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Stimulatory Effect of CMV Immunoglobulin on Innate Immunity and on the Immunogenicity of CMV Antigens

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Background. Cytomegalovirus (CMV) immunoglobulin (CMVIG) is used for the prophylaxis of CMV infection after transplantation. Beyond providing passive CMV-specific immunity, CMVIG exerts enhancing and suppressive immunomodulatory functions. Although the anti-inflammatory activities of CMVIG have been extensively documented, its immunostimulatory activities remain poorly characterized. **Methods.** This exploratory study analyzed the capacity of CMVIG to modulate cell-mediated innate and adaptive immunities in vitro on freshly isolated peripheral blood mononuclear cells (PBMCs) of CMV-seropositive and -seronegative healthy individuals, using interferon- γ (IFN- γ) enzyme-linked immunospot and intracellular cytokine staining assays. **Results.** We showed that CMVIG treatment increases the number of IFN- γ -secreting PBMCs of both CMV-seronegative and -seropositive individuals, indicating a global stimulatory effect on innate immune cells. Indeed, CMVIG significantly increased the frequency of natural killer cells producing the T helper cell 1–type cytokines tumor necrosis factor and IFN- γ . This was associated with the induction of interleukin-12–expressing monocytes and the activation of cluster of differentiation (CD) 4⁺ and CD8⁺ T cells, as measured by the expression of tumor necrosis factor and IFN- γ . Interestingly, stimulation of PBMCs from CMV-seropositive subjects with CMVIG-opsonized CMV antigens (phosphoprotein 65, CMV lysate) enhanced CD4⁺ and CD8⁺ T-cell activation, suggesting that CMVIG promotes the immunogenicity of CMV antigens. **Conclusions.** Our data demonstrate that CMVIG can stimulate effector cells of both innate and adaptive immunities and promote the immunogenicity of CMV antigens. These immunostimulatory properties might contribute to the protective effect against CMV infection mediated by CMVIG.

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INTRODUCTION

Cytomegalovirus (CMV) infection is highly prevalent in the general population, with a CMV seroprevalence ranging from 66% in European countries to 90% in Eastern Mediterranean regions.¹ Following a primary infection, CMV establishes a lifelong latency and periodically reactivates. In healthy individuals, CMV infection and reactivation are usually asymptomatic owing to an effective control by both humoral and cellular immunities.^{2,3}

Cellular immunity against CMV is driven by a complex interplay between innate immune cells (natural killer [NK] cells and antigen-presenting cells [APCs] like monocytes, macrophages, or dendritic cells [DCs]) and CMV antigen-specific T cells. T-cell responses to immunodominant CMV antigens (including the phosphoprotein 65 [pp65]) are mediated mainly by cluster of differentiation (CD) 4⁺ T helper (Th) cells and CD8⁺ cytotoxic T lymphocytes (CTLs).^{2,3} Upon CMV infection and reactivation, innate and adaptive immune cells promote a Th1-type response,

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characterized by the secretion of Th1-polarizing immunostimulatory cytokines (tumor necrosis factor [TNF], interferon- γ [IFN- γ], interleukin [IL]-2, IL-12), whereas the Th2-polarizing immunosuppressive cytokines (IL-4, IL-10) remain low.²⁻⁹

As opposed to immunocompetent hosts, immunocompromised patients, such as transplant recipients, are at high risk of uncontrolled CMV reactivation, leading to both direct (CMV end-organ disease) and indirect (including chronic inflammatory conditions, increased risks of graft rejection and of opportunistic infections) complications.⁹⁻¹³ Thus, CMV remains a serious cause of morbidity and mortality in immunocompromised patients, in particular after transplantation.

