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

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Abstract

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.

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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.

Keywords

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 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.

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 di$H_2$0, and resuspended in di$H_2$0 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 Fortessa (BD) and analyzed by using FlowJo software (Tree Star), with comparisons with isotype-matched antibody controls.

RNA extraction and real-time PCR

Total RNA was extracted from cell populations by using the RNAqueous-4 PCR Isolation Kit (Ambion, Foster City, Calif), followed by cDNA synthesis with the qScript cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, Md). The relative abundance of transcripts was measured by using quantitative RT-PCR with primers (see Table E3 in this article's Online Repository at www.jacionline.org) with PerfeCTa SYBR Green Super Mix (Quanta Biosciences). Results for ILCs were normalized to *Actb* (β-actin) mRNA and presented as relative expression (or abundance) compared with that of total PBMCs.

ELISAs

Sera collected from patients with CVID and control subjects were analyzed with BD OptEIA human INF-γ ELISA set (BD Biosciences), the ELISA MAX set for human IL-17A (BioLegend, San Diego, Calif), and ELISA Ready-SET-Go! for human IL-22 (eBioscience). Cytokines were measured in 1:10 diluted serum in picograms per milliliter, according to the manufacturer's instructions, and by recording absorbance at 450 nm.

Statistical analysis

Values were expressed as means ± SEMs or means ± SDs. Statistical significance was assessed with the 2-tailed Student *t* test and 1-way ANOVA, unless otherwise specified. A Mann-Whitney *U* test and the Kruskal-Wallis test with the Dunn multiple comparison test for matched pairs were used for analysis of nonparametric data. Correlations between data pairs were examined by using the Spearman rank order coefficient. Results were analyzed with GraphPad Prism software (version 5; GraphPad Software, La Jolla, Calif), and *P* values of less than .05 were considered significant.

RESULTS

Microarray analysis of interferon-related genes

RNA microarray studies previously showed that whole blood of 47 patients with CVID with inflammatory features contained a significant upregulation of numerous interferon-responsive genes not found in 44 subjects without these conditions, control subjects, or patients with X-linked agammaglobulinemia.[7] Examining IFN-γ and α1, α2, and β1 gene transcripts in microarray data of these 91 subjects (http://www.ncbi.nlm.nih.gov/geo) demonstrated upregulation of only IFN-γ transcripts, which were significantly increased in blood of patients with CVIDc ($P < .01$). As activated, peripheral blood T cells of these subjects produced little IFN-γ.[7] We hypothesized that other populations might be responsible for the interferon signature.

CyTOF analyses

As an unbiased approach, we used CyTOF to seek IFN-γ^+ lymphoid cells in the peripheral blood of patients with CVID with chronic inflammatory disease. For this, neighboring cells are grouped by using unsupervised hierarchical clustering with spanning-tree progression analysis of density-normalized events algorithm in which nodes are linked by a minimum-spanning tree. These studies revealed a population of cells bearing surface markers associated with ILCs, including CD127 (IL-7 receptor) and the pan-ILC marker CD161, but lacking the lineage markers CD8, CD4, CD11c, CD14, and CD19 and having low levels of CD56 (Fig 1). These cell populations expressed various amounts of surface CD25 and CD117 (c-kit) and, as expected, were positive for intracellular IFN-γ, T-bet, RORγt, and IL-22, suggesting that ILCs were present in blood of patients with CVID. Other markers examined by using CyTOF are shown in Fig E1 in this article's Online Repository at www.jacionline.org.

Phenotypic characteristics of circulating ILCs

ILCs are further categorized by both cytokine capability and transcriptional features,[12] and thus we tested the cell populations of 55 patients with CVID with panels of antibodies using fluorescence-activated cell sorting. The upper panels of Fig 2, *A*, show the expanded, lineage-negative, $CD127^+$ ILC population in a representative patient with CVIDc compared with a patient with CVID and a healthy control subject. This cell population was both IFN-γ^+ and IL-$17A^+$, as shown (Fig 2, *A*, bottom panel). Overall, ILC counts were found to be significantly increased in the blood of these 55 patients with CVID (0.62% of $CD45^+$ lymphocytes) compared with those in 22 healthy donors (0.03%). However, these cells were significantly increased in the blood of 31 patients with CVIDc (mean, 3.7%) compared with a mean of 1.6% in the 24 patients with CVID without complications ($P < .001$, 1-way ANOVA; Fig 2, *B*). ILCs are categorized based on both cytokine capability and transcriptional features.[8] The ILCs identified in the circulation of patients with CVID contained both T-bet and RORγt, correlating with a proinflammatory type 3 innate lymphoid cell (ILC3) population (Fig 2, *C*).[13,14] These cells also contained large amounts of intracellular IFN-γ, IL-17, and especially IL-22 compared with levels in T cells, NK T cells, or conventional NK cells of the same patients with CVID (Fig 2, *D*). Comparing ILC3s from patients with CVID with NK cells from the same 55 patients, ILCs from patients with CVID showed less expression of the natural cytotoxicity receptors NKp44 and NKp46 and the activating NK receptor NKG2D (Fig 2, *E*, upper row).[13,15] Comparing the features of ILC3s from patients with CVID with those of T cells from the same subjects,[11,16,17] ILCs showed detectable levels of the chemokine receptors CXCR3 and CCR6,[18,19] with some expression of CD25 and the integrin CD49d (Fig 2, *E*, middle row). As expected, circulating ILCs, as in previous studies, showed significant levels of c-Kit[9,17] and other markers of innate cells, such as the integrin αE (CD103) and Thy-1,[20] and demonstrated the capacity for antigen presentation (MHC-II; Fig 2, *E*, bottom row).[9]

