

Inflammation in common variable immunodeficiency is associated with a distinct CD8⁺ response to cytomegalovirus

Sayed Mahdi Marashi, PhD,^{a*} Mohammad Raeiszadeh, MSc,^{b*} Sarita Workman, MSc,^b Afsar Rahbar, PhD,^d Cecilia Soderberg-Naucler, PhD,^d Paul Klenerman, PhD,^e Ronnie Chee, MBBS,^c A. David Webster, MD,^c Richard S. B. Milne, PhD,^a and Vincent C. Emery, PhD^a London and Oxford, United Kingdom, and Stockholm, Sweden

Background: Common variable immunodeficiency is the most common primary immunodeficiency. A subset of patients has debilitating inflammatory complications.

Objectives: We investigated the role of cytomegalovirus (CMV), and the T-cell response targeted at this virus, in this inflammatory disease.

Methods: Phenotypic and functional assays were used to profile CMV-specific T cells in patients with common variable immunodeficiency with and without inflammatory complications. Highly sensitive immunohistochemistry was used to detect CMV antigens at sites of inflammation.

Results: Cytomegalovirus was significantly associated with inflammatory disease, which occurred in 31 of 43 (72%) virus-exposed patients and 8 of 31 (26%) naive patients ($P = .0001$). CMV pp65-NLVPMTATV epitope-specific CD8⁺ T-cell frequencies were significantly elevated in inflammatory patients, but these cells did not show evidence of exhaustion, with low levels of programmed death-1 and high T-cell receptor avidity. Rather, they showed features consistent with high *in vivo* functionality and proliferative activity including reduced levels of the anti-inflammatory marker CD73 (1.67% of NLV⁺ cells

were CD73⁺ vs 42.01% in noninflammatory patients; $P = .004$) and increased Ki-67 expression (37% vs 2% in noninflammatory patients; $P < .0001$). *In vitro*, the CMV-specific T cells showed high antigen-specific proliferative potential compared with cells from noninflammatory patients. By using sensitive immunohistochemistry, we detected for the first time viral antigen at the sites of inflammation, indicative of active viral replication.

Conclusion: Our data strongly support a direct role for CMV and a hyperreactive CMV-specific immune response in the debilitating chronic inflammatory complications of common variable immunodeficiency. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Cytomegalovirus, common variable immunodeficiency, inflammation, CD8⁺ T cells

Common variable immunodeficiency (CVID) is the most common clinically relevant primary antibody deficiency. Patients usually present after their second decade of life with defective antibody production and increased susceptibility to infection.¹⁻³ Monogenic defects have been described in a minority of patients.⁴ Complications include recurrent and chronic infections, chronic inflammatory diseases, autoimmunity, and lymphoid malignancies. The inflammatory phenotype in CVID is expressed as organ specific involvement that may lead to fibrosis and granuloma formation or as multisystem granulomatous disease. The organs most commonly affected are the lung, liver, spleen, skin, and gastrointestinal tract. Persistent viral infection has been suggested as a cause of granulomatous disease in CVID.²

Although CVID is a B-lymphocyte-related disease, reports have described alterations in the phenotype and function of T cells.⁵ Patients with CVID have not been considered at risk of serious viral infections; however, some develop persistent enteroviral infection.⁶ In addition, patients with CVID infected with hepatitis C virus by contaminated immunoglobulin batches have an aggressive clinical course of hepatitis.⁷

The betaherpesvirus human cytomegalovirus (CMV) infects between 60% and 90% of the population worldwide. After primary infection, the virus establishes latency, with periodic reactivations that are controlled in most infected individuals despite the virus producing many immunomodulatory proteins.⁸⁻¹⁰ Although there is considerable literature describing defective cellular immunity in CVID,⁵ we reported robust CD8⁺ T-cell responses to CMV and EBV in patients with CVID, with CMV responses elevated compared with healthy controls.¹¹

From ^athe Medical Research Council Centre for Medical Molecular Virology and ^bthe Department of Immunology, Division of Infection and Immunity, Royal Free Campus, University College London Medical School; ^cthe Department of Clinical Immunology, Royal Free Hospital NHS Trust, London; ^dthe Department of Medicine, Center for Molecular Medicine, Karolinska Institute, Stockholm; and ^ethe Peter Medawar Building for Pathogen Research, University of Oxford.

*These authors contributed equally to this work.

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Reprint requests: Vincent C. Emery, PhD, Medical Research Council Centre for Medical Molecular Virology, Division of Infection and Immunity, University College London Medical School, Royal Free Campus, Rowland Hill Street, London, NW3 2QG UK. E-mail: vemery@medsch.ucl.ac.uk.

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Abbreviations used

CFSE: Carboxyfluorescein diacetate succinimidyl ester
 CMV: Cytomegalovirus
 CVID: Common variable immunodeficiency
 EBV: Epstein Barr virus
 FITC: Fluorescein isothiocyanate
 IEA: Immediate-early antigen
 LA: Late antigen

Here, we investigated the function of CMV-specific T cells in patients with CVID and addressed the hypothesis that a distinct CMV-specific CD8⁺ T-cell phenotype coupled with CMV replication in affected organs contributes to the inflammation observed in a subset of patients with CVID.

METHODS**Patients and control subjects**

A total of 74 patients with CVID (33 male; mean age, 50 ± 15 years) attending the Royal Free Hospital diagnosed according to established criteria^{12,13} were recruited. All but 1 patient were receiving immunoglobulin therapy.

A total of 27 healthy subjects (9 male; mean age, 40 ± 12 years) were recruited and their CMV status determined by using a CMV-specific IgG assay (VIDAS; BioMérieux, Marcy L'Etoile, France). Exposure to CMV in patients with CVID was assessed by measuring IFN-γ production by CD4⁺ T cells stimulated with viral lysate. A value of >0.05% positive cells (2 SDs above the mean for healthy CMV-negative subjects) was taken as evidence of CMV exposure, and the patients with CVID classified on this basis. (All healthy subjects also fulfilled this criterion.) Epstein Barr virus (EBV) exposure was assessed by GLC pentamer staining (see below). An HLA-A*0201–positive subset of CMV-exposed patients participated in a more detailed study of T-cell function. Of these, 9 were inflammatory and 16 noninflammatory (based on the definitions described in the next section). None of these patients had transmembrane activator and calcium-modulator and cyclophilin ligand interactor mutations. Because of limitations on the amount of blood that could be obtained, some subjects were not included in all of the assays presented in this article.

All participants gave informed consent, and the study was approved by the local ethics committee (reference 04/Q0501/119).

Inflammatory disease definitions in patients with CVID

Patients with any of the following features were categorized as inflammatory, the remainder as noninflammatory:

1. Hepatitis without evidence of hepatitis B or C virus infection: only those with raised blood alkaline phosphatase levels (>200 IU/L) were included, although usually alanine and aspartate transaminase levels were also moderately raised; most had had a liver biopsy showing mild/moderate periportal lymphocyte infiltration and cholestasis and evidence of nodular regenerative hyperplasia; granulomas were present in a few cases, and some patients had early signs of cirrhosis and/or portal hypertension.
2. Splenomegaly defined as a clinically enlarged spleen on palpation and/or >13 cm in length on ultrasound. Splenomegaly was included as a marker of inflammation because large spleens surgically removed from patients with CVID often show granulomatous infiltration,¹⁴ and enlargement is also an early marker of portal hypertension secondary to liver inflammation. However, the splenomegaly associated with autoimmune blood dyscrasias in CVID may not show inflammatory changes, so it is important to note that splenomegaly may overestimate the frequency of inflammatory disease in the patient groups.

