

# Influence of cytomegalovirus infection on immune cell phenotypes in patients with common variable immunodeficiency

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**Background:** A subset of patients with common variable immunodeficiency (CVID) have debilitating inflammatory complications strongly associated with cytomegalovirus (CMV) infection and a hyperproliferative CMV-specific T-cell response. **Objectives:** We studied the T-cell response to CMV and the global effect of this virus on immune effector cell populations in patients with CVID.

**Methods:** Antibody staining, peptide stimulation, and proliferation assays were used to profile CMV-specific T-cell function.

**Results:** CMV infection drives the CD4/CD8 ratio inversion that is characteristic of CVID. The late effector CD8<sup>+</sup> T-cell subset is expanded in CMV-infected patients with CVID. This expansion is largely attributable to CMV-specific cells and correlates with inflammatory disease; within the CMV-specific population, the frequency of late effector cells correlates inversely with the frequency of cells expressing programmed death 1. Supernatants from proliferating CMV-specific CD8<sup>+</sup> cells from patients with inflammatory disease can confer proliferative potential on cells from patients with noninflammatory CVID and healthy subjects. Blocking experiments showed that this proliferation is mediated in part by IFN- $\gamma$  and TNF- $\alpha$ .

**Conclusions:** These data strengthen the association of CMV with inflammatory pathology in patients with CVID, explain

some of the well-known T-cell abnormalities associated with this condition, and provide a plausible mechanism for the documented therapeutic activity of anti-TNF- $\alpha$  and antiviral chemotherapy in managing CVID-associated inflammatory disease. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

**Key words:** Human cytomegalovirus, common variable immunodeficiency, programmed death 1, late effector T cell, CD27, CD28, polyfunctional T cell, inflammatory disease

Common variable immunodeficiency (CVID), a primary immunodeficiency, is characterized by defective antibody production and a consequent increased susceptibility to infection.<sup>1-4</sup> A variety of T-cell abnormalities are also present, and patients have suboptimal responses to keyhole limpet hemocyanin. Two subgroups can be defined: one with no major defects in circulating T cells and another with variable degrees of CD4 T-cell lymphopenia, activated CD8 T cells, and inversion of the CD4/CD8 ratio. We have recently shown that cytomegalovirus (CMV), a betaherpesvirus that infects between 60% and 90% of the adult population and is a major pathogen in the T-cell immunocompromised host, is also intimately associated with CVID-associated inflammatory disease. The latency and persistence of CMV in the human host is facilitated by a number of viral proteins that interfere with adaptive and innate immunity,<sup>5-7</sup> and the virus has the ability to modulate a range of cellular functions, including the cell cycle, apoptosis, and proteins involved in the inflammatory vascular response, including matrix metalloproteinases, leukotrienes, and tissue inhibitors of metalloproteinases.<sup>8</sup> Taken together, these data emphasize the proinflammatory nature of CMV infections, and the human host devotes a substantial proportion of its adaptive and innate immune systems to the control of infection to minimize such effects. In patients with CVID with concurrent inflammatory disease, we have found that the CMV CD8 T-cell response is increased further over that detected in healthy subjects and is skewed toward the production of IFN- $\gamma$  and TNF- $\alpha$ . These CMV CD8 T cells are highly proliferative both *in vivo* and *ex vivo* and express low levels of both programmed death 1 (PD-1) and the anti-inflammatory marker CD73. This phenotype contrasts with that found in transplant recipients at risk of CMV replication, in whom CD8 T cells are generally PD-1 high but express relatively low levels of IFN- $\gamma$ . To complement our previous studies in patients with CVID, we have investigated the role that CMV infection and inflammation plays in shaping the B- and T-cell phenotype and identify the contribution of specific proinflammatory cytokines involved in the hyperproliferation of

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**Abbreviations used**

APC: Allophycocyanin  
 CMV: Cytomegalovirus  
 CVID: Common variable immunodeficiency  
 FITC: Fluorescein isothiocyanate  
 PD-1: Programmed death 1  
 PE: Phycoerythrin

CMV CD8 T cells seen in patients with CVID with concurrent inflammation.

**METHODS****Study populations**

Seventy-one patients with CVID (35 male patients; age, 24–86 years; median, 52 years) attending the Royal Free Hospital and 19 healthy subjects (7 male subjects; age, 25–71 years; median, 45 years;  $P > .05$  vs patients with CVID) were analyzed. CVID was defined according to the International Union of Immunological Societies criteria.<sup>9,10</sup> All patients with CVID received standard immunoglobulin therapy, and blood samples for T-cell studies were collected before immunoglobulin treatment. CMV status was determined by means of serology for healthy subjects or, for patients with CVID in whom results of serologic assays are uninformative, by means of measurement of virus-specific CD4<sup>+</sup> T-cell responses, as previously described.<sup>10,11</sup> Three patients (1 CMV-positive and 2 CMV-negative patients) were excluded from analysis because of the presence of mutations in the TNF receptor family member transmembrane activator and cyclophilin ligand interactor gene (*TNFRSF13B*). A subset of the CMV-positive patients (25 patients with CVID and 14 control subjects) were included in further studies of T-cell function. All were HLA-A\*0201 positive, and there was no significant difference in age or sex between the CVID group (age, 40–69 years; median, 55 years; 57% male) and the healthy group (age, 34–60 years; median, 49 years; 60% male). All participants had CMV and EBV infections. The CVID group was subdivided into patients with ( $n = 9$ ) and without ( $n = 16$ ) inflammatory disease based on established criteria.<sup>10</sup>

**Ethics statement**

This research was approved by the local research ethics committee of the Royal Free Hospital (reference no. 04/Q0501/119). Written informed consent was obtained from all patients.

**Antibodies, peptides, and MHC pentamers**

Peptides representing the HLA-A\*0201-restricted viral epitopes NLVPMVATV (NLV), which is derived from the CMV tegument protein ppUL83(pp65), and GLCTLVAML (GLC), which is derived from the EBV lytic protein BMLF1, as well as phycoerythrin (PE)-conjugated HLA class I pentamers containing these peptides, were obtained from ProImmune (Oxford, United Kingdom).

The following mAbs were used:

1. *T-cell cocktail*: anti-CD3–peridinin-chlorophyll protein, anti-CD4–fluorescein isothiocyanate (FITC), anti-CD8–allophycocyanin (APC), anti-CD25–PE, anti-CD4–peridinin-chlorophyll protein (BD Biosciences, Oxford, United Kingdom), anti-CD45RO–APC, anti-CD45RA–APC, anti-CD27–FITC, anti-CD28–PE, anti-CD31–PE, anti-T-cell receptor  $\alpha\beta$ –PE, anti-CD127–FITC, and anti-CXCR5–PE (BD Biosciences).
2. *CD8 T-cell phenotyping*: anti-CD8 PerCp-cyanin 5.5 (Cy5.5), anti-PD-1 FITC, anti-granzyme B–APC, anti-IFN- $\gamma$ –APC, anti-TNF- $\alpha$ –PE, anti-IL-2–FITC, and anti-CD28 (all from BD Biosciences).
3. *B-cell surface phenotyping*: anti-CD19–phycoerythrin-cyanin 7 (Beckman Coulter, High Wycombe, United Kingdom); anti-CD21–PE, anti-CD38–FITC, and anti-IgM–cyanin 5 (BD Biosciences);

anti-CD27–FITC (Dako Cytomation, Glostrup, Denmark); and anti-IgD–PE (Oxford Biotechnology, Oxford, United Kingdom).

