authorship

Ponpan Matangkasombut and Muriel Pichavant performed analysis of iNKT cells and contributed to the writing and critical revision of the manuscript. Evelina Mazzolari and Andrea Finocchi were involved in clinical care of patients 2, 3, 4 and 5. Doris E. Saez performed the the in vitro V(D)J recombination analysis, under the supervision of Patricia Cortes. Silvia Giliani performed mutation analysis at the *RAG* loci in patients 2, 3 and 4, Anna Villa and Cristina Sobacchi identified the mutations in patient 5. Dale Umetsu and Luigi D. Notarangelo conceived the study and wrote the manuscript.

The authors declare they have no potential conflict of interest.

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**Legend to Figure**

**Figure 1**. **Flow cytometric analysis of peripheral blood iNKT cells from patients with hypomorphic RAG mutations.** Mononuclear cells (PBMC) from a healthy control (representative of 6 donors) (a) and five patients with hypomorphic mutations of the *RAG* genes (b) were analyzed for the presence of iNKT cells. Leukocytes were enumerated by gating on $CD45^+CD3^+$ cells. iNKT cells were identified using PBS57-loaded CD1d tetramers (right panel) using cells stained with unloaded CD1d tetramers as control (left panel).
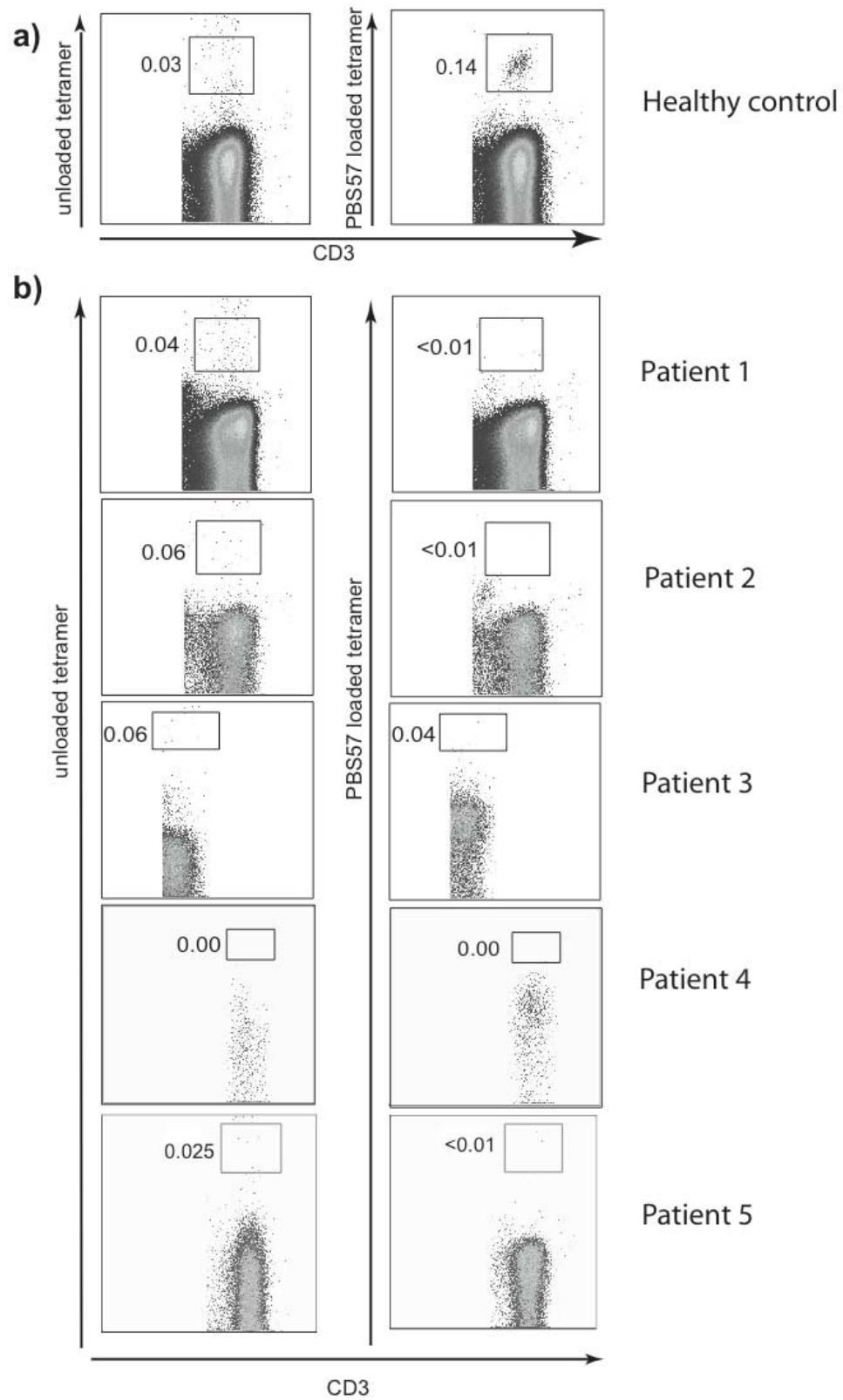
**Figure 1**

**Table 1 – Clinical and laboratory features of five infants with hypomorphic *RAG* mutations**

| | Patient number | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Gene mutations | *RAG1* deletion (encompassing also *RAG2*); **M435V** | *RAG2* **G139R;G139R** | *RAG1* **G392E**;L732fs | *RAG1* R332X;**R561H** | *RAG1* **L526R**, S117fs |
| VDJ recombination activity (% of wild-type)[a] | n.d. | 1.5 | 1.5 | n.d. | n.d. |
| Age at onset | first month | 6 months | birth | 5 months | 2 months |
| Erythrodermia/pachydermia | + | + | + | - | + |
| Liver/ Spleen enlargement | - | - | + | - | + |
| Lymphadenopathy | - | - | + | - | - |
| Diarrhea | + | + | + | + | - |
| Oedemas | + | - | + | - | - |
| Infections | Achromoacter xylosoxidans sepsis | Pneumonia, sepsis | Candida, Klebsiella | Candida; Pseudomonas | Interstitial pneumonia |
| ALC (cells x$10^{-9}$/L) | 62.3 | 0.3 | 3.7 | 0.4 | 25.7 |
| CD3 (%) | 96 | 20.9 | 83.9 | 47.4 | 91 |
| CD4 (%) | 32 | 10.4 | 69.6 | 19.0 | 77 |
| CD8 (%) | 62 | 9.0 | 12.9 | 6.3 | 10 |
| CD19 (%) | 0 | 1.1 | 0.4 | 0.1 | 0 |
| CD16 (%) | 2 | 60.0 | 15.6 | 42.7 | 4 |
| CD4+ CD45R0+/CD4+ (%) | n.d. | 82.3 | 96.0 | 85.8 | 100 |
| Response to PHA (cpm x$10^{-3}$) | 39.2 (179) | 1.6 (121) | 8.0 (134) | 6.5 (112) | 9.4 (57) |
| IgG (mg/dL) | 41 | <100 | 121 | 14 | 240 |
| IgA (mg/dL) | <7 | <5 | <6 | 6.1 | 10 |
| IgM (mg/dL) | <4 | <5 | 25 | 4 | 24 |
| IgE (kU/L) | 128 | n.d. | n.d. | <19 | >5000 |

[a]For patients #2 and 3, V(D)J recombination activity was evaluated using a plasmid-based colony formation assay, as described[14].

Hypomorphic mutations are indicated in bold. Values of proliferative response to PHA in normal controls are indicated in parentheses.

ALC: absolute lymphocyte count; PHA: phytoemagglutinin.