Ex vivo culture and transcriptional features of isolated ILCs

ILCs from peripheral blood of 4 patients with inflammatory complications were sorted and cultured *ex vivo* into $CD56^+$ and $CD5^{dim}$ populations to compare them with ILC3s described in human spleens.[11] As for these cells, $CD56^+$ ILC subsets from patients with CVID were capable of surviving for 5 days in culture when supplemented with IL-7, IL-1β, or both (Fig 3, *A*). We also compared the transcriptional features of freshly sorted ILCs from patients with CVID with similarly sorted $CD56^+$ and $CD5^{dim}$ ILCs isolated from spleens of immunocompetent donors.[11] Although ILC3s were $CD56^+$, perforin-1 mRNA was detected solely in the sorted $CD127^-CD56^+$ NK cells but not in the $CD127^+$ ILC3 cells from blood or normal spleen tissue of patients with CVID, differentiating these ILC3s from cytotoxic NK cells (Fig 3, *B*).

Further comparing transcriptional features of ILCs from patients with CVID with sorted splenic ILC3 populations, $CD56^{dim}$ ILC3s from patients with CVID were found to have higher amounts of the transcriptional regulator inhibitor of DNA binding 2 (Id-2) and comparable levels of RORγt (Fig 3, *C*).[15,17,21] In contrast, ILC3s from patients with CVID had higher amounts of promyelocytic leukemia zinc finger (PLZF) when compared with splenic ILC3s, indicating a different developmental stage (Fig 3, *C*).[22] Although splenic

ILC3s contained greater amounts of T-bet, both subsets of ILCs from patients with CVID contained this transcription factor (Fig 3, *C*). Displaying their unique signature and in concert with intracellular staining, both $CD56^+$ and $CD56^{dim}$ subsets of ILCs from patients with CVID contained higher levels of mRNA transcripts for the proinflammatory cytokines IFN-γ, IL-17A, IL-22, and IL-26 when compared with peripheral T cells or NK cells of the same subjects or splenic ILC3s (Fig 3, *D*). $CD56^+$ and $CD56^{dim}$ ILC3s from patients with CVID also contained higher levels of aryl hydrocarbon receptor (important in IL-22 production) than splenic ILC3, and only ILC3s from patients with CVID were positive for IL-23 receptor (IL-23R), a part of the inflammatory IL-17/IL-23 axis (Fig 3, *E*).[23,24]

ILCs are found in gastrointestinal and pulmonary tissues

In healthy human subjects about 0.01% to 0.1% of circulating lymphocytes are $CD127^+$ cells, and these are mostly nonactivated type 2 ILCs.[9] In contrast, ILC3s, with a few exceptions,[25,26] are very rare in the circulation and are generally considered to be restricted to the mucosa, where they appear to play important roles in immunity to fungal, bacterial, and viral microbes, as well as pathologic states of inflammation.[9,11,27] Thus we examined tissues from mucosal sites, where patients with CVID have significant tissue damage. Immunofluorescence analysis of small bowel biopsy specimens of patients with enteropathy revealed a distinct population of $CD3^-ROR\gamma t^+IFN\text{-}\gamma^+$ cells, which are not present in noninflamed gastrointestinal tissues of control subjects undergoing unrelated surgery or mucosa of patients with active Crohn disease, where $ROR\gamma t^+$ cells were $CD3^+$ (Fig 4, *A*). $CD3^-IFN\text{-}\gamma^+$ populations were detected in other portions of the small bowel in other patients with CVID who had enteropathy (see Fig E2 in this article's Online Repository at www.jacionline.org).

Examining lung biopsy samples of patients with CVID given a diagnosis of lymphocytic interstitial lung disease also showed $CD3^-IFN\text{-}\gamma^+ROR\gamma t^+$ cells, which are suggestive of inflammatory ILC3s. Cells of this sort were not noted in the lungs of an immunocompetent subject given a diagnosis of nodular lymphoid hyperplasia,[28] examined here as a pertinent control subject (Fig 4, *B*, and see Fig E3 in this article's Online Repository at www.jacionline.org).

Detection of IFN-γ in serum of patients with CVID

With the significant mRNA IFN-γ signature in blood and expanded IFN-γ ILC3s in the circulation, we then tested sera of patients with CVID for levels of IFN-γ, IL-17A, and IL-22. Sera from patients with CVIDc had significantly increased amounts of IFN-γ ($P < .001$, 1-way ANOVA; Fig 5, *A*). Although IL-17A was detected in sera of a few subjects, no significant differences were noted between groups (Fig 5, *B*). IL-22, which was also measured, was not detectable in these sera (data not shown).