3. Biopsy and clinical evidence of chronic enteropathy involving small and/or large bowel not explained by infection or gluten sensitivity.
4. Interstitial lung disease not explained by infection. These patients had evidence of deteriorating CO gas transfer in the lungs and computerized tomography scan features of interstitial disease (ground glass appearance, micronodularity, and fibrosis).

CD8⁺ T-cell phenotyping

PBMCs were purified by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) centrifugation.

1. Cytokine production and degranulation. PBMCs were stimulated with a peptide pool encompassing the CMV ppUL83(pp65) protein (JPT, Germany). Cytokine production¹⁵ and CD107 expression¹⁶ were measured by using published methods.
2. Class I HLA pentamers and phenotyping. A total of 1 × 10⁶ PBMCs were stained with phycoerythrin-conjugated HLA-A*0201 pentamers (ProImmune, Oxford, United Kingdom) for the NLVPMVATV epitope (from CMV ppUL83[pp65], abbreviated to NLV) or the GLCTLVAML epitope (from the EBV BMLF1 protein, abbreviated to GLC) at 10 μg/mL for 15 minutes at room temperature. Cells were washed with phosphate buffered saline (0.1% sodium azide) and stained with peridinin-chlorophyll proteins-cyanine 5.5 (PerCp-Cy5.5)–labeled anti-CD8 and, as appropriate, anti-PD-1 fluorescein isothiocyanate (FITC), anti-CD57 FITC (BD Biosciences, Oxford, United Kingdom), anti-CD73 allophycocyanin (eBiosciences, Hatfield, United Kingdom), and anti-human leukocyte antigen D-related (HLA DR) allophycocyanin (R&D Systems, Oxford, United Kingdom). For Ki-67 staining, thawed cryopreserved cells were pentamer/CD8-stained as described, then fixed, permeabilized (Fix & Perm; Invitrogen, Paisley, United Kingdom) and stained with anti-Ki-67–FITC (BD Biosciences). Isotype controls were from BD Biosciences. Data were acquired on a FACScalibur (Becton Dickinson, Oxford, United Kingdom) and analyzed with FlowJo (Tree Star Inc, Ashland, Ore).
3. Differential avidity class I HLA tetramers. NLV epitope tetramers with differential avidity were synthesized and refolded as described.¹⁷ The CD8-null tetramer has reduced avidity because of a mutated CD8 binding domain.¹⁸ A total of 1 × 10⁶ cells were stained with 4 μg phycoerythrin-labeled tetramers in 0.5 mL, then washed and surface-stained with anti-CD8 antibodies.

T-cell proliferation assays

Cell proliferation was analyzed by using carboxyfluorescein diacetate succinimidyl ester (CFSE).¹⁹ Cells (10⁷/mL) were labeled with 2.5 μmol/L CFSE (Invitrogen) for 5 minutes at room temperature in the dark. Labeled cells were cultured in 96-well plates at a concentration of 1 × 10⁵ cells/200 μL in RPMI containing 10% fetal calf serum for 5 days in 37°C 5% CO₂.

CMV NLV peptide, EBV GLC peptide (ProImmune), CMV pp65 peptide pool, and phytohemagglutinin (Sigma, Poole, United Kingdom) were used at 10 μg/mL to stimulate the cells. After 5 days, cells were stained with pentamers and anti-CD8 antibody as described. ModFit LT 3.0 (Verity Software, Topsham, Me) was used to quantify cycles of cell division.

Immunohistochemical detection of CMV in inflamed tissues

Formalin-fixed, paraffin-embedded colon biopsies from patients with CVID, chronic diarrhea, and histologic evidence of mild and moderate colitis were analyzed for CMV immediate-early (IEA) and late (LA) antigens by immunohistochemistry using a recently developed protocol with highly optimized tissue preparation and blocking.^{20–22} Control colon biopsies were healthy tissue removed during resection surgery for bowel cancer. Tissue sections (6 μm) were deparaffinized, rehydrated, and postfixed with 4% formalin. Sections were treated with pepsin (Biogenex, San Ramon, Calif) at 37°C for 3 minutes, then treated with antigen retrieval solution (BioSite, San Diego,

Calif). Nonspecific peroxidase was blocked with 3% H₂O₂ (Sigma), Fc receptors were blocked with Fc-receptor blocker (Innovex, Richmond, Calif), biotin and avidin were blocked with the Biotin/Avidin Blocking Kit (DakoCytomation, Glostrup, Denmark), and slides were treated with Background Buster (Innovex). Primary antibodies were mouse anti-CMV IEA, anti-CMV LA, and antihuman α -actin (Chemicon, Billerica, Mass). Incubation with primary antibodies was overnight at 4°C. Antibodies were detected with biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase. Slides were developed with chromogen diaminobenzidine (Innovex) and counterstained with hematoxylin (Sigma). Images were captured at room temperature by using a Leica Microsystems AB, Type DC 480 microscope and a Leica DFC320 camera. Adobe Photoshop was used for image acquisition, formatting, and annotation.

Statistical analysis

The Fisher exact test was used to compare frequencies of clinical complications between the CMV-exposed and naive patients with CVID. The Mann-Whitney test was used for comparing data sets that were nonnormally distributed. For normally distributed data, mostly phenotypic analyses, the Student *t* test was used. *P* values ≤ 0.05 were regarded as significant.

RESULTS

CMV infection is associated with inflammatory disease in CVID

We asked whether exposure to CMV was associated with the inflammatory complications characteristic of a subset of patients with CVID. By using CD4⁺ T-cell responses to CMV-infected cell lysates¹¹ to identify CMV-infected patients because serologic confirmation of CMV exposure is impossible in immunoglobulin-deficient individuals, we found a highly significant association between CMV exposure and inflammatory complications (see this article's Table E1 in the Online Repository at www.jacionline.org). Thirty-one of 43 (72%) CMV-exposed patients had 1 or more features of chronic inflammation compared with 8 of 31 (26%) CMV-naive patients. The CD4/CD8 lymphocyte ratio was lower in CMV-exposed patients, and malignancy was more common in this group. In contrast, there was no difference in the incidence of autoimmune disease between the CMV-exposed and naive patients with CVID. Importantly, when the CMV-exposed patients were stratified according to the presence or absence of inflammatory disease, there was no significant difference in the CMV-specific CD4⁺ T-cell responses between the 2 groups, so CD4 responses were not further investigated.