**Isolation of PBMCs from peripheral blood**

PBMCs were separated from fresh whole blood (20–25 mL) containing preservative-free heparin by using Ficoll-Paque centrifugation (778g for 22 minutes at room temperature) and resuspended in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated FCS.

**Flow cytometric analysis of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets**

Routine monitoring of immunologic parameters (total cell counts and T-cell and B-cell phenotyping) was performed by using the Clinical Immunology Service of the Royal Free Hospital with fresh EDTA blood. All data were acquired on a FACSCalibur 2 (Becton Dickinson) by using the Cell Quest Pro software. Twenty thousand gated events were collected for each sample. For quality control, CaliBRITE Beads (BD Biosciences) were used. Isotype controls were analyzed in parallel with each batch of staining.

**Peptide stimulation of T cells**

Fresh PBMCs ( $1 \times 10^6$ /1 mL) were stimulated with 10  $\mu$ g/mL CMV or EBV peptides or no peptide as a negative control and incubated for 16 hours at 37°C in a 5% CO<sub>2</sub> atmosphere in the presence of anti-CD28 (10  $\mu$ g/mL) with the addition of 10  $\mu$ g/mL brefeldin A (Sigma, Poole, United Kingdom) after 2 hours. PBMCs were then stained with the anti-CD8 antibody, permeabilized/fixed with Caltag A and B reagents (Invitrogen, Paisley, United Kingdom), and stained with anti-IFN- $\gamma$ , anti-TNF- $\alpha$ , and anti-IL-2 at room temperature for 15 minutes; then fixed in 4% paraformaldehyde; and analyzed immediately on a FACSCalibur flow cytometer. A minimum of 50,000 events were acquired for each sample. All antibody assays were validated by using appropriate isotype controls. Subsequent analysis was with FlowJo software (Tree Star, Inc, Ashland, Ore). Boolean gating was used for combination analysis of the 7 possible subsets cells producing all 3 cytokines: IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 (ILT); those producing 2 cytokines: IFN- $\gamma$  and IL-2 (IL), IFN- $\gamma$  and TNF- $\alpha$  (IT), or IL-2 and TNF- $\alpha$  (LT); and those producing only 1 cytokine: IFN- $\gamma$  (I), IL-2 (L), or TNF- $\alpha$  (T).

**Class I HLA pentamer staining**

PBMCs ( $1 \times 10^6$ ) were stained with the appropriate HLA pentamer (10  $\mu$ g/mL) for 15 minutes at room temperature, washed with PBS containing 0.1% sodium azide, stained (15 minutes at room temperature) for surface markers (CD8 and PD-1), and then fixed and permeabilized with Caltag A and B and stained for granzyme B.

**CD8 T-cell culture supernatant transfer experiments**

Proliferation of carboxyfluorescein diacetate succinimidyl ester-labeled T cells after peptide stimulation was measured as previously described.<sup>10</sup> For supernatant transfer experiments, supernatants were taken from peptide-stimulated PBMC cultures at 48 hours after stimulation. One hundred microliters of filtered supernatant was included during stimulation of freshly isolated carboxyfluorescein diacetate succinimidyl ester-labeled PBMCs. Supernatants from PBMCs from healthy subjects or patients with noninflammatory CVID served as controls in these experiments. Cells were analyzed after 5 days of incubation. In some experiments purified anti-human TNF- $\alpha$  (0.08–0.10  $\mu$ g/mL) and anti-human IFN- $\gamma$  (0.13–0.20  $\mu$ g/mL) polyclonal antibodies (Peprotech, London, United Kingdom) or TNF- $\alpha$  and IFN- $\gamma$  (100 ng/mL) were included.

**Statistical analysis**

GraphPad Prism software (GraphPad Software, Inc, La Jolla, Calif) was used for plotting graphs and for statistical analysis, and the Student *t* test and

**TABLE I.** Demographic and laboratory characteristic of patients with CVID and healthy control subjects\*

|                                                                     | Patients with CVID    |                       | <i>P</i> value | Healthy control subjects (n = 19) |
|---------------------------------------------------------------------|-----------------------|-----------------------|----------------|-----------------------------------|
|                                                                     | CMV positive (n = 44) | CMV negative (n = 27) |                |                                   |
| Demographics                                                        |                       |                       |                |                                   |
| Age (y)                                                             | 56 (32-86)            | 48 (24-75)            | NA             | 45 (25-71)                        |
| Sex                                                                 |                       |                       |                |                                   |
| No. of male subjects                                                | 25                    | 10                    | NA             | 12                                |
| No. of female subjects                                              | 19                    | 17                    | NA             | 7                                 |
| Laboratory findings                                                 |                       |                       |                |                                   |
| CD3                                                                 |                       |                       |                |                                   |
| Absolute count†                                                     | 1.16 (0.3-4.09)       | 0.9 (0.5-5.41)        | .2             | 1.14 (0.8-2.07)                   |
| CD4                                                                 |                       |                       |                |                                   |
| Absolute count                                                      | 0.56 (0.1-1.36)       | 0.57 (0.27-1.36)      | .7             | 0.67 (0.4-1.36)                   |
| CD8                                                                 |                       |                       |                |                                   |
| Absolute count                                                      | 0.58 (0.15-2.8)       | 0.3 (0.1-1.3)         | .01            | 0.46 (0.2-0.85)                   |
| CD4 <sup>+</sup> /CD8 <sup>+</sup>                                  |                       |                       |                |                                   |
| % Lymphocytes                                                       | 3 (1-16)              | 3 (1-8)               | .5             | 2 (1-3)                           |
| CD19 <sup>+</sup> B cells                                           |                       |                       |                |                                   |
| % Lymphocytes                                                       | 10 (0-40)             | 11 (0-47)             | .7             | 15 (12-22)                        |
| Absolute count                                                      | 0.19 (0-1.77)         | 0.12 (0-0.77)         | .3             | 0.18 (0.05-0.5)                   |
| % IgM memory (CD27 <sup>+</sup> IgD <sup>+</sup> )                  | 10.13 (0.4-56)        | 10.19 (2-34.2)        | .3             | 11.41 (7.4-29.54)                 |
| % Switched memory (CD27 <sup>+</sup> IgD <sup>+</sup> )             | 1.49 (0-17.4)         | 3.2 (0.2-9.7)         | .6             | 15 (6.6-23.4)                     |
| % Naive (CD27 <sup>+</sup> IgD <sup>+</sup> [% CD19 <sup>+</sup> ]) | 83.8 (41.1-97.4)      | 80.3 (55.9-91.6)      | .9             | 73.59 (42.9-81.7)                 |
| % Transitional B cells                                              | 2.9 (0-35.1)          | 4.3 (0.25-17.3)       | .7             | 1.66 (0.17-3.3)                   |
| % Plasmablasts                                                      | 0.36 (0-5.2)          | 0.79 (0-9.3)          | .1             | 1.18 (0.4-3.3)                    |

*P* values refer to pairwise comparisons (Student *t* test) of indicated variables between the CMV-positive and CMV-negative groups.