DISCUSSION

One of the more puzzling features of patients with CVID is clinical heterogeneity, with a significant number of subjects whose symptoms clinically stable for years receiving replacement immunoglobulin with very little need for additional therapy. In contrast, up to

50% of subjects have a series of often interlinked complications with the hallmarks of immune dysregulation.[4,5,29–31] These complications require treatment with immunosuppressants, immunomodulators, or, in the worst cases, stem cell transplantation.[32–34] Seeking to understand the underlying differences in CVID groups, investigators have examined immunologic profiles, clinical phenotypes, cell populations and functions,[4,6,35] molecular aspects,[36] and cytokines, as previously reviewed.[37] Our recent work used a modular whole blood RNA approach to probe the inflammatory signature in patients with CVID.[7] We found that the most overactive genes were those in interferon pathways and showed that this signature identified subjects with inflammatory complications. Examination of interferon gene transcripts showed that of these cytokines, only levels of IFN-γ were upregulated and significantly increased in patients with CVIDc. Although cytokine dysregulation has been a recurring theme in patients with CVID, many studies have shown that activated T cells from patients with CVID might produce less IFN-γ than control cells.[37] Nevertheless, *IFNG* mRNA was previously found to be enriched in both gastrointestinal and liver tissues of patients with CVID with enteropathy or nodular regenerative hyperplasia.[38–40] Here we investigated the possibly that an expanded IFN-γ^+ ILC population might be responsible for the IFN-γ signature observed.

ILCs have emerged as a family of developmentally related hematopoietic effector cells involved in innate immunity, tissue development, and remodeling; however, these cells appear to be essential players in inflammatory diseases and capable of rapid and potent cytokine production. ILCs are divided based on phenotypic and functional characteristics: group 1 contains typical NK cells and other noncytotoxic ILCs, which secrete IFN-γ and express T-bet; group 2 cells depend on GATA-3 and produce IL-5 and IL-13; and group 3 ILCs produce IL-17 and IL-22 and contain the transcription factor RORγt, and depending of the cytokine environment, might produce IFN-γ.[8,9] Perhaps reflecting their defensive/inflammatory role, ILCs have been described in the oral mucosa, respiratory and gastrointestinal tracts, and spleen.[17,23,26,41–43] In health ILCs secrete cytokines needed for neutrophil recruitment and promote an equilibrium between mucosal immunity and microbial colonization. However, in disease states, such as eczema, psoriasis, Crohn disease, asthma, or multiple sclerosis, these cytokines can foster mucosal damage, alteration of microbial flora, or even tumorigenesis.[27]

In contrast to a previous study that suggested reduced numbers of peripheral blood IL-17^+CD127^+Lin$^-$ cells in patients with CVID,[44] in peripheral blood and tissues of patients with CVIDc, we identified a markedly expanded population of Lin$^-$ IL-7 receptor–positive cells that contain IFN-γ, IL-17A, and IL-22, characteristics of ILC3s described in spleen and other lymphoid tissues, skin, brain, and the gastrointestinal and respiratory mucosa.[8,9,11,45] Peripheral blood ILCs in patients with CVID, being CD127^+ and containing intracellular IFN-γ, IL-17A, IL-22, and the transcription factors T-bet and RORγt, are similar to the proinflammatory “double-negative” ILC3 population previously described.[13]

Although ILCs have been most extensively investigated at mucosal sites, very rare ILC populations have been detected in peripheral blood. In healthy human subjects about 0.01% to 0.1% of lymphocytes are CD127^+ cells, mostly classified as nonactivated type 2 ILCs.[9] However, ILC3s in the circulation in patients with CVID were much more numerous, and

subjects with inflammatory complications had significantly more of these cells (mean, 3.7%) than subjects without these complications. On sorting ILCs and culture with appropriate cytokines, these cells displayed the distinctive transcriptional features associated with ILCs: Id-2 and PLZF, as well as IL-26, aryl hydrocarbon receptor, and IL-23R.[9,13,22] We also found numerous $CD3^-$ IFN-γ^+ and RORγt^+ cells in mucosal biopsy specimens of patients with CVID with enteropathy and interstitial lung disease. Because biopsy specimens were obtained from patients with chronic mucosal disease, we suspect that these are likely to play a role in ongoing mucosal inflammation.

We conclude that inflammatory conditions in patients with CVID are characterized by an expansion of activated mucosal and circulating ILC3s, which might contribute to the interferon mRNA gene signature and cytokine levels in whole blood. Recombination-activating gene 1–deficient mice, lacking T and B cells, also have increased ILC numbers and increased IL-22 secretion, suggesting that adaptive immunity exerts controls on innate cell populations, which proliferate when these are defective.[46] On this basis, we predict that an expansion of ILCs will be a feature of other primary immune deficiency states in which there are inflammatory complications. Potentially, the loss of mucosal immunity leading to infections and/or alteration of the microbiome, or bacterial translocation,[47] could drive recruitment of ILC3s to these sites. Once recruited, the inflammatory nature of these cells is likely to contribute to further tissue damage.[9] Although immunoglobulin treatment reversed the $CD4^+$ T-cell defects ascribed to absorbed bacterial endotoxins in patients with CVID,[47] we and others have not found that immunoglobulin therapy alters the clinical course of the noninfectious inflammatory complications in patients with CVID.[5,34,40] We also did not find that immunoglobulin therapy prevented the appearance of the whole blood interferon signature in these subjects.[7] As the inflammatory potential of ILCs has been increasingly recognized, therapeutic means to control these cells and re-exert homeostasis have not emerged. Further studies to understand the emergence of the ILC population and therapeutic measures to better address these inflammatory complications are required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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

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Clinical implications

The IFN-γ signature in patients with CVIDc is correlated with a highly proinflammatory ILC population in circulation and mucosal tissues.

