Expansion of inflammatory innate lymphoid cells in patients with common variable immune deficiency

Montserrat Cols, PhD,[a,b] Adeeb Rahman, PhD,[b] Paul J. Maglione, MD, PhD,[a,b] Yolanda Garcia-Carmona, PhD,[a,b] Noa Simchoni, PhD,[a,b] Huai-Bin M. Ko, MD,[d] Lin Radigan,[a,b] Andrea Cerutti, MD, PhD,[a,b] Derek Blankenship, PhD,[c] Virginia Pascual, MD,[c] and Charlotte Cunningham-Rundles, MD, PhD[a,b] *New York, NY, and Dallas, Tex*

Background: Common variable immunodeficiency (CVID) is an antibody deficiency treated with immunoglobulin; however, patients can have noninfectious inflammatory conditions that lead to heightened morbidity and mortality.

Objectives: Modular analyses of RNA transcripts in whole blood previously identified an upregulation of many interferon-responsive genes. In this study we sought the cell populations leading to this signature.

Methods: Lymphoid cells were measured in peripheral blood of 55 patients with CVID (31 with and 24 without inflammatory/autoimmune complications) by using mass cytometry and flow cytometry. Surface markers, cytokines, and transcriptional characteristics of sorted innate lymphoid cells (ILCs) were defined by using quantitative PCR. Gastrointestinal and lung biopsy specimens of subjects with inflammatory disease were stained to seek ILCs in tissues.

Results: The linage-negative, CD127$^+$, CD161$^+$ lymphoid population containing T-box transcription factor, retinoic acid–related orphan receptor (ROR) γt, IFN-γ, IL-17A, and IL-22, all hallmarks of type 3 innate lymphoid cells, were expanded in the blood of patients with CVID with inflammatory conditions (mean, 3.7% of PBMCs). ILCs contained detectable amounts of the transcription factors inhibitor of DNA binding 2, T-box transcription factor, and RORγt and increased mRNA transcripts for IL-23 receptor (IL-23R) and IL-26, demonstrating inflammatory potential. In gastrointestinal and lung biopsy tissues of patients with CVID, numerous IFN-γ$^+$RORγt$^+$CD3$^-$ cells were identified, suggesting a role in these mucosal inflammatory states.

Conclusions: An expansion of this highly inflammatory ILC population is a characteristic of patients with CVID with inflammatory disease; ILCs and the interferon signature are markers for the uncontrolled inflammatory state in these patients. (J Allergy Clin Immunol 2016;137:1206-15.)

Key words: *Common variable immunodeficiency, inflammatory complications, mucosal disease, innate lymphoid cells*

Common variable immunodeficiency (CVID), one of the more prevalent primary immunodeficiency diseases, is characterized by low levels of serum IgG, IgA, and/or IgM and lack of production of specific IgG antibodies.[1] Mutations in autosomal genes have been identified in a few patients, but for the majority, genetic or other causes of B-cell failure remain unknown.[2] Although replacement immunoglobulin given at frequent intervals reduces the number of infections, it appears to do little to prevent or treat the inflammatory/autoimmune complications that occur in almost 50% of subjects.[3-5] These complications include autoimmunity, granulomatous infiltrations, interstitial lung disease, lymphoid hyperplasia, lymphoma, liver disease, and enteropathy. In aggregate, these can lead to an 11-fold increased morbidity and mortality over time in these subjects compared with those seen in patients with CVID who have not had these conditions.[5] Although patients with fewer B cells and isotype-switched memory B cells or more impaired T-cell functions are at greater risk for the development of complications,[4-6] mRNA transcriptional profiling allowed us to distinguish patients with CVID with and without inflammatory diseases from each other and from control subjects.[7] Modular analysis of the differentially expressed RNA transcripts identified a marked upregulation of interferon-related genes, as well as a significant downregulation of both B cell–and T cell–related genes.