NA, Not applicable.

\*Except where indicated, data shown are median values followed by interquartile ranges in parentheses.

<sup>†</sup>Absolute cell counts are  $\times 10^9/L$ .

2-way ANOVA were used as appropriate. Data in the table are reported as medians and interquartile range. *P* values of .05 or less were considered significant.

## RESULTS

### CMV infection is associated with inversion of the CD4/CD8 T-cell ratio in patients with CVID

The cellular immunologic profiles of the patients with CVID and healthy control subjects are shown in Table I. Differences between patients with CVID and healthy subjects are well documented<sup>12-15</sup>; here we separate them according to CMV status. Frequencies of switched memory and transitional B cells and plasmablasts were marginally reduced in CMV-positive patients compared with CMV-negative patients, but these changes were not statistically significant.

CD4 T-cell numbers in patients with CVID did not vary according to CMV status. Notably, however, CD8 T-cell numbers were increased in CMV-positive patients with CVID. Presumably, as a consequence of these changes, the mean frequency of CD4<sup>+</sup> T cells within the peripheral blood lymphocyte population was significantly reduced in CMV-positive patients with CVID compared with that seen in CMV-negative patients and healthy control subjects (mean  $\pm$  SEM: 35.39%  $\pm$  1.5% for CMV-positive patients vs 46.2%  $\pm$  2.3% for CMV-negative patients, and 46.26%  $\pm$  1.97% for healthy subjects; *P* = .0001; Fig 1, A). In contrast, CD8<sup>+</sup> T-cell frequencies were significantly increased in CMV-positive patients (38%  $\pm$  2.2% for CMV-positive patients vs 26.8%  $\pm$  2.03% for CMV-negative patients, and 27.1%  $\pm$  1.2% for healthy subjects; *P* = .0008 and *P* = .002, respectively; Fig 1, B).

Accordingly, the CD4/CD8 ratio was significantly reduced in patients with CMV infection (1.1  $\pm$  0.1 vs 1.9  $\pm$  0.18; *P* < .0001; Fig 1, C).

### Influence of CMV infection on CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets in patients with CVID

Given the association between CMV infection and inversion of the CD4/CD8 ratio, we next explored whether CD4<sup>+</sup> or CD8<sup>+</sup> T-cell subsets were altered in CMV-positive patients with CVID. CD4<sup>+</sup> memory T-cell (CD4<sup>+</sup>CD45RO<sup>+</sup>) frequencies were significantly higher in CMV-positive than in CMV-negative patients (79.3%  $\pm$  2.2% vs 70.1%  $\pm$  3.1%, *P* = .01), with frequencies in both groups of patients with CVID significantly higher compared with those seen in control subjects (Fig 2, A). This difference was not observed for CD4<sup>+</sup>CD45RO<sup>+</sup>CXCR5<sup>+</sup> T follicular helper cells (19.3%  $\pm$  1.6% for CMV-positive patients vs 20.1%  $\pm$  1.8% for CMV-negative patients; *P* > .05; Fig 2, B), although again this population of cells was significantly increased in patients with CVID (irrespective of CMV status) compared with that seen in healthy subjects (Fig 2, B).

Subsequently, we assessed the distribution of CD8<sup>+</sup> T cells between the effector (CD27<sup>+</sup>CD28<sup>-</sup>) and late effector (CD27<sup>-</sup>CD28<sup>-</sup>) subsets. The frequency of late effector CD8<sup>+</sup> T cells was significantly higher in CMV-positive patients with CVID compared with that seen in both CMV-negative patients with CVID (41.8%  $\pm$  3.6% vs 16.29% vs 3.04%, *P* < .0001) and healthy control subjects (41.8%  $\pm$  3.6% vs 21.26%  $\pm$  2.8%; *P* = .0004; Fig 2, C). In contrast, the frequency of effector CD8<sup>+</sup> T cells was significantly higher in CMV-negative

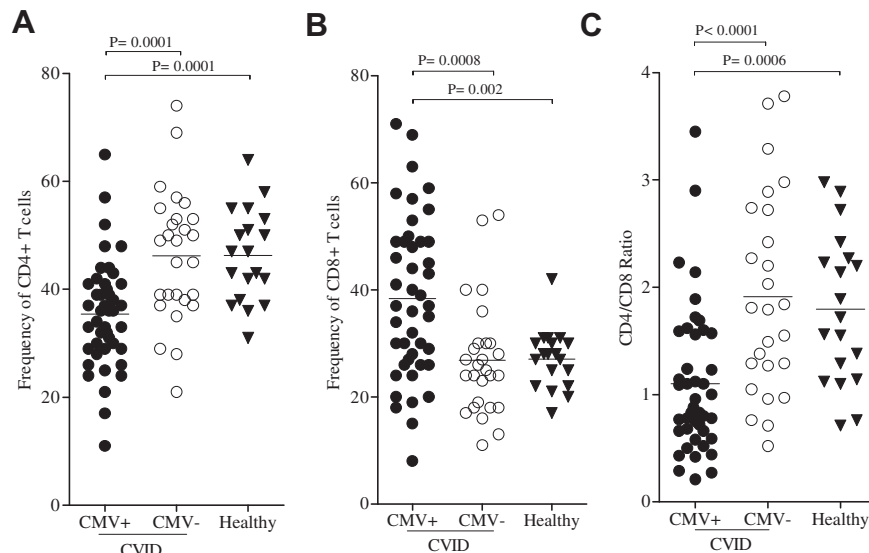


**FIG 1.** Analysis of CD4<sup>+</sup>/CD8<sup>+</sup> T-cell ratio. The frequency of CD4<sup>+</sup> (A) and CD8<sup>+</sup> (B) T cells as a percentage of total lymphocytes and the CD4<sup>+</sup>/CD8<sup>+</sup> ratio (C) are shown for patients with CVID (with and without CMV infection) and healthy control subjects. Bars indicate the mean percentage values. *P* values are for the indicated pairwise comparisons.

patients with CVID than in CMV-positive patients with CVID ( $17.76\% \pm 2.6\%$  vs  $11.3\% \pm 1.1\%$ ,  $P = .03$ ) and healthy subjects ( $17.76\% \pm 2.6\%$  vs  $9.0\% \pm 0.9\%$ ;  $P = .002$ ; Fig 2, D).