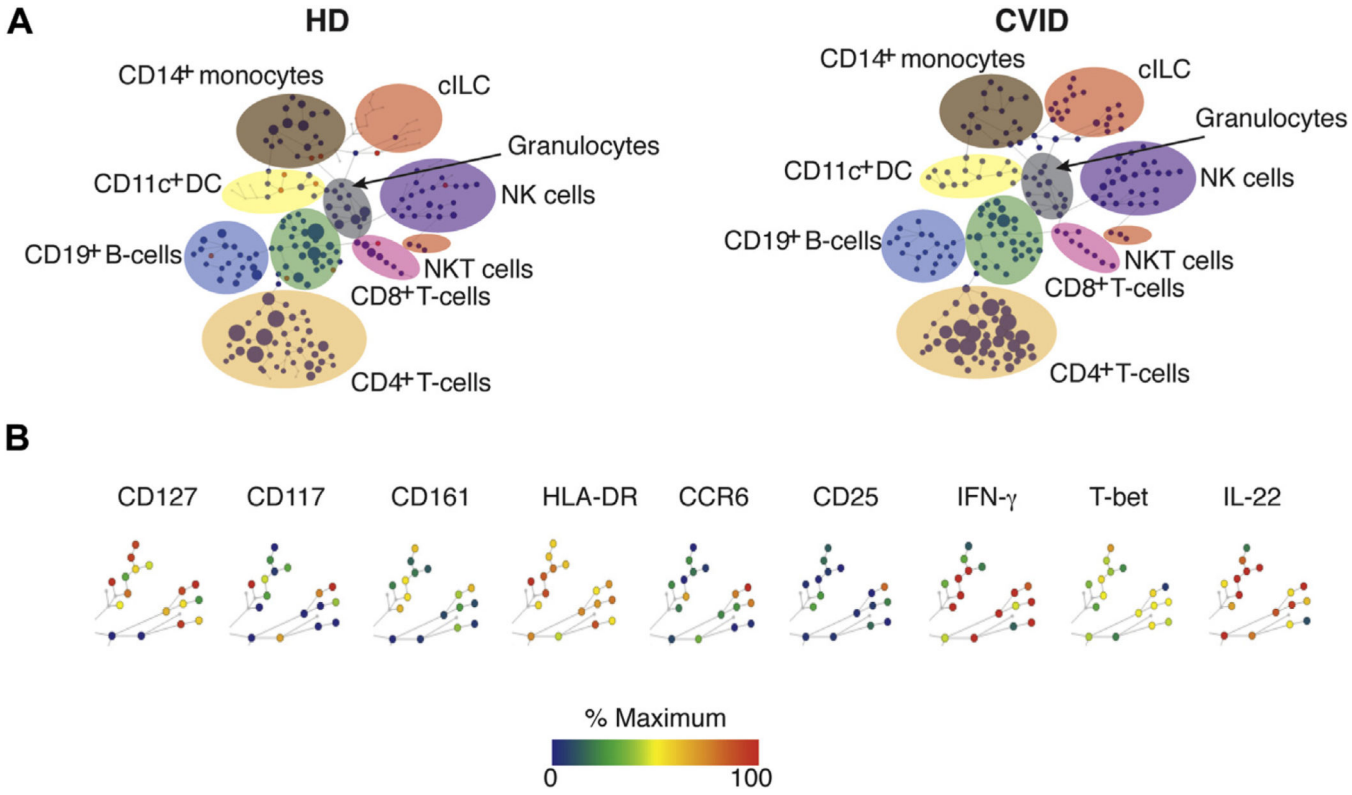

FIG 1.

CyTOF analyses. **A,** CyTOF was used to compare cell lineages in the blood of 4 patients with CVIDc compared with control subjects *(HD)*. **B,** Lin$^-$ cells positive for CD127 and CD161 were observed in samples from patients with CVID. Other markers tested are shown here. ILC populations are highlighted. Node color is scaled to the median intensity of marker expression. One representative experiment is displayed.