Functional integrity of CMV-specific CD8⁺ T cells from patients with CVID

To assess the functional potential of the CMV specific CD8⁺ T cells, we used a pp65 peptide pool to analyze responses irrespective of class I HLA haplotype. Mobilization of CD107a/b molecules and simultaneous production of IFN- γ and TNF- α by CD8⁺ T cells after stimulation were determined in CMV-exposed patients with CVID and CMV IgG-positive healthy controls. This article's Fig E1, A, in the Online Repository at www.jacionline.org shows that in all samples, the majority of CMV-specific CD8⁺ T cells produced both cytokines; however, the frequency of double producer cells in peripheral blood of patients with CVID, in particular those with inflammatory complications, was significantly higher than the frequency of these cells in healthy individuals (noninflammatory, 1.09%;

inflammatory, 5.05%; healthy, 0.67%; Fig E1, B). The double producer cells in both groups were capable of degranulation, as shown by mobilization of CD107a/b molecules to the cell surface (Fig E1, C).

CMV-specific CD8⁺ T-cell frequency is elevated in patients with inflammatory CVID

To investigate more subtle aspects of the CD8⁺ T-cell response in patients with inflammatory CVID, we focused on the HLA-A*0201-positive subsets of our panel of patients with CVID (*n* = 25) and healthy controls (*n* = 14) whose CD8⁺ T cells would be expected to recognize the relevant restricted peptides in CMV pp65 (NLVPMVATV) and, for comparison, the EBV BMLF1 protein (GLCTLVAML). Enumeration of HLA class I pentamer-specific CD8⁺ T-cell frequencies showed a significantly increased frequency of CMV NLV-specific CD8⁺ T cells in PBMCs from patients with CVID compared with healthy controls (CVID: mean frequency, 2.6%, SD, 0.49%; healthy: mean frequency, 0.66%, SD, 0.2%; *P* = .005; Fig 1, A). There was no difference in the frequency of EBV GLC-specific CD8⁺ T cells between the 2 groups (CVID: mean frequency, 0.18%, SD, 0.04%; healthy: mean frequency, 0.32%, SD, 0.19%; *P* = .35; Fig 1, A). Within the CVID patient group, the frequency of CMV-specific cells was significantly higher than the frequency of EBV-specific cells, with an average difference in frequency of 14.4-fold. This was much greater than the difference observed between the same responses to these 2 viral epitopes in healthy individuals (2.1-fold).

Nine of the 25 HLA-A*0201-positive patients with CVID had inflammatory disease, and these patients had a significantly higher frequency of CMV NLV-specific CD8⁺ T cells compared with noninflammatory CMV-exposed patients with CVID (mean frequency, 4.8%, SD, 0.77%, vs mean frequency, 1.4%, SD, 0.39%; *P* = .0002; Fig 1, B). Moreover, in the latter group of patients with CVID, CMV NLV-specific CD8⁺ T-cell frequencies were not significantly different from those in healthy CMV-positive individuals (Fig 1, B). CMV NLV-specific CD8⁺ cell frequencies were broadly consistent in individuals over the course of the study (Fig 1, C). Frequencies of EBV GLC-specific CD8⁺ T cells were similar in all groups (Fig 1, D).

Because CMV NLV-specific CD8⁺ T-cell frequencies were elevated in the inflammatory patients, we next investigated the surface phenotype of these cells. We found no significant differences between the inflammatory and noninflammatory groups in expression levels or expression frequencies of PD-1, a marker associated with T-cell exhaustion, on CMV or EBV pentamer⁺ CD8⁺ T cells. The frequency of PD-1⁺ cells in the noninflammatory group was elevated compared with the healthy controls for CMV and EBV pentamer⁺ cells, but in general, all CD8⁺ T cells expressed low levels of PD-1 (Fig 2, A and B; see this article's Fig E2, A and B, in the Online Repository at www.jacionline.org). However, patients with inflammatory CVID showed significantly higher frequencies of Ki-67 (a marker of *in vivo* cycling)-positive CD8⁺ T cells than noninflammatory patients or healthy controls (inflammatory patients: mean, 2.4%, SD, 0.37%, vs noninflammatory patients: mean, 0.73%, SD, 0.14%, *P* = .0002; and vs healthy controls: mean, 0.32%, SD, 0.03%, *P* = .002; Fig 2, C). This distinction was greatly enhanced when Ki-67 expression in CMV NLV pentamer⁺ CD8⁺ T cells was analyzed, with healthy controls and noninflammatory patients showing less than 2% and

