Defect of regulatory T cells in patients with Omenn syndrome

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Background: Omenn syndrome (OS) is an autosomal-recessive disorder characterized by severe immunodeficiency and T-cell–mediated autoimmunity. The disease is caused by hypomorphic mutations in recombination-activating genes that hamper the process of Variable (V) Diversity (D) Joining (J) recombination, leading to the generation of autoreactive T cells. We have previously shown that in OS the expression of autoimmune regulator, a key factor governing central tolerance, is markedly reduced.

Objective: Here, we have addressed the role of peripheral tolerance in the disease pathogenesis.

Methods: We have analyzed forkhead box protein P3 (FOXP3) expression in peripheral blood T cells of 4 patients with OS and in lymphoid organs of 8 patients with OS and have tested the suppressive activity of sorted $CD4^+$ $CD25^{high}$ peripheral blood T cells in 2 of these patients.

Results: We have observed that $CD4^+CD25^{high}$T cells isolated *ex vivo* from patients with OS failed to suppress proliferation of autologous or allogenic $CD4^+$ responder T cells. Moreover, despite individual variability in the fraction of circulating $FOXP3^+$ CD4 cells in patients with OS, the immunohistochemical analysis of FOXP3 expression in lymph nodes and thymus of patients with OS demonstrated a severe reduction of this cell subset compared with control tissues.

Conclusion: Overall, these results suggest a defect of regulatory T cells in OS leading to a breakdown of peripheral tolerance, which may actively concur to the development of autoimmune manifestations in the disease. (J Allergy Clin Immunol 2010;125:209-16.)

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Omenn syndrome (OS) is a peculiar form of combined immunodeficiency presenting with early-onset generalized erythrodermia, failure to thrive, alopecia, lymphadenopathy, hepatosplenomegaly, and intractable diarrhea.[1] The immunologic phenotype of OS is characterized by a normal to increased number of autologous T lymphocytes that express activation markers, whereas circulating B cells are usually low or absent. Immunoglobulin serum levels are very low, with the notable exception of IgE levels, which are often increased.[2,3]

OS is most frequently associated with hypomorphic mutations in the recombination-activating genes (*RAG*s), which impair but do not complete abolish V(D)J recombination process, leading to the generation only few productive antigen receptor gene rearrangements. As a consequence, T-cell repertoire is highly restricted.[4-7] More recently, additional gene defects that severely reduce but do not completely ablate T-cell differentiation have been shown to account for OS in a proportion of patients.[8] Whatever the molecular defects, oligoclonal and activated T cells infiltrate various organs, including skin, gut, spleen, and liver, resulting in profound tissue damage.[9-11] We have previously reported that the thymus from patients with OS is markedly abnormal, with lack of corticomedullary demarcation and absence of Hassall bodies. In addition, the expression of autoimmune regulator (AIRE) and AIRE-dependent tissue-restricted antigens is severely reduced.[12] On the basis of these findings, it has been hypothesized that loss of central tolerance may contribute to the immunopathology of OS. More recently, the lack of invariant natural killer T cells (iNKT) has been proposed to contribute to the immunopathology of OS.[13] However, the possibility that defects in other mechanisms of tolerance might be affected in OS has not been tested so far. Peripheral dominant control of autoreactive T cells is primarily mediated by natural occurring $CD4^+CD25^+$forkhead box protein P3 $(FOXP3)^+$ regulatory T (Treg) cells.[14-17] Indeed, several human autoimmune diseases have been associated with alterations in Treg-cell functions.[18] The thymic development of this cell population is critically dependent on the expression of the transcription repressor gene *FOXP3*,[19] which also represents a valuable marker to identify both thymic and peripheral Treg cells.[20] In human beings, there is evidence that in the thymic medulla Treg cells are generated from thymocytes on high-affinity T-cell receptor–peptide–MHC—mediated interaction with a subset of activated dendritic cells, driving these cells to acquire proper regulatory function.[21] Although FOXP3 is a marker for human Treg cells, it cannot be

Abbreviations used
AIRE: Autoimmune regulator
FOXP3: Forkhead box protein P3
HD: Healthy donor
OS: Omenn syndrome
RAG: Recombination-activating gene
Treg: Regulatory T

considered specific for this population because it can also be expressed, along with CD25, by activated $CD4^+$ cells.[22-24]

In the current study, we have analyzed FOXP3 expression in peripheral blood T cells of 4 patients with OS and in lymphoid organs of 8 patients with OS and have tested the suppressive activity of sorted $CD4^+$ $CD25^{high}$ $CD127^{low/-}$ peripheral blood T cells in 2 of these patients. Our results provide for the first time evidence of impaired development and function of Treg cells in OS, implying that both central and peripheral tolerance are compromised in this disease.

METHODS

Patients

Eight patients with RAG1 defects and 1 patient with a RAG2 defect previously described (patient 5[12]) were included in this study. The clinical, immunologic, and molecular features of the patients, consistent with OS, are outlined in Tables I and II. Briefly, patient 1 was a boy who presented in the first days of life with erythrodermia, lymphadenopathy, spleen, and liver enlargement. Laboratory analysis showed leukocytosis (59×10^3 cells/μL) with marked eosinophilia (40×10^3 cells/μL) and the presence of T and natural killer cells but an absence of B cells. In patient 2, a diagnosis of OS was made at 2 months of age after observation of erythrodermia and lymphadenopathy. Immunologic studies showed hypogammaglobulinemia, high levels of IgE (>5000 IU/mL), and impaired lymphocyte proliferative response to mitogens (anti-CD3, 6000 counts per minute; PHA, 2000 cpm). Patients 3 and 4 were previously described.[13] Patient 3 presented at the age of 5 months with diarrhea and failure to thrive. Cow's milk protein intolerance was suspected, but a dairy-free diet remained ineffective. In the following weeks, the child developed interstitial pneumonia and dermatitis. Laboratory analysis revealed lymphopenia (1.2 cells $\times 10^3$ cells/μL), a low number of T cells (CD3, 47%), a relative increase of natural killer cells (42%), and an absence of B cells. Maternal engraftment was undetectable by HLA chimerism analysis. In patient 4, clinical manifestations of OS were observed at 2 weeks of life. In particular, exudative erythrodermia, cervical lymphadenopathy, and hepatosplenomegaly were detected. Blood testing showed agammaglobulinemia and circulating T cells expressing activation markers. All patients were compound-heterozygous for RAG1 gene as described in Tables I and II.