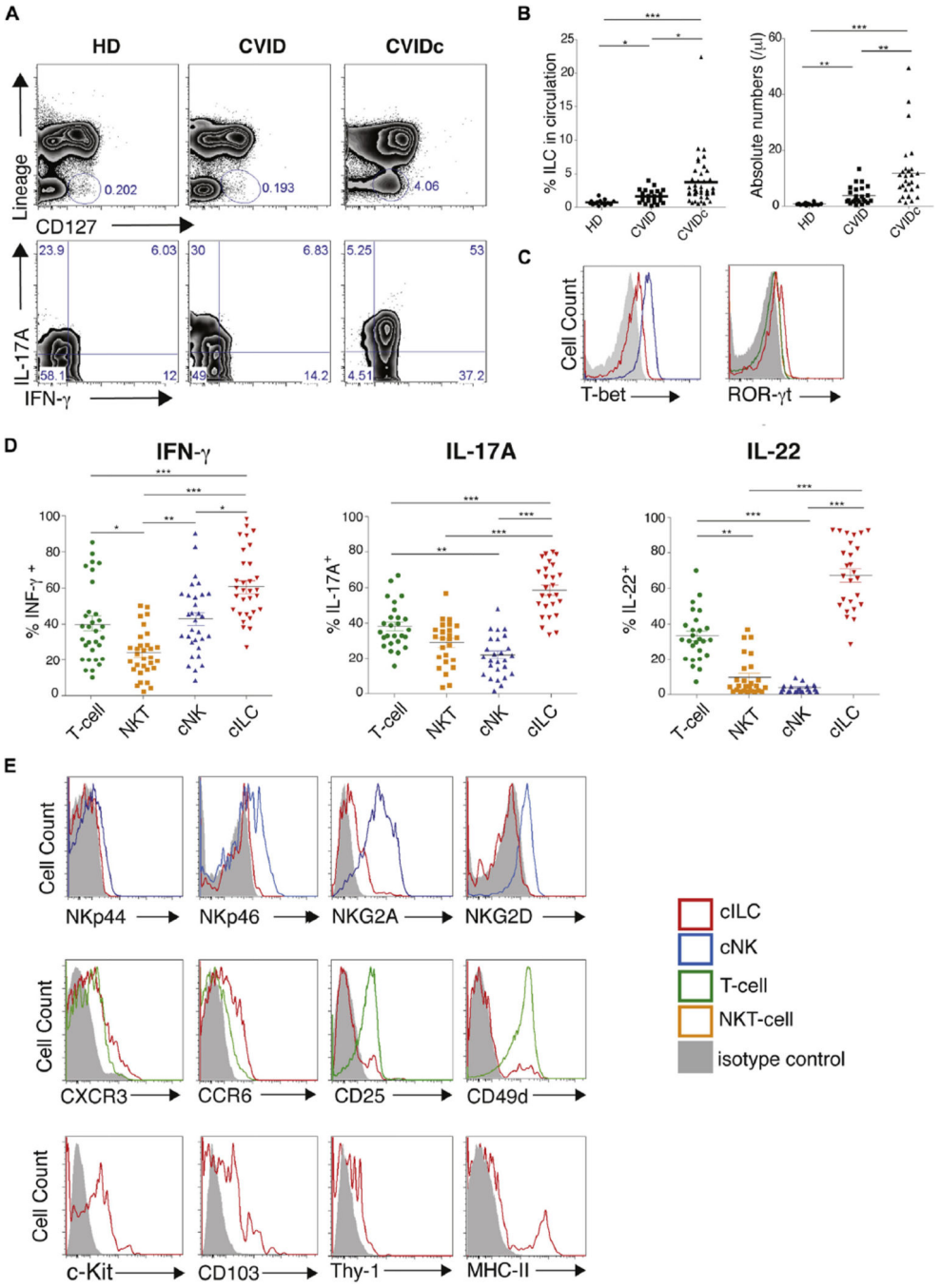

FIG 2.

Immunophenotyping of circulating ILC3s. **A,** Fluorescence-activated cell sorting analyses show Lin^{-}IFN-γ^{+} and IL-17A^{+}CD127^{+} cells in peripheral blood in patients with CVIDc. **B,** ILC3s were significantly increased in these patients with CVID. **C,** Intracellular T-bet and RORγt of ILC3 populations (*red*), NK cells (*blue*), NK T cells (*green*), or T cells (*orange*). **D,** Percentages of IFN-γ^{+}, IL17-A^{+}, and IL-22^{+} immune cells in these patients with CVID. **E,** Fluorescence-activated cell sorting comparison of ILC3s from patients with CVID with NK cells (NKp44, NKp46, and NKG2D). *Middle row*, CXCR3, CCR6, CD25, and CD49d

levels for ILC3s from patients with CVID to T cells. *Bottom row*, ILC3s from patients with CVID are c-Kit^{+}, CD103^{+}, and Thy-1^{+}. Representative plots from 22 healthy control subjects, 31 patients with CVID with complications, and 24 patients with CVID without complications are shown in Fig 2, *A, C*, and *E*. For Fig 2, *B* and *D*: $*P < .05$, $**P < .01$, and $***P < .001$ (1-way ANOVA). *HD*, Healthy donors.