### Polyfunctional phenotype of total and CMV-specific CD8 T cells in patients with CVID

Previously, we showed an association between CMV infection, in particular an increased CD8<sup>+</sup> T-cell response to CMV, and the inflammatory phenotype of CVID.<sup>10</sup> When we analyzed the T- and B-cell phenotyping data described above in the context of this inflammatory phenotype, we observed a significant increase in the overall frequency of late effector CD8<sup>+</sup> T cells in patients with CVID with inflammatory diseases ( $P < .001$ ; Fig 3, A and B).

For a more subtle analysis of the CD8<sup>+</sup> T-cell response in this context, we focused on the HLA-A\*0201-restricted responses to CMV and, as a control, EBV. We observed a highly significant inverse correlation between the proportion of CMV NLV pentamer-positive cells expressing granzyme B or PD-1 and the overall frequency of late effector cells (Fig 3, C and D). No such correlations were seen with EBV GLC-specific cells (Fig 3, E and F) or between the percentage of late effector cells and the overall frequency of PD-1- or granzyme B-positive CD8<sup>+</sup> T cells.

There was also a significant inverse correlation between the frequency of PD-1-expressing NLV pentamer-positive cells and the frequency of NLV pentamer-positive late effector cells (Fig 3, G). Interestingly, CMV NLV pentamer-positive late effector CD8<sup>+</sup> T-cell frequencies were significantly higher in patients with inflammatory disease than in those without ( $65.6\% \pm 5.8\%$  vs  $24.16\% \pm 4.8\%$ ;  $P < .0001$ ; Fig 3, H).

We showed previously that the majority of CMV pp65-specific CD8<sup>+</sup> T cells in patients with CVID produce IFN- $\gamma$  and TNF- $\alpha$ .<sup>10</sup> Here we extended this observation to show that patients with CVID with inflammatory disease had significantly higher frequencies of IFN- $\gamma$ - and TNF- $\alpha$ -producing CD8<sup>+</sup> T cells compared with the noninflammatory group (IFN- $\gamma$ :  $3.0\% \pm 0.4\%$  for

the inflammatory group vs  $0.8\% \pm 0.20\%$  for the noninflammatory group;  $P < .0001$  [see Fig E1, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)]; TNF- $\alpha$ :  $1.46\% \pm 0.17\%$  for the inflammatory group vs  $0.52\% \pm 0.11\%$  for the noninflammatory group;  $P < .0001$  [see Fig E1, C, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)]). Frequencies of IFN- $\gamma$ - and TNF- $\alpha$ -producing CD8<sup>+</sup> T cells in the patients with CVID without inflammatory disease were similar to those observed in healthy control subjects (IFN- $\gamma$ :  $0.79\% \pm 0.20\%$  for the noninflammatory group vs  $0.49\% \pm 0.14\%$  for the healthy subjects;  $P = .23$  [see Fig E1, A]; TNF- $\alpha$ :  $0.52\% \pm 0.11\%$  for the noninflammatory group vs  $0.26\% \pm 0.07\%$  for the healthy subjects;  $P = .08$  [see Fig E1, C]). No differences were observed in frequencies of IFN- $\gamma$ - or TNF- $\alpha$ -producing CD8<sup>+</sup> T cells between any of the patient groups after EBV peptide stimulation (see Fig E1, B and D), and frequencies of IL-2-producing CD8<sup>+</sup> T cells were similar regardless of the stimulating peptide (CMV and EBV) or patient group (see Fig E1, E and F). IFN- $\gamma$  and TNF- $\alpha$  but not IL-2 production also correlated positively with numbers of CMV NLV pentamer-positive late effector CD8<sup>+</sup> T cells (see Fig E1, G-I).

Polyfunctional profiling of the CD8<sup>+</sup> T-cell cytokine response to pp65 NLV peptide stimulation revealed a striking focus among the patients with inflammatory CVID on IFN- $\gamma$ -producing cells, either as single producers or IFN- $\gamma$ /TNF- $\alpha$  double producers, with these 2 subsets comprising 79.8% of the total population of CMV NLV-specific CD8<sup>+</sup> T cells. There was no such focus in the patients with noninflammatory CVID, among whom the cytokine production profile was the same as that seen in healthy subjects (see Fig E2, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

Levels of IFN- $\gamma$  production (but not TNF- $\alpha$  or IL-2) by NLV-stimulated CD8<sup>+</sup> cells were generally increased in patients with CVID compared with those seen in healthy subjects. Notably, the triple cytokine-producing cells, which comprised a minimal proportion of the total response in all 3 sample groups, produced significant levels of IFN- $\gamma$  (see Fig E2, C).