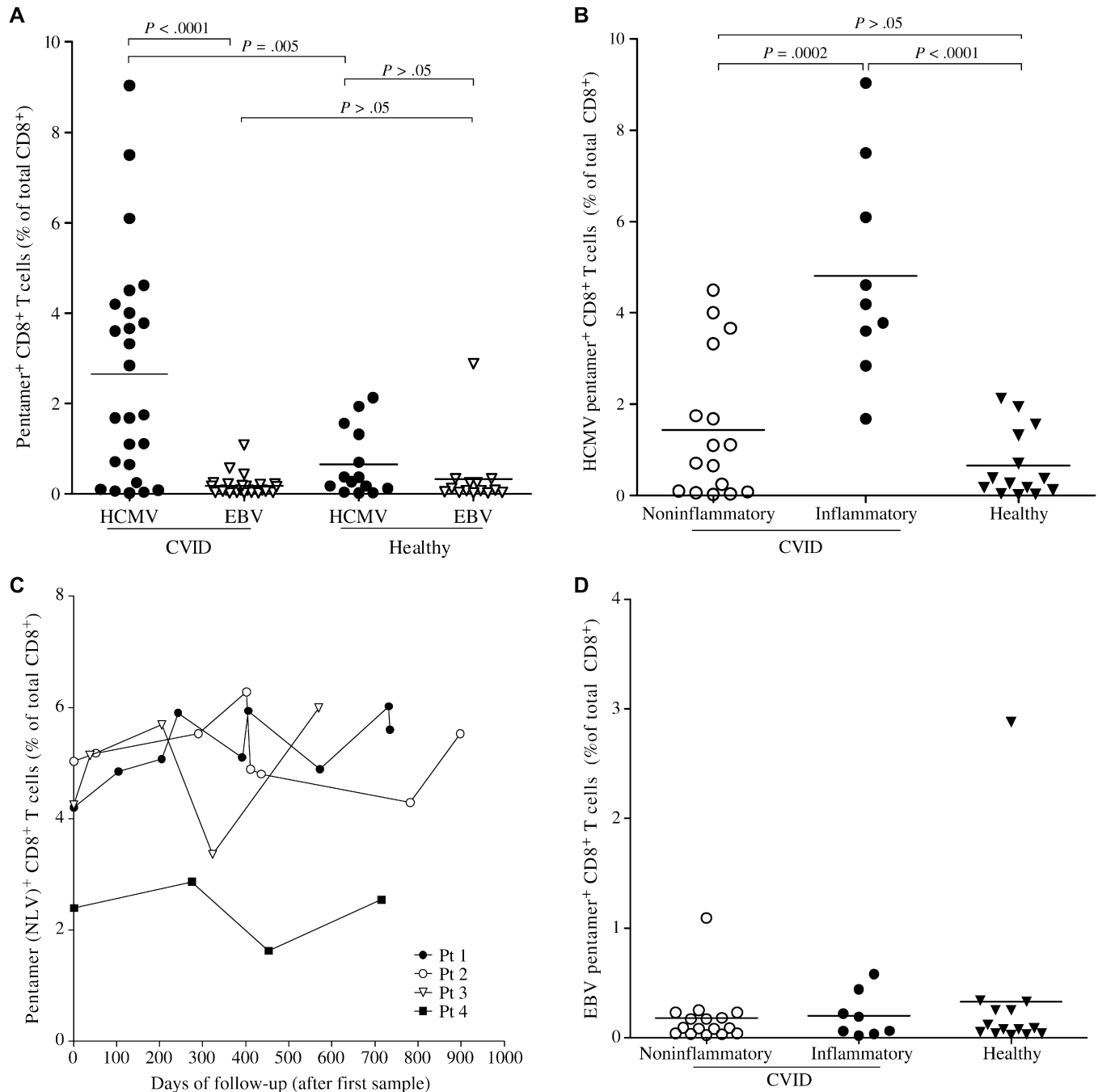


FIG 1. A, Percentage of CMV (NLV) or EBV (GLC) pentamer⁺ cells in patients with CVID or healthy controls. B and D, Data from A showing inflammatory and noninflammatory groups. C, Frequency of CMV (NLV) pentamer⁺ cells in serial samples from 4 patients (Pt). Lines in A, B, and D indicate means.

inflammatory patients around 40% Ki-67 positive (Fig 2, D; Fig E2, C). This elevation was not seen with EBV GLC pentamer-positive cells (data not shown). Frequencies of CD57⁺ CMV NLV pentamer⁺, but not EBV GLC pentamer⁺, CD8⁺ T cells were elevated in the inflammatory subgroup (Fig E2, D and E), as were HLA-DR⁺ CD8⁺ T cells and HLA DR⁺ CMV NLV pentamer⁺ CD8⁺ T cells (Fig 2, E and F).

To investigate further the association between activated cycling CMV-specific CD8⁺ T cells and inflammatory CVID, we measured expression of CD73, a marker of anti-inflammatory

function. The 2 CVID patient groups and the healthy controls had similar frequencies of CD73⁺ CD8⁺ T cells (Fig 2, G). However, we saw a striking distinction between the 2 CVID groups with CD73⁺ NLV pentamer⁺ CD8⁺ T-cell frequencies significantly lower in inflammatory patients than in noninflammatory patients or in healthy controls (inflammatory: mean, 1.67%, SD, 0.89; noninflammatory: mean, 42.01%, SD, 8.7%, $P = .004$; healthy: mean, 19.89%, SD, 7.94%, $P = .03$; Fig 2, H). There was no such distinction on EBV GLC pentamer⁺ CD8⁺ T cells (data not shown).

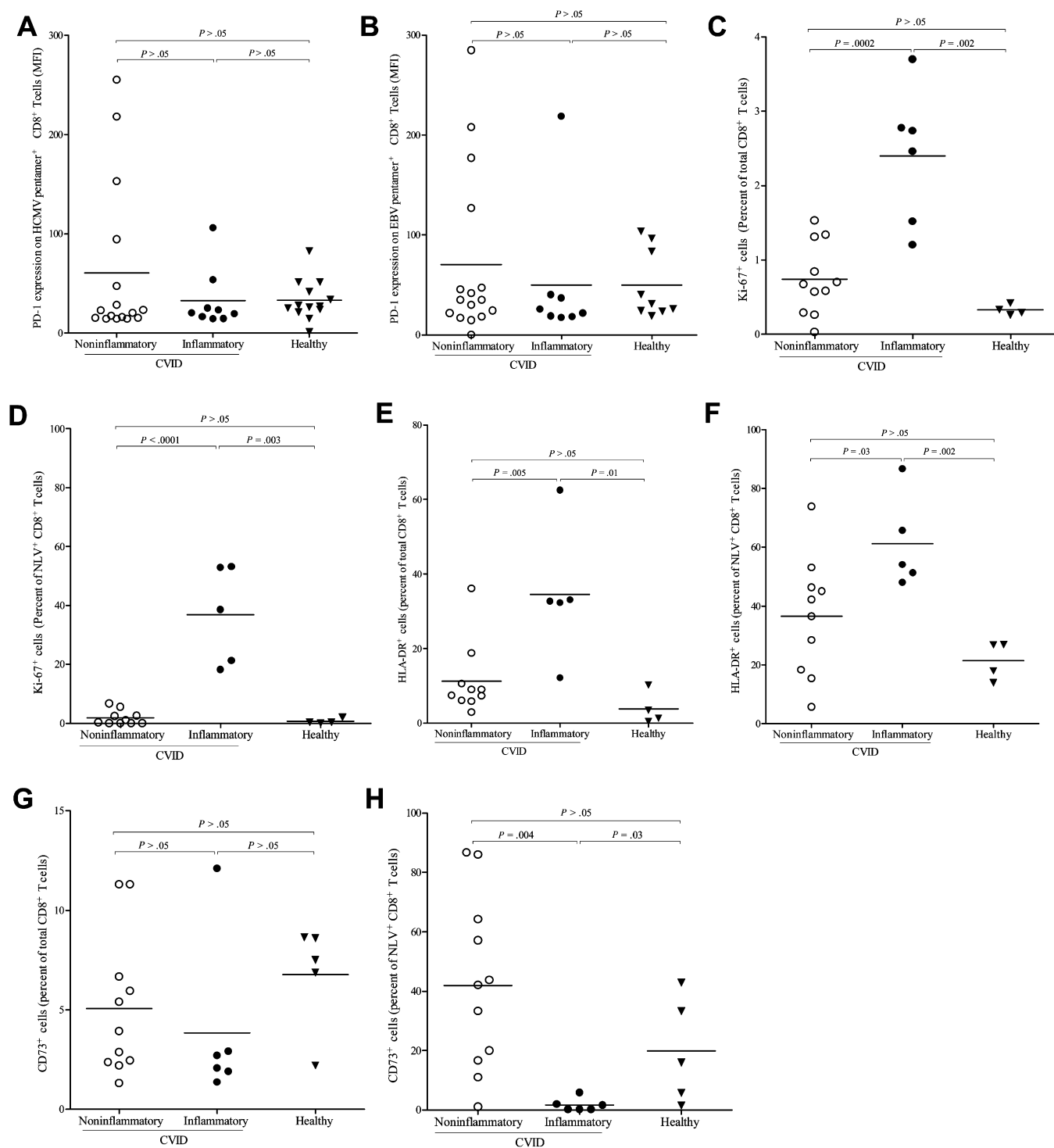


FIG 2. Phenotyping of CMV NLV-specific CD8⁺ T cells. **A and B**, PD-1 (median fluorescence intensity [MFI]). **C and D**, Ki-67. **E and F**, HLA-DR. **G and H**, CD73. Bars indicate mean percentage values.