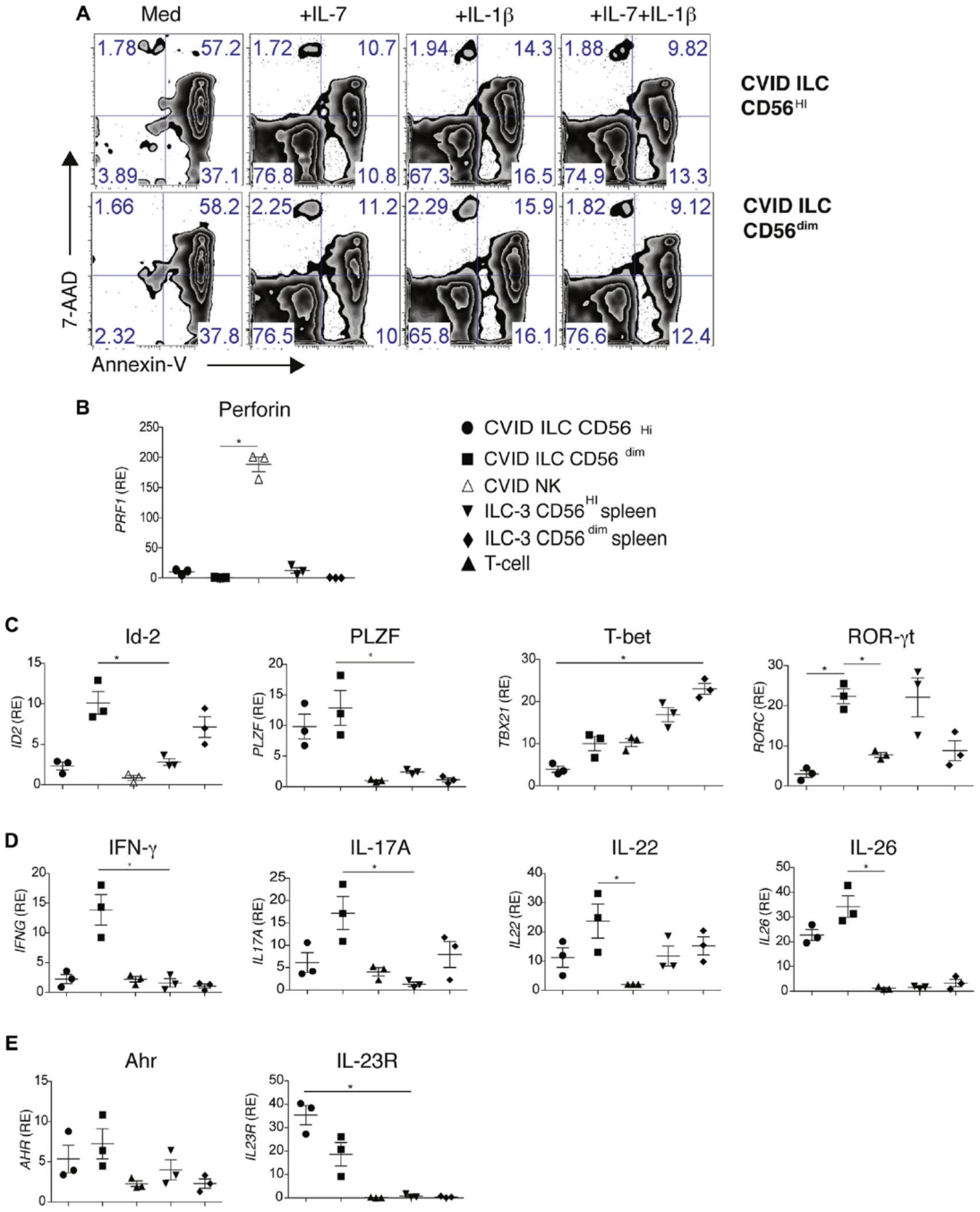

FIG 3.

CD56 expression and transcriptional features. **A,** Viability of sorted CD56^{hi} and CD56^{lo} ILC3s from patients with CVIDc cultured with medium alone or IL-7, IL-1β, or both × 5 days, as assessed by using fluorescence-activated cell sorting. **B,** RT-PCR for perforin (*PRF1*) mRNA expression of sorted cILC3s or NK cells from spleens of patients with CVID or control subjects. **C,** Quantitative RT-PCR analysis of mRNA for Id-2 (*ID2*), PLZF, T-bet (*TBX21*), and RORγt (*RORC*) in sorted ILC3s, NK cells, or CD3^{+} T cells. **D,** Quantitative RT-PCR mRNA encoding for IFN-γ, IL-17A, IL-22, and IL-26. Results were normalized

and expressed as above (Fig 3, *C*). **E,** Quantitative RT-PCR mRNA for aryl hydrocarbon receptor and the cytokine receptor IL-23R. All RT-PCR results were normalized to β-actin mRNA expressed as relative expression *(RE)* to PBMCs. Data are from 3 experiments. For Fig 3, *B–E*: **P* < .05, 1-way ANOVA. *Error bars* = SEMs.