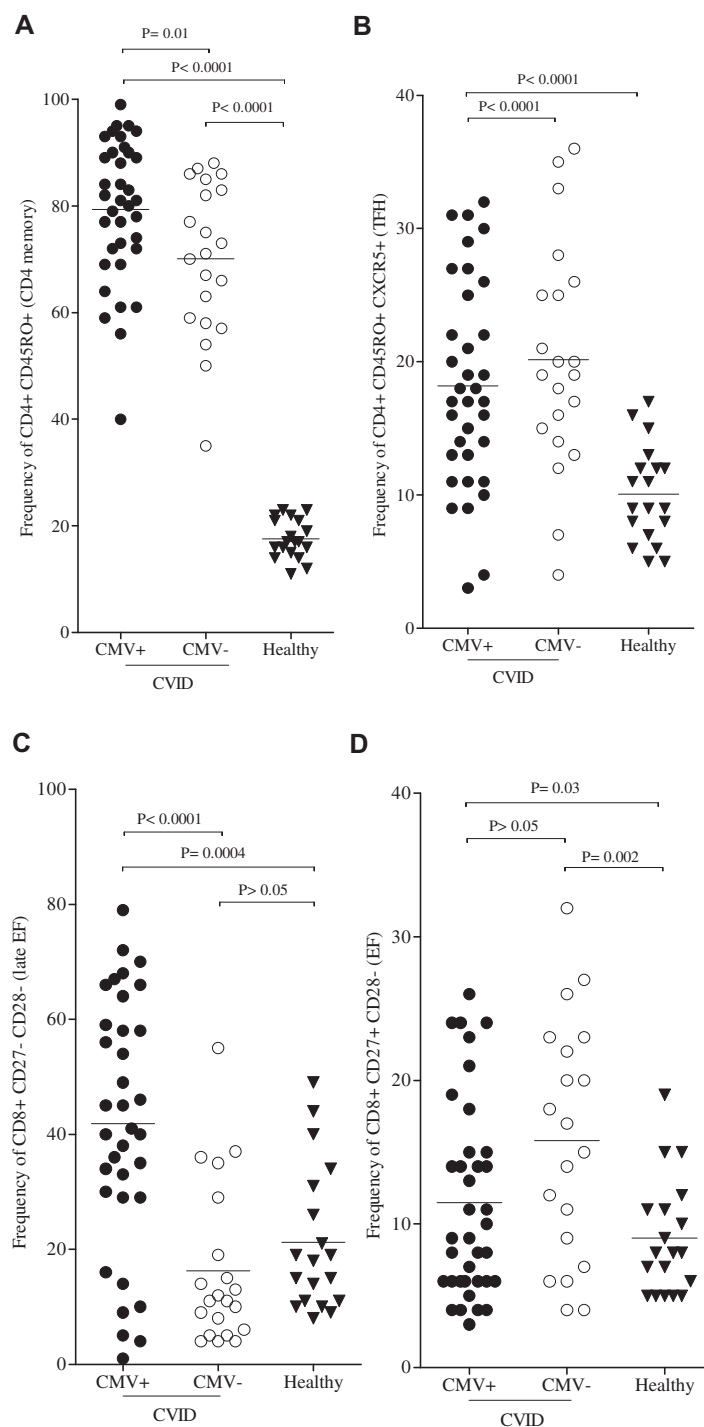
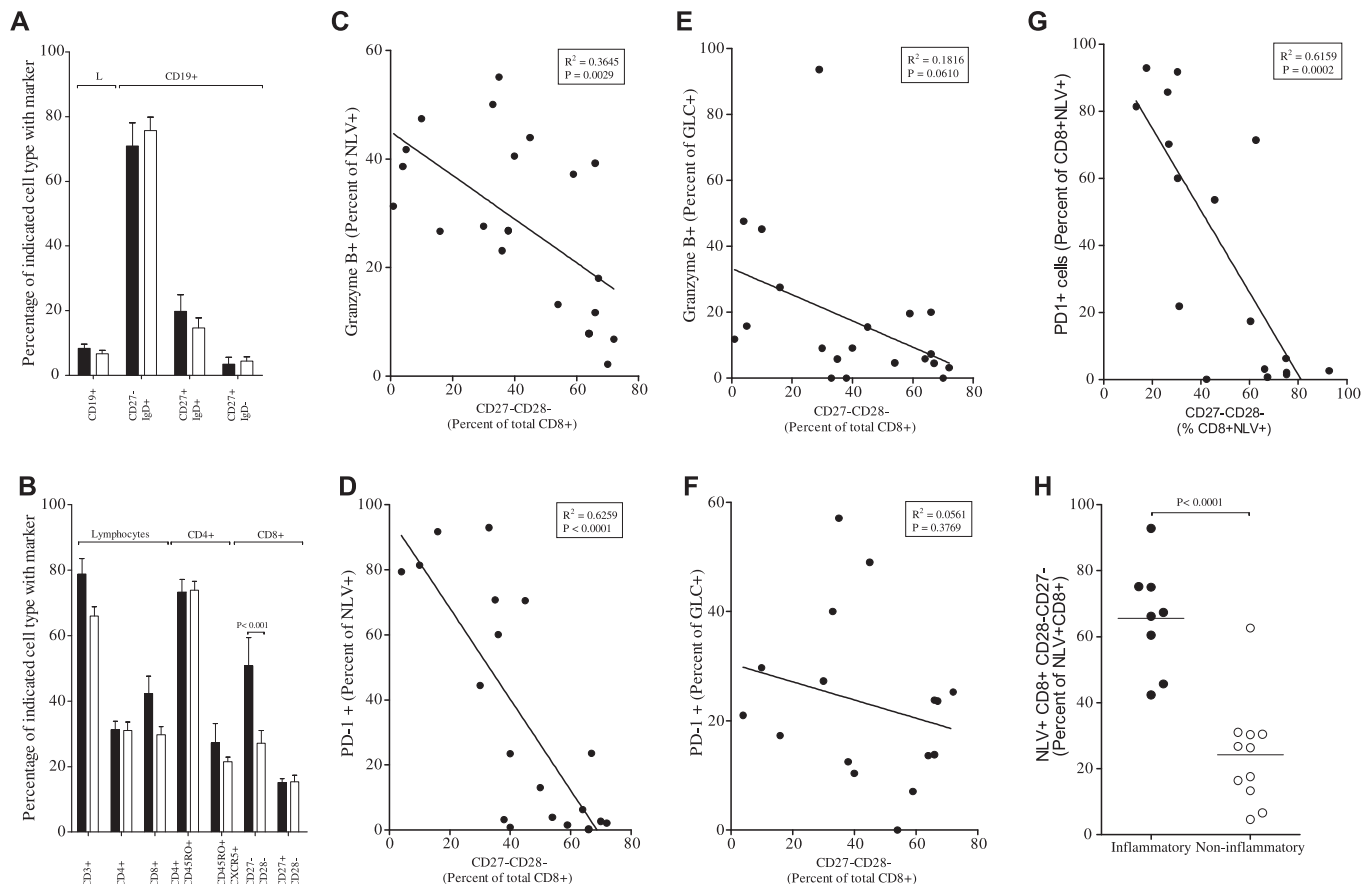


**FIG 2.** Analysis of selected CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets. Whole blood was analyzed for CD4<sup>+</sup> memory cells (**A**), T follicular helper cells (**B**), late effector CD8<sup>+</sup> T cells (**C**), and effector CD8<sup>+</sup> T cells (**D**). Bars indicate mean values. *P* values are for the indicated pairwise comparisons.

Analysis of the response to the EBV GLC peptide showed no differences between the 2 groups of patients with CVID, emphasizing that the focus seen in the CMV-specific response in the patients with CVID with inflammatory disease does not reflect a general increase in IFN- $\gamma$ -producing CD8<sup>+</sup> T-cell populations in these patients (see Fig E2, B).

### Role of IFN- $\gamma$ and TNF- $\alpha$ in peptide-driven proliferation of CMV CD8<sup>+</sup> T cells in patients with inflammatory CVID

Recently, we showed that CD8<sup>+</sup> T cells from patients with inflammatory CVID but not those from patients with noninflammatory CVID or healthy control subjects can proliferate



rapidly after stimulation with CMV peptides in the absence of exogenous cytokines or other costimulators.<sup>10</sup> In light of the CD8<sup>+</sup> T-cell polyfunctional cytokine profiles summarized above, we reasoned that the cytokines important for this proliferation might be reflective of their cytokine profile. To investigate this further, we transferred different amounts of supernatants from cultures of proliferating cells from inflammatory patients with CVID at 2 days after stimulation to *ex vivo* CD8<sup>+</sup> T-cell cultures from patients with noninflammatory CVID and healthy control subjects. Supernatants from patients with inflammatory CVID enhanced proliferation of CMV-specific CD8<sup>+</sup> T cells of both healthy subjects and patients with noninflammatory CVID in a dose-dependent manner (see Fig E3, A and C, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org); data shown are for recipient cells from patients with noninflammatory CVID). Although the expansion was not as substantial as that observed in the T-cell cultures from patients with inflammatory CVID, it was CMV specific, as judged by the relevant HLA tetramer (see Fig E3, D). Stimulation with a pp65 peptide pool produced similar results to those observed with NLV peptide stimulation (see Fig E3, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). No expansion was detected after stimulation with EBV (GLC) peptide after supernatant transfer (see Fig E3, A) or when

supernatants were transferred from T-cell cultures from patients with noninflammatory CVID (see Fig E3, B).