In this study we identified an expanded population of innate lymphoid cells (ILCs) with a pronounced IFN-γ signature in both peripheral blood and gastrointestinal and lung tissues of these patients with CVID. ILCs are of lymphoid linage but lack recombination-activating gene–dependent rearranged antigen receptors and myeloid and dendritic cell markers (lineage negative). Because of inflammatory cytokine secretion, transcriptional properties, and location in mucosal tissues, ILCs appear to play important roles in immunity to fungal, bacterial, and viral microbes; wound healing; and inflammation.[8] ILC functions are tightly regulated because uncontrolled activation and proliferation can contribute to inflammation and damage in different organs.[9] The expansion of IFN-γ$^+$, IL-17A$^+$, and IL-22$^+$ ILCs in peripheral blood in patients with CVID with inflammatory

From [a]the Department of Medicine, [b]the Immunology Institute, and [d]the Department of Pathology, Icahn School of Medicine at Mount Sinai, New York, and [c]the Baylor Institute for Immunology Research, Dallas.

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Corresponding author: Charlotte Cunningham-Rundles, MD, PhD, Departments of Medicine and Pediatrics, Mount Sinai School of Medicine, 1425 Madison Ave, New York, NY 10029. E-mail: Charlotte.Cunningham-Rundles@mssm.edu.

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Abbreviations used
CVID: Common variable immunodeficiency
CVIDc: CVID with inflammatory disease
CyTOF: Mass cytometry
Id-2: Inhibitor of DNA binding 2
ILC: Innate lymphoid cell
ILC3: Type 3 innate lymphoid cell
IL-23R: IL-23 receptor
PLZF: Promyelocytic leukemia zinc finger
ROR: Retinoic acid–related orphan receptor γt
T-bet: T-box transcription factor

disease (CVIDc) suggests a role for these cells in both inflammation and mucosal damage.

METHODS

Patients and blood and sample processing

Peripheral blood samples were obtained from 22 healthy adult volunteers and 55 patients with CVID (30 female and 25 male patients; age, 14-68 years) by using a protocol and consent approved by the Institutional Review Board of the Icahn School of Medicine at Mount Sinai (IRB#03-1008). One blood sample was used for immediate cell immunophenotyping and another for serum isolation. PBMCs were separated on Ficoll-Histopaque (Pharmacia, Uppsala, Sweden). Thirty-one patients with CVID had inflammatory/autoimmune manifestations (hematologic or organ-specific autoimmunity, biopsy-proved granulomatous disease, interstitial lung disease leading to impaired lung function, lymphoid hyperplasia with splenomegaly, or noninfectious gastrointestinal inflammatory disease), whereas 24 had a history of respiratory or gastrointestinal infections but lacked these conditions (Table I). All subjects were free from concurrent infections and were not taking antibiotics or immune-modifying medications at the time of the study. Blood was taken before interval intravenous immunoglobulin infusions or between subcutaneous immunoglobulin administrations.

TABLE I. Demographics and clinical parameters for 55 patients with CVID

Infections only	24
Subjects with inflammatory/autoimmune complications	31
Autoimmunity: ITP and/or AIHA	18
Interstitial lung disease	18
Splenomegaly/lymphadenopathy	16
Chronic noninfectious gastrointestinal disease	9
Granulomatous disease	7
Splenectomy	6
Nodular regenerative hyperplasia of the liver	3
Other autoimmunity (rheumatoid arthritis/nephritis)	2

AIHA, Autoimmune hemolytic anemia; *ITP*, immune thrombocytopenic purpura.

Immunophenotyping by using flow cytometry

Absolute B-cell, T-cell, and natural killer (NK) cell numbers in peripheral blood were determined by using flow cytometry with CountBright beads (Thermo Fisher, Waltham, Mass) with an Fc-blocking reagent after incubation at 4°C and gating on live $CD45^+$ cells. ILC populations were identified as $CD3^-CD14^-CD11c^-CD19^-CD117^+CD127^+$ cells (ILCs); $CD3^-CD14^-CD19^-CD117^-CD127^-CD56^+$ cells were identified as conventional NK cells. For analysis of intracellular transcription factors, PBMCs were permeabilized, fixed with Transcription Factor Buffer Set (eBioscience, San Diego, Calif) and labeled with mAbs to human retinoic acid–related orphan receptor (ROR) γt or T-box transcription factor (T-bet; antibodies are shown Table E1 in this article's Online Repository at www.jacionline.org). For intracellular cytokine staining, PBMCs were cultured for 4 hours in RPMI medium with GolgiStop (BD PharMingen, San Jose, Calif). Those cells were stained with antibodies to specific surface molecules, fixed, and permeabilized (Cytofix/Cytoperm Kit, BD PharMingen). Cells were examined by using LSR Fortessa (BD) and analyzed with FlowJo software (Tree Star, Ashland, Ore).