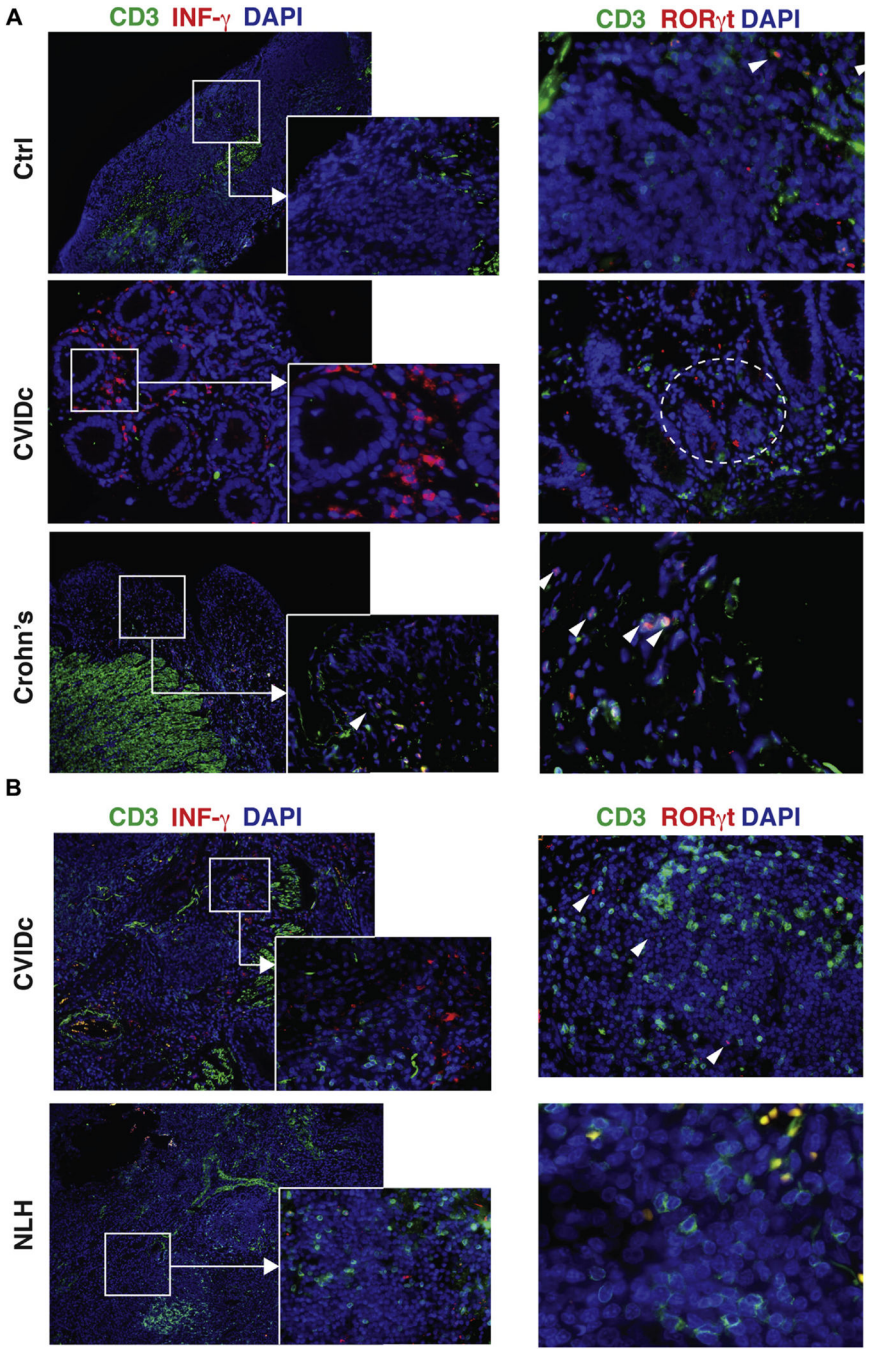

FIG 4.

ILC3s are detected in mucosal tissues. **A,** Ileal biopsy specimens of a patient with CVID with enteropathy were compared with tissue from a nonimmunodeficient donor *(top)* and a patient with Crohn disease *(bottom)*. Tissue was stained for $CD3^+$ T cells *(green)*, IFN-γ *(left panels)*, and RORc *(right panels; red)* and counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride *(DAPI)* for nuclei *(blue)*. *Solid squares*, Area of magnification; *dashed circles*, IFN-γ^+ and $RORc^+$ $CD3^-$ cells. $CD3^+RORc^+$ double-positive T cells *(arrowheads)* in noninflamed control ileum *(upper rightmost panel)* and T cells in

patients with Crohn disease. Magnification ×10; inset magnification ×40. Magnification ×20 and ×40 for Crohn disease. **B,** Lung biopsy specimen of a patient with CVID with lymphoid hyperplasia (CVIDc). The control subject was a nonimmunodeficient patient with nodular lymphoid hyperplasia. Tissue was stained as in Fig 4, *A*. Magnification ×10; inset magnification ×40. *Right panel* magnification ×20 and ×40 for nodular lymphoid hyperplasia. Data are from one 4 experiments.