Having shown that patients with inflammatory CVID have increased frequencies of IFN- $\gamma$ - and TNF- $\alpha$ -producing CMV-specific CD8<sup>+</sup> T cells (see Fig E1), we asked whether either of these cytokines was involved in the control of proliferation. Using blocking antibodies against each of these cytokines, we observed a dose-dependent inhibition of proliferation after peptide stimulation of PBMCs from patients with inflammatory CVID (see Fig E3, E and F, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org), shows data from a representative experiment). No such inhibition was seen with an anti-IL-2 antibody. Addition of exogenous IFN- $\gamma$  and TNF- $\alpha$  also conferred a modest but consistent proliferative capacity to cells from patients with noninflammatory CVID (see Fig E3, G).

## DISCUSSION

CVID is the most common clinically relevant primary immune deficiency. A subset of patients experience debilitating inflammatory complications that are strongly associated with CMV infection. We previously described a distinct hyperproliferative CD8<sup>+</sup> T-cell phenotype in these patients. In this study we have extended our previous observations to profile the global effect of

CMV on immune effector cell populations in patients with CVID, refine our description of the functional phenotype of the CD8<sup>+</sup> T-cell response to CMV in this setting, and define the mechanism of hyperproliferation.

Initially, given the variety of abnormalities in immune cells observed in patients with CVID, we studied the effect of CMV infection on the immune cell phenotypes in these patients. Because the absolute number of CD4<sup>+</sup> T cells was comparable between the CMV-infected and uninfected patients with CVID, the increase in CD8<sup>+</sup> T cells resulted in the inversion of the CD4/CD8 ratio. This inversion has been described as a consistent feature of a subset of patients with CVID over many years,<sup>13,15</sup> and our data provide compelling evidence that this is predominantly driven by CMV infection.

Of particular note was the significantly increased frequency of CD8<sup>+</sup>CD27<sup>−</sup>CD28<sup>−</sup> late effector cells in the CMV-infected patients with CVID. This subset is enriched in the CMV-specific response during chronic CMV infection.<sup>16</sup> Given the generally high frequencies of CMV-specific CD8<sup>+</sup> T cells, it seems likely that the increase we see in the patients with CVID is attributable entirely to CMV-specific cells rather than to a more general effect. Regardless, these data emphasize the global effects of CMV infection on cellular immunity.

Interestingly, when we segregated patients according to inflammatory disease rather than CMV status, the late effector subset was the only one that differed significantly, with increased overall frequencies in the inflammatory patient group, and this increase was mirrored in the CMV-specific population. These observations argue that many of the immune cell differences observed in patients with CVID are driven by CMV infection rather than a consequence of the inflammatory disease, which is itself intimately associated with CMV infection.<sup>10</sup> Indeed, we showed previously that CMV antigens, which are indicative of active virus replication, were present at sites of inflammation.<sup>10</sup>

When we investigated relationships between CMV-specific CD8<sup>+</sup> T cells and global CD8<sup>+</sup> T-cell phenotypes, we observed a highly significant negative correlation between CMV NLV pentamer-specific cells expressing either PD-1 or granzyme B and the total frequency of late effector cells. To our knowledge, this relationship has not been observed before in patients with CMV infection and was not apparent when we assessed the same cells in healthy subjects or EBV-specific responses in the same patient group.

This observation and the correlation we found between PD-1<sup>−</sup>positive and late effector frequencies in the CMV NLV pentamer-specific population are consistent with impaired cytotoxic potential and a reduced propensity for programmed cell death in the late effector subset, which is consistent with the remarkable accumulations of activated CD8<sup>+</sup> T cells commonly seen in patients with CMV infection.

The underlying reasons why these CMV-specific CD8<sup>+</sup> T cells should be so highly correlated with the total frequency of the late effector cells are unclear. It might reflect the significant numbers of highly proliferative (PD-1<sup>low</sup>, perforin<sup>hi</sup>, granzyme<sup>hi</sup>) CD8<sup>+</sup> cells that are directed toward CMV in these patients with CVID but might also imply that patients with CVID with low levels of PD-1, granzyme, or both on their CMV-specific CD8<sup>+</sup> T cells would have correspondingly higher levels of CD8<sup>+</sup>CD27<sup>+</sup>CD28<sup>−</sup> effector cells, which might control CMV replication while being less prone to exacerbating an inflammatory environment. Our observation of a positive correlation between IFN- $\gamma$  and TNF- $\alpha$  production

and late effector cell frequencies would be consistent with this suggestion. Regardless, the low PD-1 expression is of interest. This might be driven by persistent replication of tissue-resident CMV, which we previously documented in this patient group.<sup>10</sup> Other plausible explanations include an unrecognized viral coinfection or an underlying genetic defect. (We note that in our cohort the genetic basis for the CVID is not known.)

Consistent with our previous data on the cytokine profile of CMV-specific CD8<sup>+</sup> T cells in patients with CVID with inflammation,<sup>10</sup> the polyfunctional phenotype of these CD8 T cells was skewed toward IFN- $\gamma$  and TNF- $\alpha$  production, although the level of expression of these cytokines on a per-cell basis only showed significantly increased expression of IFN- $\gamma$  in the triple-producing cells of patients with inflammation. We found no significant differences in the EBV-specific polyfunctional signature between patients with CVID with or without inflammation, arguing that the skew toward an IFN- $\gamma$ /TNF- $\alpha$  phenotype is associated specifically with CMV.

In principle, the hyperproliferative CD8<sup>+</sup> phenotype that we describe might be driven by this cytokine production, persistent antigen stimulation from virus at sites of inflammation, or both. Our supernatant transfer experiments implicate soluble factors in the initiation, maintenance, or both of proliferation, and suppression of proliferation with anti-cytokine antibodies and enhancement by exogenous cytokines are consistent with a role for IFN- $\gamma$  and TNF- $\alpha$  in this phenomenon. We note that these cytokines would not normally be expected to mediate proliferation, but the enhanced proliferation of the CMV CD8<sup>+</sup> T cells might reflect increased TNF receptor display on these cells, protection against apoptosis, or both. We are currently investigating the mechanisms underlying the proliferation. Regardless, a block to proliferation of these proinflammatory CMV-specific CD8 T cells is a plausible mechanism for the documented clinical effects of anti-TNF- $\alpha$  therapy (infliximab) in reducing CVID-associated inflammatory disease.<sup>17,18</sup> It will be interesting to determine whether the CD4/CD8 ratio inversion, the increase in late effector CD8<sup>+</sup> T-cell numbers, or both resolve in patients receiving either anti-TNF- $\alpha$  therapy or in those who are receiving the anti-CMV drug ganciclovir.

In summary, our results strengthen the evidence base for controlled clinical trials of anti-TNF- $\alpha$  and antiviral therapy in the management of inflammatory CVID and emphasize the emerging but enigmatic role of CMV in patients with CVID-associated inflammatory disease, and more generally, the global imprint that CMV infection can have on immune effector cell populations.