Proliferative potential of CMV-specific CD8⁺ T cells in patients with CVID

In view of the high proportion of cycling CMV NLV-specific CD8⁺ T cells in the patients with inflammatory CVID, we next investigated their proliferative potential. Initially, in a conventional assay using 5-day stimulation of CFSE-labeled PBMCs

with the pp65 peptide pool in the presence of exogenous IL-15, we observed expansion of CMV-specific T cells in both groups of patients with CVID (data not shown). We next assessed proliferation of CD8⁺ T cells in response to peptide stimulation in the absence of exogenous cytokines or costimulators. PBMCs from patients with inflammatory (n = 6) or noninflammatory (n = 8)

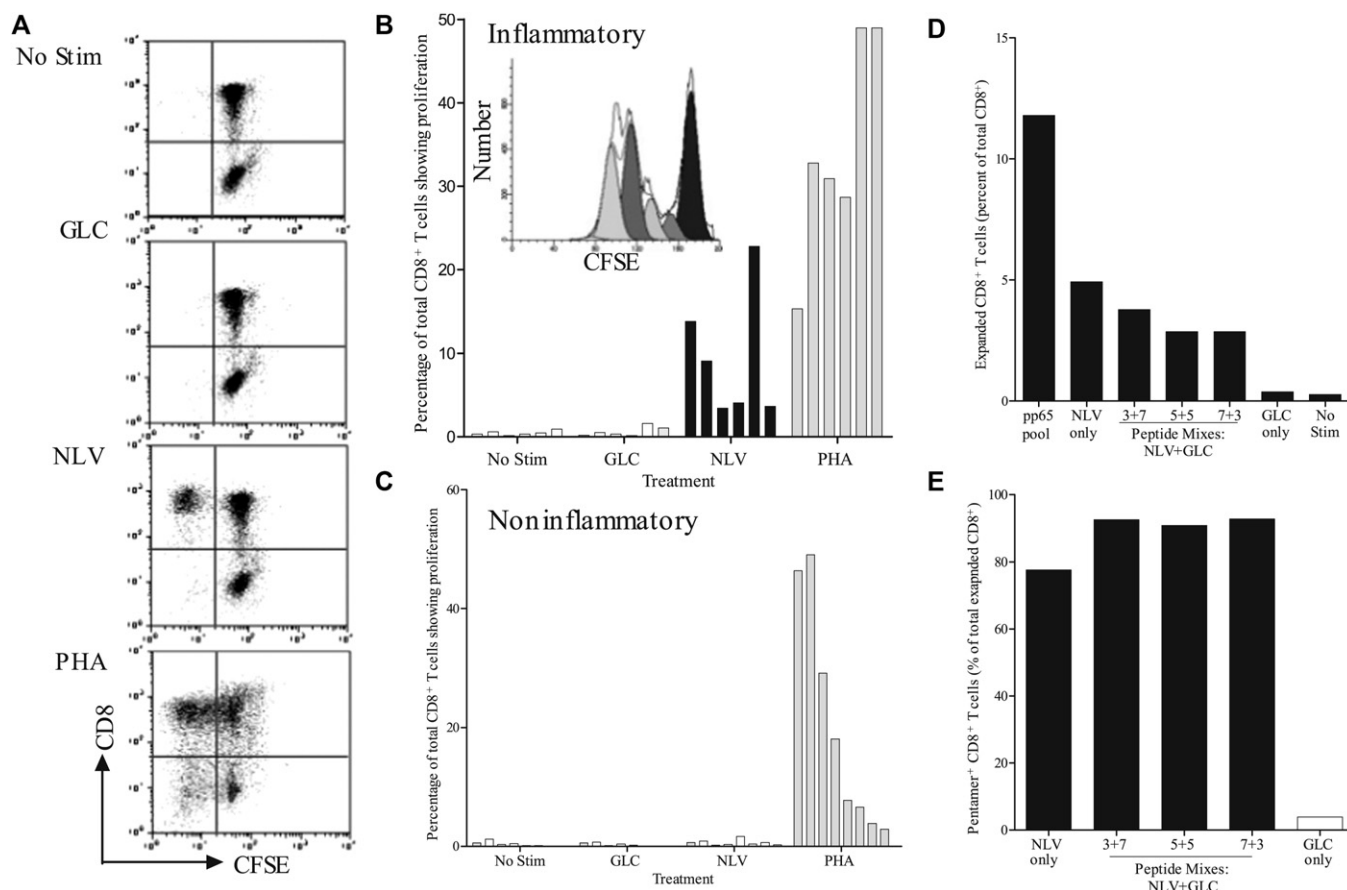


FIG 3. Proliferation of CMV-specific CD8⁺ T cells. **A**, Representative fluorescence-activated cell sorting plots. **B and C**, Analysis of inflammatory (n = 6) and noninflammatory (n = 8) samples. Insert in **B** shows generational analysis after NLV stimulation. **D**, Mixed peptide stimulation. **E**, Specificity (CMV, black; EBV, white) of expanded cells from **D**. Data obtained 5 days after stimulation. *PHA*, Phytohemagglutinin; *Stim*, stimulation.

CVID were stimulated with phytohemagglutinin, CMV NLV peptide, or EBV GLC peptide. NLV peptide-driven CD8⁺ T-cell proliferation was observed in all samples from patients with inflammatory CVID after 5-day stimulation with CMV NLV peptide (Fig 3, A and B). This autonomous proliferation was not observed in samples from patients with noninflammatory CVID (Fig 3, C) or healthy controls (data not shown). The extent of CD8⁺ T-cell proliferation was equivalent to 5 divisions, consistent with a doubling time of approximately 24 hours (Fig 3, B, insert). Similar results were obtained after stimulation with pp65 peptide pool (data not shown). No expansion was observed after stimulation with EBV GLC peptide (Fig 3, A-C). Combined stimulation of PBMCs from inflammatory patients using different ratios of the CMV and EBV peptides showed that the majority (>95%) of the proliferated cells were CMV-specific and that, by extension, the EBV-specific CD8⁺ T cells did not proliferate even in an environment favoring proliferation of CMV-specific cells (Fig 3, D and E).

Binding avidity of CMV-specific CD8⁺ T cells in patients with CVID

The capacity of CMV NLV-specific CD8⁺ T cells from inflammatory patients to undergo autonomous proliferation in response

to peptide stimulation may indicate an enhanced functional sensitivity.²³ To assess this, we used CD8 binding site-mutated (null) and normal class I HLA tetramers refolded with the NLV peptide to estimate the avidity of the HLA class I-T-cell receptor interactions on CD8⁺ T-cell populations present in the different groups of patients with CVID (see this article's Fig E3 in the Online Repository at www.jacionline.org). In 4 of 5 patients with inflammatory CVID studied (Fig E3, A and B), the normal and null CMV tetramers identified similar numbers of cognate CD8⁺ T cells, indicating that these are mainly high-avidity cells (ie, CD8-independent). In contrast, in patients with noninflammatory CVID (Fig E3, C and D), the cognate CD8⁺ T cells were mostly CD8-dependent (ie, did not bind the null tetramer).