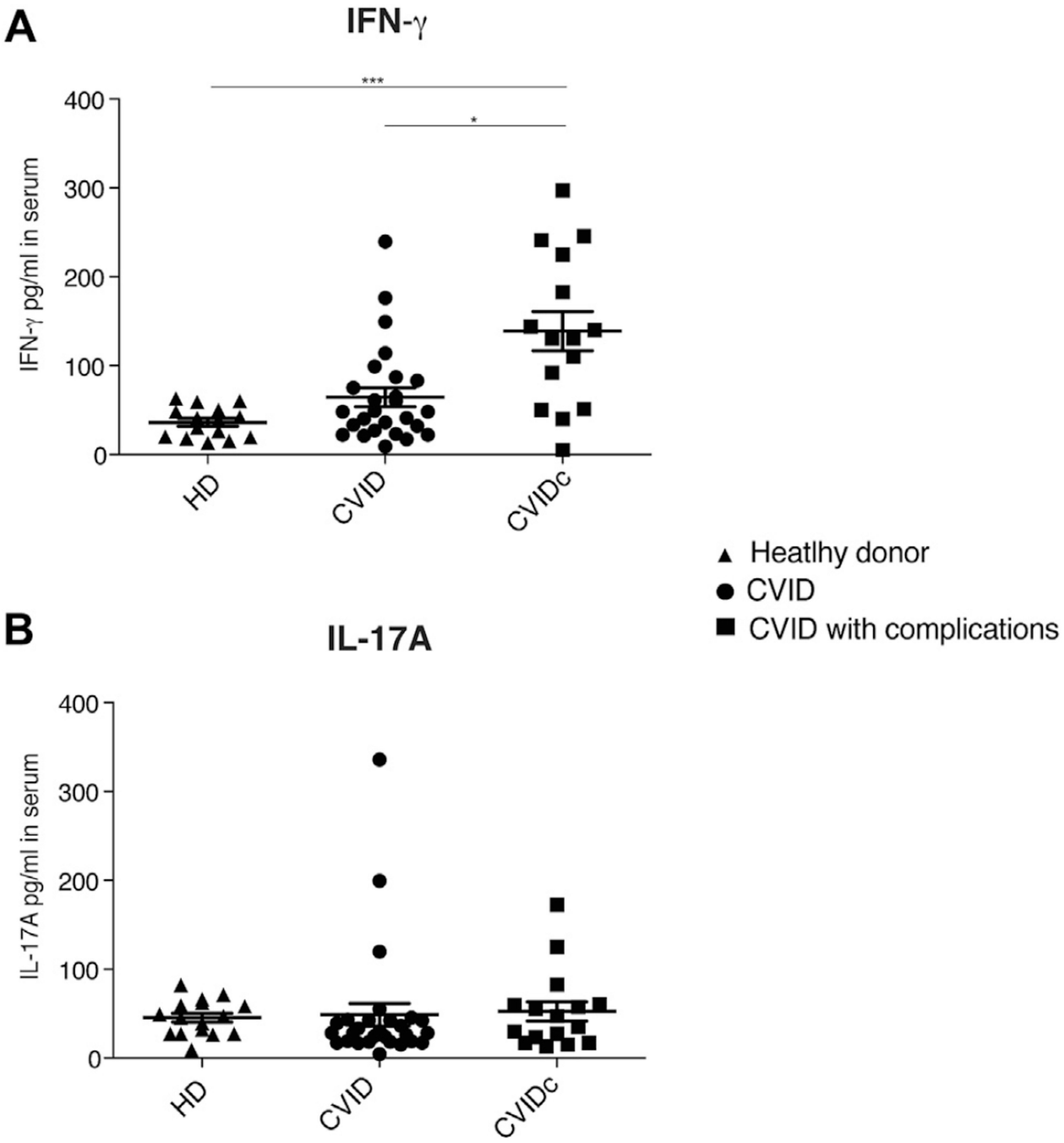

FIG 5.

Cytokine profile of patients with CVID. IFN-γ **(A)** and IL-17A **(B)** levels in serum, as measured by using ELISA. **P* < .05 and ****P* < .001, 1-way ANOVA, followed by the Dunn multiple comparison *post hoc* test). Data for 15 healthy subjects *(HD)*, 26 patients with CVID without inflammatory complications, and 16 patients with CVID with such complications were pooled for Fig 5, *B*. *Bars* denote means with SEMs.

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.

TABLE II

Pathology samples from patients with CVID and control subjects

Source	Tissue	Pathologic diagnosis	Fixation
CVID, 33-year-old man	Terminal ileum	Patchy, mildly active nonspecific colitis with marked depletion of plasma cells	FFPE
CVID, 47-year-old woman	Small bowel, jejunum	Small intestine with moderate intraepithelial lymphocytosis, mild villous atrophy, moderate lymphocytic and eosinophilic infiltration, with focal aggregation of the lymphocytes; marked decrease of plasma cells; no granuloma or parasites	FFPE
Control, 38-year-old man	Terminal ileum	Obstructed, not inflamed	FFPE
Control, 27-year-old woman	Terminal ileum	Crohn disease, mildly inflamed	FFPE
Control, 39-year-old man	Right colon	Active Crohn enteritis with transmural chronic inflammation	FFPE
CVID, 49-year-old mam	Lung	Interstitial lung disease, lymphoid hyperplasia, organizing pneumonia, and occasional poorly formed granulomas	FFPE
CVID, 49-year-old woman	Lung	Interstitial lung disease, lymphoid hyperplasia, organizing pneumonia, and occasional poorly formed granulomas	FFPE
CVID, 46-year-old man	Lung	Reactive lymphoid hyperplasia, interstitial lung disease and few poorly formed nonnecrotizing granulomas	FFPE
Control, 47-year-old woman	Lung	Lung nodule: nodular lymphoid hyperplasia	FFPE
18-year-old man	Spleen	Resection after trauma	Fresh
34-year-old man	Spleen	Resection after trauma	Fresh

FFPE, Formalin fixed and paraffin embedded.