Blood samples and inguinal lymph node biopsies were obtained from the patients during the clinical setting, whereas control reactive lymph nodes were obtained from patients with unrelated nonimmunologic diseases. OS thymus (patient -5) was retrieved during postmortem examination within 36 hours of death,[12] and control normal human thymus tissues were obtained anonymously from an infant with no known immunologic abnormalities during heart surgery, according to the protocol approved by the Institutional Review Board of the Spedali Civili, Brescia, Italy.

Fluorescence-activated cell sorting analysis

After PBMC purification by standard density gradient technique, cells were stained with anti-CD4 PerCP-Cy5.5-conjugated mAb (BD Pharmingen, San Diego, Calif) in combination with 1 of the following antihuman phycoerythrin-conjugated mAbs: CD25 (clone BC96; Biolegend, San Diego, Calif), CCR7 (R&D Systems, Minneapolis, Minn), and CD45RA and HLA-DR (BD Pharmingen). After 20 minutes of room temperature incubation, cells were washed, and the intracellular staining for antihuman FOXP3 was performed by using an Alexa 488-conjugated mAb (clone 259D; Biolegend) according to the manufacturer's protocol. Samples were acquired the same day of the staining using a FACSCalibur flow cytometer (Becton Dickinson) and analyzed by using FlowJo software (TreeStar Inc, Ashland, Ore).

In vitro suppression assays

$CD4^+CD25^{high}CD127^{low/-}$ Treg cells [25] and $CD4^+CD25^-CD127^+$ responder T cells from patients with OS and age-matched healthy controls were isolated from PBMCs by fluorescence-activated cell sorting. In both cases, the purity was ≥95%. Suppression assays were performed as follows: 5×10^4 responder T cells were stimulated in U-bottom 96-well plates with 1 μg/mL soluble anti-CD3 mAbs (Orthoclone OKT3; Jansssen-Cilag, Milan, Italy) in the presence of an equal number of allogeneic accessory cells (APCs), in a final volume of 200 μL complete medium. $CD4^+CD25^{high}CD127^{low/-}$ Treg cells were added at a ratio of 1:0.5 (responder/suppressor). Accessory cells were obtained by immunomagnetic depletion of $CD3^+$ cells by using CD3-coated beads (Miltenyi, Bergisch Gladbach, Germany) from PBMCs of healthy controls, followed by 30 minutes of treatment with Mitomycin C (40 μg/mL; Sigma Aldrich, St Louis, Mo). After 72 hours of coculture, 50 μL culture supernatant was collected to test for IFN-γ production by ELISA, and cells were pulsed for 16 hours with 1 μCi per well [^{3}H]thymidine (GE Healthcare, Little Chalfont, UK). Cells were harvested and counted in a scintillation counter. Considering the limited number of cells plated per well and the individual variability in the proliferation rate, which may affect the results, the assay reliability was carefully ensured by testing the suppressive activity, in either autologous or allogeneic settings, of a cohort of healthy donors (HDs; n = 8/9).

Immunohistochemical studies

Four-μm-thick sections of lymph nodes and thymus tissues were taken from formalin-fixed, paraffin-embedded blocks and subjected to routine hematoxylin and eosin staining and immunohistochemical analysis. Briefly, sections were dewaxed and rehydrated and endogenous peroxidase activity blocked with 0.3% H_2O_2 in methanol for 15 minutes. Heat induced epitope retrieval was performed by treatment in 1.0 mmol/L EDTA buffer pH 8.0 in a thermostatic bath for 40 minutes at 98 °C. Sections were then cooled, washed in a TRIS-base buffer at pH 7.4, preincubated in blocking buffer containing 5% normal goat serum in TRIS-HCl for 5 minutes, and incubated for 1 hours with primary antibody (rat antihuman Foxp3, 1:200; eBioscience, San Diego, Calif) in TRIS/1% BSA. Sections were then washed again before incubation for 30 minutes with the appropriate secondary antibody (biotinylated rabbit antirat, 1:100; Vector, Burlingame, Calif). Reactivity was revealed by incubation in streptavidin–horseradish peroxidase and diaminobenzidine DAB (DAKO Cytomation, Golstrup, Denmark) and slides counterstained with hematoxylin. Images were acquired by an Olympus DP70 camera mounted on an Olympus Bx60 microscope using Cell^F imaging software (Soft Imaging System GmbH).

Real-time PCR

RNA was purified from whole frozen thymus using the guanidinium thiocyanate–phenol-chloroform method according to the instruction manual (RNAwiz; Ambion Inc, Austin, Tex). One microgram of deoxyribonuclease-treated total RNA was used to synthesize the first strand of cDNA with the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, Calif). For real-time PCR analysis, Assays-on-Demand products (20×) and TaqMan Master Mix (2×) from Applied Biosystems were used to amplify FOXP3 and GAPDH genes according to the manufacturer's instructions. Reactions were run on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Expression levels of FOXP3 were normalized to GAPDH levels in each sample.