Detection of CMV in inflamed tissues

Using sensitive immunohistochemistry, we analyzed colon biopsies from 2 patients with histologic features of mild/moderate colitis and granulomatous disease, classified as inflammatory CVID. All biopsies were positive for CMV immediate-early (IE) and late antigens, the latter indicative of active virus replication, with 40% to 70% of cells in any given field expressing viral IE antigen in their nuclei and 5% to 10% cells expressing viral late antigen. A variety of cells expressed viral antigens, including

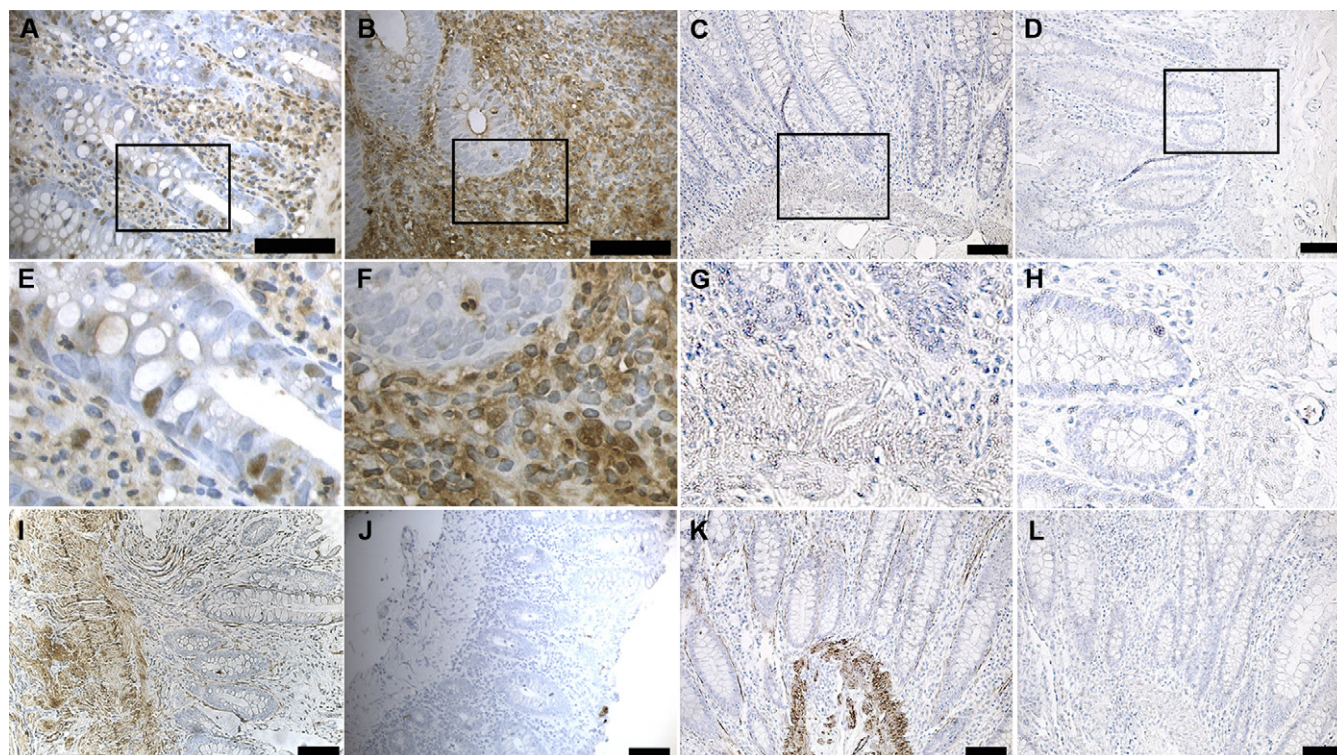


FIG 4. Immunohistochemical detection of CMV antigens. Colon tissue from representative patient with inflammatory CVID (**A, B, E, F, I, J**) or healthy control subject (**C, D, G, H, K, L**) was stained for CMV IEA (**A, C, E, G**) or LA (**B, D, F, H**); α -actin (**I, K**) or processed without primary antibody (**J, L**). **E, F, G, and H** are enlargements of areas boxed in **A, B, C, and D**, respectively. Brown staining (diaminobenzidine chromogen) indicates presence of antigen. Original magnification $\times 40$ (**A and B**); $\times 20$ (**C, D, I-L**). Bars are 50 μ m.

vascular endothelium, glandular epithelium, smooth muscle cells in the vessel wall, and inflammatory cells, all known targets for CMV (Fig 4). We have seen a similar pattern of staining in a liver biopsy from a patient with CVID with liver disease (data not shown). Three control colon biopsies from healthy bowel (Fig 4) as well as kidney biopsies from CMV-negative renal transplant recipients (data not shown) were uniformly negative.

DISCUSSION

Inflammatory disease is a significant and persistent burden for many patients with CVID. In this study, we have demonstrated that (1) inflammatory disease is significantly more prevalent in patients with evidence of CMV exposure; (2) CMV-specific CD8⁺ T-cell frequencies are specifically elevated in patients with inflammatory CVID; (3) CMV-specific CD8⁺ T cells from patients with inflammatory CVID show evidence of substantial proliferation *in vivo* and respond rapidly to antigen *in vitro*; and (4) CMV antigens can be directly detected in inflamed organ systems. Taken together, these results strongly suggest that the combination of CMV replication in the organ, and a unique CMV-specific CD8⁺ T-cell response directly results in the inflammatory disease.

Until now, there has been no rational explanation for the frequent occurrence of many of the inflammatory complications associated with CVID^{14,24,25} or the persistent lymphocyte activation or oligoclonal expansions of activated CD8⁺ T cells described in a

subset of patients with CVID.²⁶⁻²⁹ This has been compounded by the inability to provide evidence of pathogens in inflamed organs by using conventional histology/immunohistochemistry despite gene array analysis of PBMCs suggesting that the T cells in these patients with CVID are responding to a persistent stimulus.³⁰ We have not detected evidence of CMV DNAemia by using sensitive quantitative PCR approaches, but CMV-associated gastrointestinal disease can occur in the absence of viraemia^{22,31} and, using an enhanced immunohistochemistry approach,^{21,32} we provide the first direct evidence of actively replicating virus in the inflamed tissues. On the basis of the data presented here, we conclude that CMV infection is a major factor accounting for the aforementioned observations. Although there were patients with inflammation in the CMV-negative group, much of this was attributable to splenomegaly. A role for other infectious agents in the inflammation or an unusually low CD4⁺ T-cell response to CMV in these patients cannot be excluded. These remain formal possibilities.

Because persistent CMV replication in inflamed tissues could lead to an environment in which CMV-specific CD8⁺ T cells are driven to immune exhaustion, we assessed the phenotype and proliferative capacity of the CD8⁺ T cells from patients with inflammation compared with patients with CVID without inflammation. T-cell exhaustion, especially of high-avidity cell populations, has been described in HIV, hepatitis C virus, and lymphocytic choriomeningitis virus infections and in other settings.³³⁻³⁵ We showed that CMV NLV-specific CD8⁺ T cells in patients with inflammation were of high avidity, predominantly

IFN- γ and TNF- α coproducers, and able to degranulate on stimulation, implying good functional capacity.^{36,37} The majority expressed HLA-DR and CD57, but not PD-1, a phenotype not consistent with an exhausted population but rather one that is able to proliferate and maintain full functionality. Frequencies of PD-1 expression on CMV NLV-specific CD8⁺ T cells in the noninflammatory patients were significantly elevated, suggesting that these cells would be less able to proliferate and mediate inflammatory processes. To investigate this further, we analyzed Ki-67 expression and found it to be very high on CMV-specific T cells (average of 37%) compared with patients with noninflammatory CVID and healthy controls, implying that a high proportion of the CMV-specific T cells were actively cycling. When PBMCs were cultured with CMV peptides in the absence of exogenous cytokines such as IL-15, we observed that CMV CD8⁺ T cells from patients with CVID with inflammation proliferated with a doubling time of approximately 1 day, whereas cells from the other groups showed no proliferation under these conditions. Interestingly, there was no proliferation in response to EBV peptides in parallel experiments, indicating that this was a CMV-specific effect. These data confirm the substantial proliferative capacity of the CD8⁺ T cells in patients with CVID and underline the fact that the CD8⁺ T-cell response is not exhausted. The mechanism behind the enhanced proliferative capacity in these experiments is unknown but could include differences in avidity, cytokine production (which could have an autocrine effect *in vitro*), cytokine responsiveness, and response to endogenous costimulation or protection against cell death.