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**Clinical implications: CMV is associated with T-cell abnormalities in patients with CVID. TNF- $\alpha$  and IFN- $\gamma$  drive T-cell proliferation in patients with CVID, providing a rationale for anti-TNF- $\alpha$  therapy in CVID management.**

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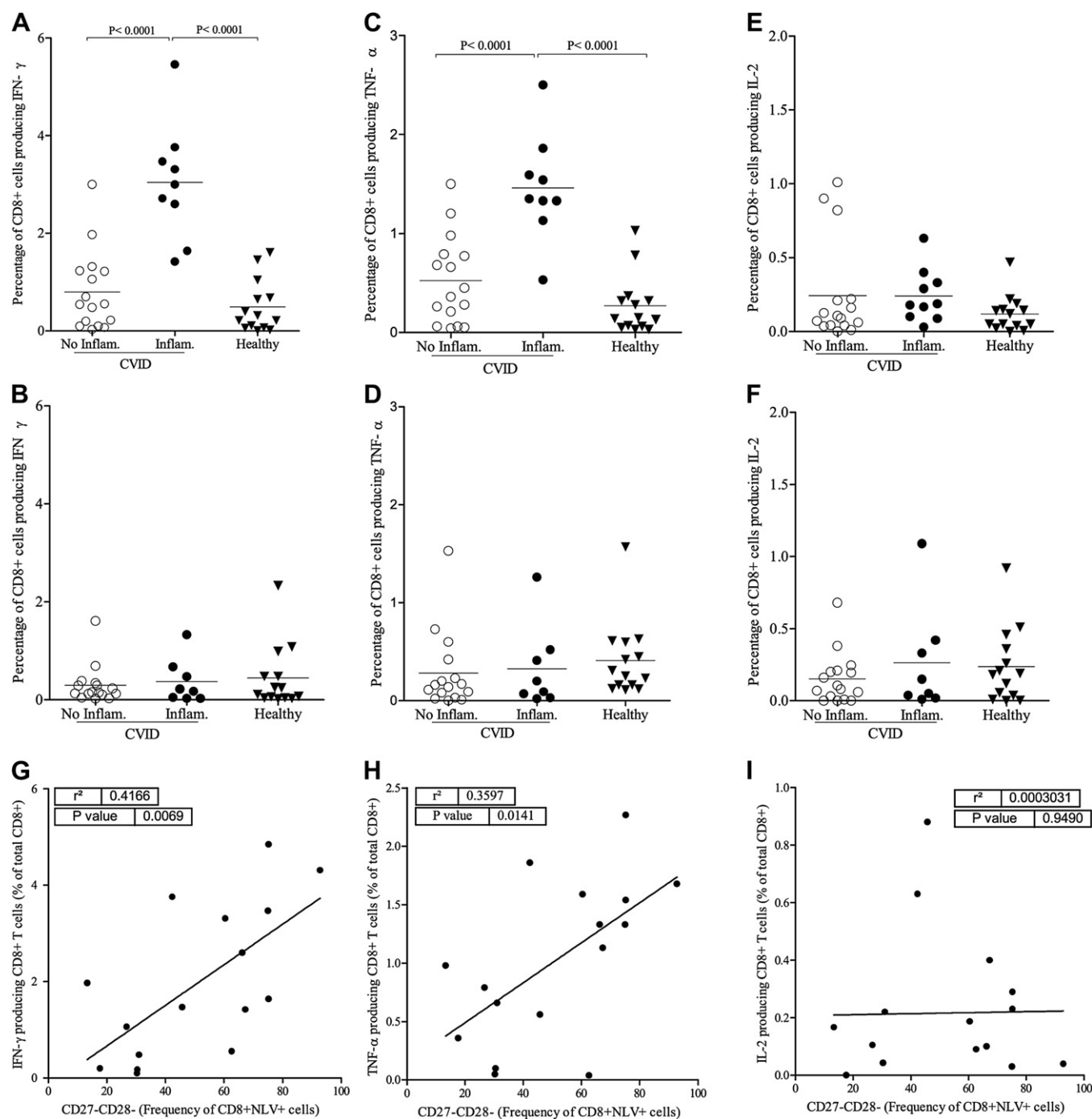


**FIG E1.** Cytokine production by CD8<sup>+</sup> T cells in patients with CVID. **A-F**, Production of IFN- $\gamma$  (Fig E1, **A** and **B**), TNF- $\alpha$  (Fig E1, **C** and **D**), and IL-2 (Fig E1, **E** and **F**) by CMV NLV (Fig E1, **A**, **C**, and **E**) or EBV GLC (Fig E1, **B**, **D**, and **F**) peptide-stimulated CD8<sup>+</sup> T cells. **G-I**, Correlation of the frequency of cytokine-producing CD8<sup>+</sup> T cells with the frequency of CD27<sup>+</sup>CD28<sup>+</sup> NLV pentamer-positive cells (Fig E1, **G**: IFN- $\gamma$ ; Fig E1, **H**: TNF- $\alpha$ ; and Fig E1, **I**: IL-2). In Fig E1, **A-F**, bars indicate mean values. *P* values for pairwise comparisons are shown for significant differences only.