Tissues and immunofluorescence

Previous biopsy samples of the lung, gastrointestinal tract, or both from patients with CVID were retrieved with permission and examined with deidentified appropriate control tissues from immunocompetent subjects (Table II). Tissue sections of 5 μm in thickness were stained with primary antibodies (see Table E2 in this article's Online Repository at www.jacionline.org) and appropriate secondary reagents: Alexa Fluor 488–conjugated anti-rat pAb, Alexa Fluor 546/488–conjugated anti-rabbit pAb, Alexa Fluor 546/647–conjugated anti-mouse pAb, and cyanine 5–conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, Pa). Nuclei were visualized with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Boehringer Mannheim, Indianapolis, Ind). Primary antibodies with irrelevant binding activity and appropriate secondary reagents were used to validate the specificity of tissue staining. Coverslips were applied with FluorSave Reagent (Calbiochem, Nottingham, United Kingdom), and images were acquired with a Zeiss Axioplan 2 microscope (Atto Instruments, Rockville, Md).

Mass cytometric analyses

PBMCs from 4 patients with CVID with interstitial lung disease, chronic enteropathy, or both or 4 healthy sex-matched adult donors were stained immediately or after a 5-hour incubation in monensin (for intracellular staining) and examined by means of mass cytometry (CyTOF).[10] In brief, 3 million cells were washed with PBS containing 0.1% BSA and incubated with antibodies against selected surface markers for 30 minutes on ice. Antibodies were preconjugated to metal tags or conjugated in house by using MaxPar X8 conjugation kits (Fluidigm, South San Francisco, Calif). Cells were incubated in cisplatin (Fluidigm) to label dead cells, washed, fixed, and permeabilized with a commercial kit (FoxP3/Transcription Buffer Staining Kit, eBioscience) and stained with antibodies against intracellular cytokines and transcription factors (see Table E2). The samples were then incubated overnight in PBS containing 1.6% formaldehyde and 1:3000 dilution of the nucleic acid Intercalator-Ir (Fluidigm), washed with PBS and diH_20, and resuspended in diH_20 with a 1:10 dilution of EQ 4 Element Calibration beads and cells acquired on a CyTOF2 Mass Cytometer (Fluidigm). Data files were concatenated and normalized by using a bead-based normalization algorithm (CyTOF software) and uploaded to Cytobank. The gated populations were clustered by using spanning-tree progression analysis of density-normalized events,[10] and cell populations were annotated based on expression of key canonical markers while preserving visualization of novel populations within the data set.

Sorting and culturing of ILCs

ILCs were sorted from fresh PBMCs as $CD117^+CD127^+CD56^+$ cells, as described elsewhere.[11] Separately, $CD3^-CD14^-CD19^-CD117^-CD127^-CD56^+$ NK cells and $CD3^+$ T cells were also sorted. For comparison with ILCs from patients with CVID and control subjects, ILCs were similarly sorted from splenocytes of fresh spleens from healthy subjects removed because of trauma.[11] Cells were stained with appropriate mixtures of fluorochrome-labeled antibodies (see Table E1) and sorted with a FACSAria II (BD Biosciences) after exclusion of dead cells by using the LIVE/DEAD Fixable Violet Cell Stain Kit (Invitrogen, Carlsbad, Calif). The purity of sorted cells was consistently greater than 97%. To further examine sorted circulating ILCs, cells (5×10^4/well) were plated in 96-well U-bottom plates and then cultured for 3 to 5 days in complete RPMI medium with 10% FBS, penicillin, and streptomycin (10 U/mL), with or without 50 ng/mL IL-7, 50 ng/mL IL-1β, or both (PeproTech, Rocky Hills, NJ), as previously described.[11] The survival of the sorted ILC population after culture was measured with the Annexin V Apoptosis Detection Kit I (BD PharMingen). Cells were acquired with an LSR