Further supporting a role of CMV-specific CD8⁺ T cells in the inflammatory disease seen in patients with CVID was our observation that the frequency of CD73⁺ CMV NLV pentamer⁺ CD8⁺ T cells was significantly reduced in inflammatory patients. CD73 is an ecto-5-nucleotidase that acts in the purine salvage pathway to generate nucleosides such as adenosine, which has anti-inflammatory properties.^{38,39} Although relatively little is known of the functional implications of CD73 expression in CD8⁺ T cells, our data suggest that downregulation of CD73 may well affect the capacity of these cells to mediate inflammatory processes. Indeed, in a recent study, *Borrelia garinii* infection of CD73^{-/-} mice was associated with joint swelling, and the authors suggested that low levels of adenosine may favor local inflammatory responses and persistent infection.⁴⁰

Although much of our data have been generated by using pp65 A*0201-restricted pentamers, where possible we have also performed experiments by using pp65 and IE1 peptide pools with multiple HLA specificities and have seen comparable results.

Our data suggest that the inflammation observed in these patients with CVID likely reflects the combination of intraorgan CMV infection and an aggressive and sustained CD8⁺ T-cell response. In this context, treatment of patients with CVID with anti-TNF- α therapy (infliximab) reduces inflammatory disease^{22,41} and inhibition of CMV replication with the antiviral drug ganciclovir has a similar effect.¹¹ Indeed, in preliminary studies, we have observed that a marked reduction in CMV NLV pentamer⁺ CD8⁺ T-cell frequencies (4% of CD8⁺ T cells pretherapy to 0.3% posttherapy) parallels clinical improvement in a patient with inflammatory CVID undergoing antiviral therapy. Thus, removal of the antigen or suppression of the inflammatory T-cell response can be effective in controlling inflammatory disease in this setting.

Conclusion

Our data provide a compelling evidence base in support of controlled clinical trials of treatment protocols targeting both sides of this virus-host interaction and emphasize the importance of considering CMV status both in patient treatment and in laboratory studies of patients with CVID. In addition, we suggest that the CD8⁺ T-cell response to CMV in CVID, and in particular the differences we have observed between the 2 CVID patient groups in the response to CMV, but not EBV, provide a powerful and tractable model of immune regulation.

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Clinical implications: The authors describe an association between CMV and inflammatory CVID. Our results provide a rationale for investigations of antiviral or anti-inflammatory interventions for management of the inflammatory complications of CVID.

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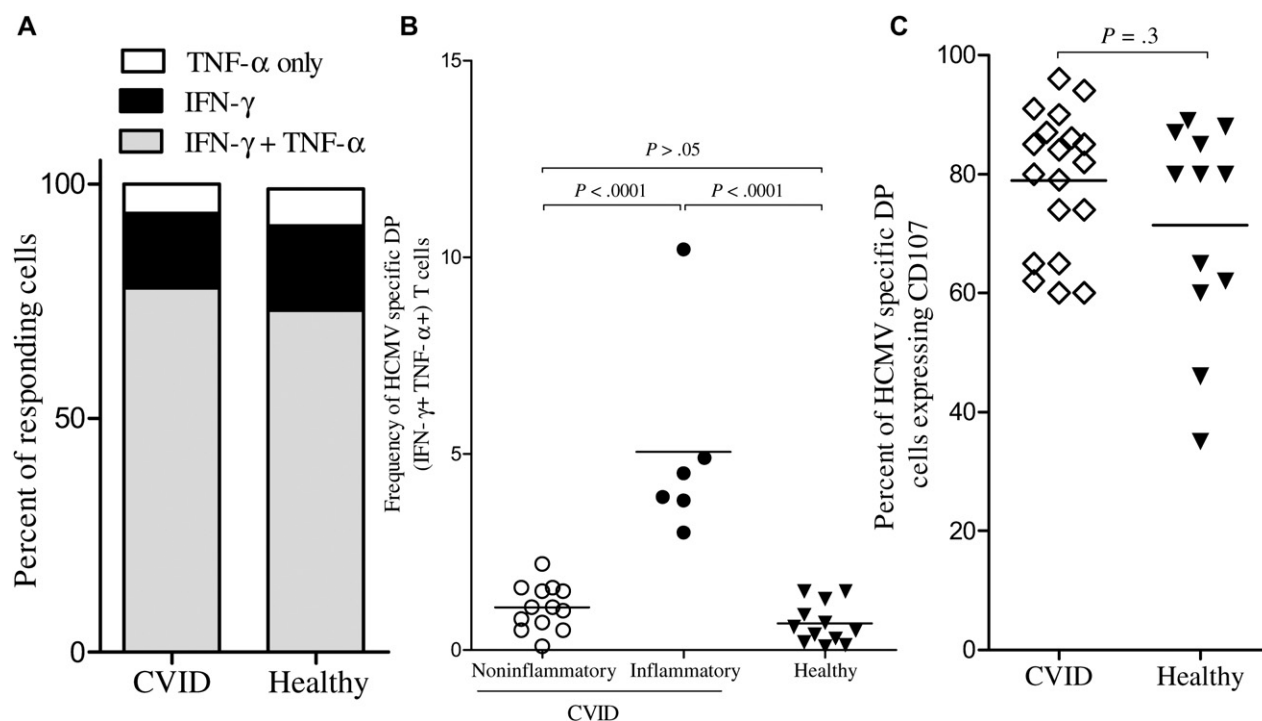


FIG E1. Cytokine production and degranulation. **A**, Proportion of responding CD8⁺ T cells producing IFN- γ , TNF- α , or both after pp65 peptide pool stimulation. (Means from 19 patients with CVID and 12 healthy controls.) **B**, Frequency of double producers (DP) among total CD8⁺ cells. **C**, Percentage of double producers that degranulated. Bars in **B** and **C** indicate means.