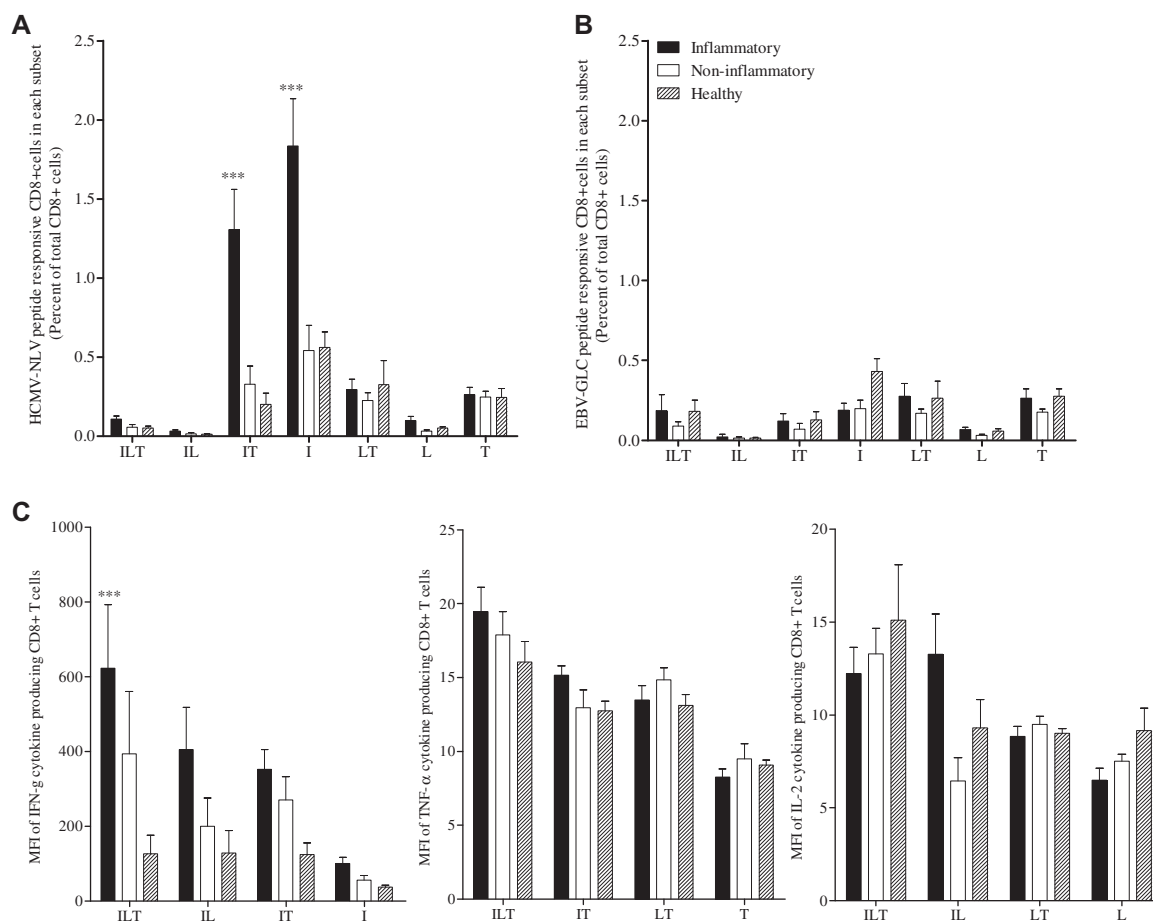


**FIG E2.** Analysis of polyfunctional CD8<sup>+</sup> T cells. **A** and **C**, Frequency (Fig E2, A) and quantity (Fig E2, C) of production of IFN- $\gamma$  (I), TNF- $\alpha$  (T), IL-2 (L), or combinations thereof by CMV NLV peptide-responsive CD8<sup>+</sup> T cells in patients with CVID with (black bars) and without (white bars) inflammation and healthy control subjects (hashed bars). **B**, Frequency data for EBV GLC-responsive cells. Bars indicate mean percentages  $\pm$  1 SEM. Asterisks indicate significantly increased values ( $P < .0001$ ).

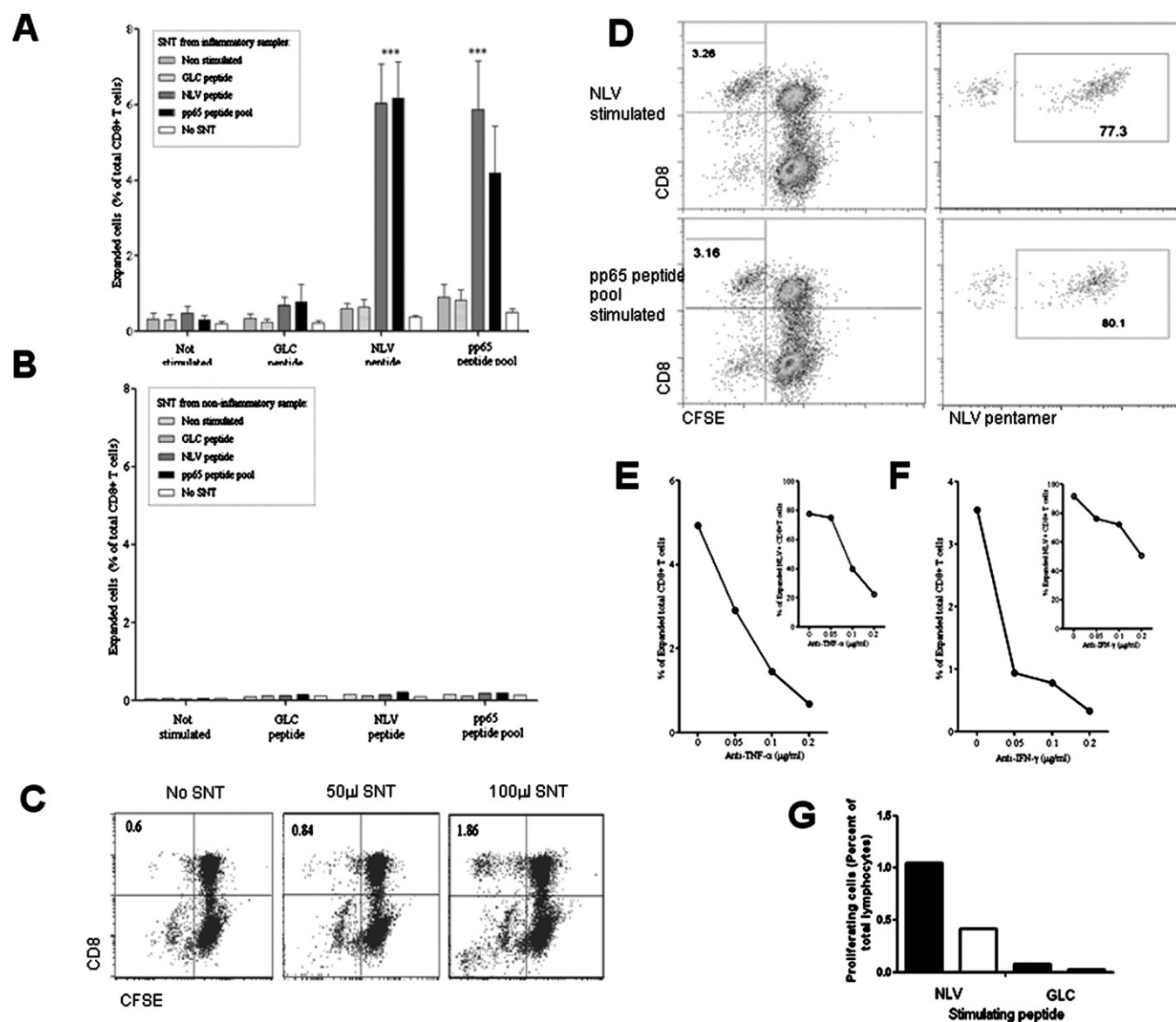


**FIG E3.** Proliferation enhancement by stimulated CD8<sup>+</sup> T-cell supernatants. **A** and **B**, Supernatants (SNT) of proliferating PBMCs from patients with inflammatory (Fig E3, **A**) but not noninflammatory (Fig E3, **B**) CVID enhance proliferation of CMV peptide-stimulated PBMCs from patients with noninflammatory CVID (mean  $\pm$  SE of 4 experiments). Asterisks indicate significantly increased values: ( $P < .0001$ ). **C** and **D**, Enhancement is dose dependent (Fig E3, **C**), and most proliferating cells are CMV NLV pentamer positive (Fig E3, **D**). **E-G**, Proliferation is inhibited by anti-IFN- $\gamma$  and anti-TNF- $\alpha$  antibodies and enhanced by exogenous IFN- $\gamma$  and TNF- $\alpha$  (data from representative experiments). In panel **G**, black bars: cytokine addition; white bars: no cytokine addition). CFSE, Carboxyfluorescein diacetate succinimidyl ester.