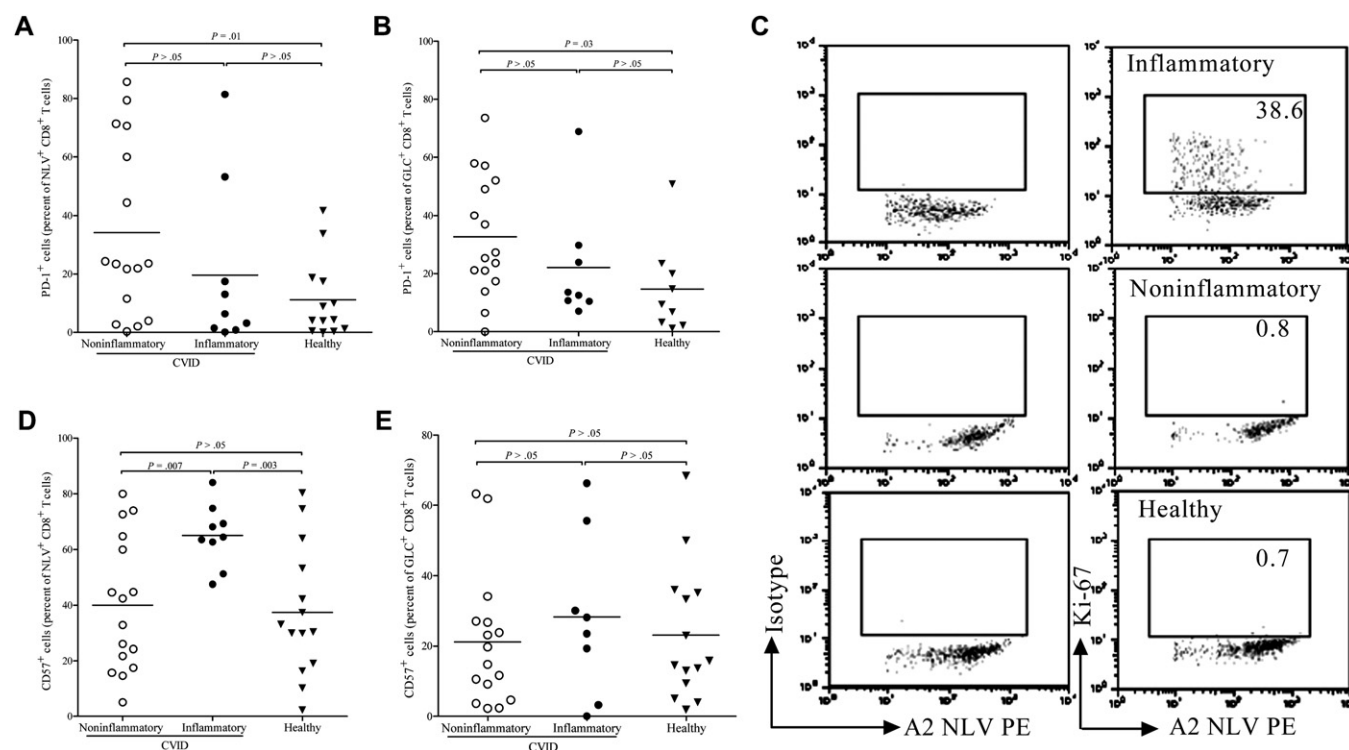


FIG E2. Phenotyping of CMV NLV-specific CD8⁺ T cells. **A and B**, PD-1 (frequency). **C**, Representative fluorescence-activated cell sorting plots for anti-Ki-67 and isotype control staining on CMV NLV pentamer⁺ CD8⁺ T cells. **D and E**, CD57 (frequency). Bars in **A**, **B**, **D**, and **E** indicate mean percentage values. PE, Phycoerythrin.

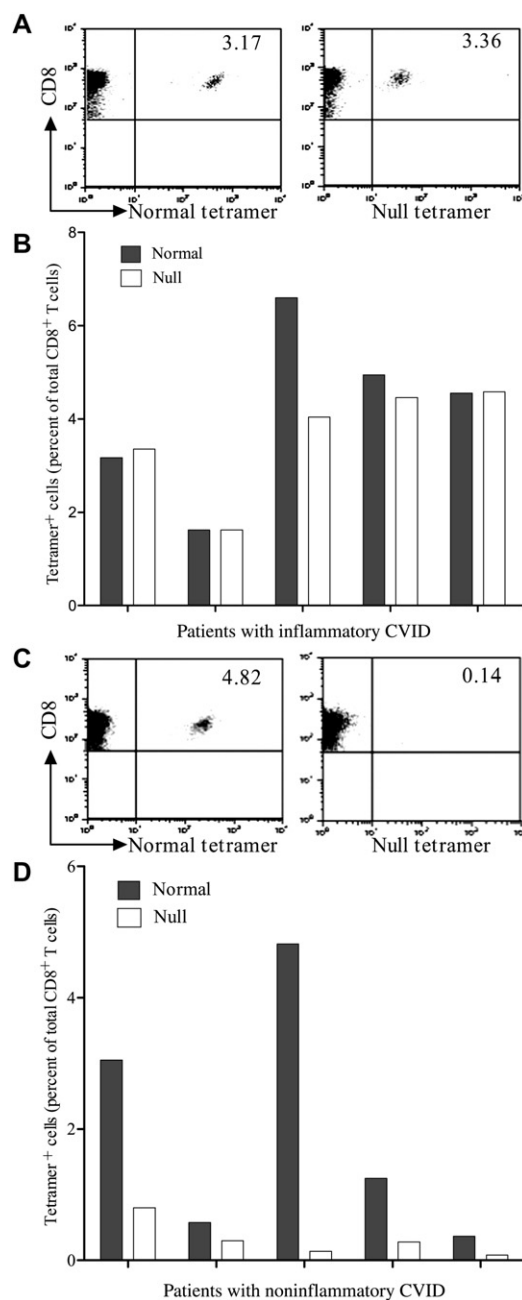


FIG E3. High-avidity CMV NLV-specific CD8⁺ cells in inflammatory CVID. **A and C,** Representative fluorescence-activated cell sorting plots of normal and null tetramer staining for an inflammatory (A) and a noninflammatory patient (C). **B and D,** Bar graphs showing the percentage of total CD8⁺ T cells recognized by the normal (black) and null (white) tetramers in patients with inflammatory (B) and noninflammatory (D) CVID.

TABLE E1. Clinical manifestations of inflammatory disease according to CMV status

Complications	CMV status		P value
	Exposed (n = 43)	Naive (n = 31)	
a. Large spleen*	19	7	.04†
b. Liver disease	11	2	.03†
c. Interstitial lung disease	10	2	.04†
d. Bowel disease	11	2	.03†
Tumors	9	1	.02†
Autoimmune disease	9	6	NS†
One or more of a, b, c, and d	31	8	.0001‡
CD4/8 ratio	1.08	1.90	.0005§

NS, Not significant.

a. Large spleen, >13 cm length and/or palpable below costal margin.

b. Liver disease, alkaline phosphatase >200 U/L (all biopsies showed abnormal histology).

c. Interstitial lung disease, unexplained fibrosis/granulomatous infiltration.

d. Bowel disease, chronic unexplained diarrhea and/or enteropathy on histology.

In the autoimmune disease category, 3 CMV-exposed and 1 CMV-naive patient with idiopathic thrombocytopenic purpura also had autoimmune hemolytic anemia.

In the tumor category, 5 of 9 tumors in the CMV-exposed patients were B-cell lymphomas.

*Two CMV-exposed and 1 CMV-naive patient had a splenectomy for autoimmune disease (spleen size not known) and were therefore excluded when comparing splenomegaly between the 2 groups.

†Fisher exact test, 1-tailed.

‡Fisher exact test, 2-tailed.

§Mann-Whitney.