Over the past years, posttransplant management strategies have been implemented and have greatly improved patients' clinical outcomes. Preemptive antiviral therapy in association with viral load monitoring and antiviral prophylaxis is classically used in posttransplant patients at risk of CMV reactivation.¹⁴ In addition, CMV immunoglobulin (CMVIG) is licensed for the prophylaxis of CMV infection and disease after solid organ and hematopoietic stem cell transplantation. CMVIG is usually used to complement antiviral prophylaxis but can also be used as monotherapy in instances of antiviral intolerance or resistance.¹⁴⁻²⁰

CMVIG is an immunoglobulin preparation formulated for intravenous administration. It is derived from pooled human plasma selected for high anti-CMV antibody titers, compared with normal IVIG preparations, which are not specific for CMV and thus contain low anti-CMV antibody titers.^{21,22} CMVIG and IVIG have pleiotropic effects. IVIG was originally developed as replacement therapy in patients with immunodeficiencies and was soon found to be beneficial beyond antibody replacement, notably as an anti-inflammatory agent in autoimmune and inflammatory diseases.²³⁻²⁶ The immunomodulatory properties of IVIG, notably its anti-inflammatory activities, have been extensively documented. Their mode of action is complex and involves multiple pathways, both Fc-dependent and -independent, including among others the modulation of expression and function of (activating and inhibitory) Fc-gamma receptors (Fc γ Rs) and the modulation of activation and function of DC, T, B, and NK cells.²⁵⁻⁴³ Primary function of CMVIG is to provide passive CMV-specific immunity by neutralizing circulating viral particles and facilitating their elimination by opsonization and phagocytosis.¹⁸ In addition, CMVIG exerts enhancing and suppressive immunomodulatory functions that might help control some of the direct and indirect effects of posttransplant CMV infection.^{18,44-51} However, to date, the immunomodulatory properties of CMVIG remain poorly characterized.

This exploratory study aimed to better characterize the immunomodulatory properties of CMVIG. We analyzed the capacity of CMVIG to modulate cell-mediated innate and adaptive immunities *in vitro*. We assessed the effects of CMVIG and of CMVIG-opsonized CMV antigens on the response of freshly isolated human peripheral blood mononuclear cells (PBMCs) from CMV-seropositive and -seronegative healthy subjects, using IFN- γ enzyme-linked immunospot (ELISpot) as well as intracellular cytokine staining and flow cytometry.

MATERIALS AND METHODS

Stimulants

CMVIG (Cytotect CP Biotest; 100 U/mL) and normal IVIG (Intratec; 50 g/L) were provided by Biotest AG (Dreieich,

Germany). The immunodominant region of CMV pp65 protein (amino acids 366 to 546, human CMV strain AD169) was provided by Mikrogen (Neuried, Germany). Lysate of CMV-infected fibroblasts (CMV lysate) was from Virion Serion (Würzburg, Germany). Phytohemagglutinin, phorbol 12-myristate 13-acetate/ionomycin, lipopolysaccharide, and cyclosporine A were from Sigma-Aldrich. Tacrolimus (Prograf) was from Astellas Pharma (Munich, Germany).

Opsonization of Stimulating CMV Antigens

Opsonization of CMV pp65 and CMV lysate was performed by preincubating 1 μ g CMV antigen with 80 μ L CMVIG (8 U) or IVIG (4mg) for 1 h at 37 °C. Equivalent concentrations of CMVIG or IVIG controls were incubated in parallel. Opsonized proteins were used as stimulants in the presence of residual (ie, unbound) CMVIG or IVIG, as to keep the total amount of CMVIG constant in each condition. In antigen titration experiments, the concentration of CMVIG was kept constant in all conditions (80 μ L/mL or 8 U/mL).

Determination of Anti-CMV Antibody Binding to CMV Antigens by Enzyme-linked Immunoassay

The binding activity of CMVIG CMV-specific antibodies to the CMV antigens pp65 and CMV lysate was verified by end-point enzyme-linked immunoassay (Figure S1, SDC, <http://links.lww.com/TXD/A374>), as detailed in Supplemental Materials and Methods (SDC, <http://links.lww.com/TXD/A374>).

Blood Collection and PBMC Preparation

Blood samples were collected in lithium heparin tubes (S-Monovette; SARSTEDT AG & Co., Nümbrecht, Germany) by venipuncture from healthy volunteers with known CMV serostatus (Table S1, SDC, <http://links.lww.com/TXD/A374>). Before blood collection, written informed consent for the use of blood for research purposes was obtained from all donors, in accordance with the Declaration of Helsinki. No institutional review board approval was required for this study. When possible, blood was repeatedly collected from the same donors across different experiments. The selected donors are specified in the respective figure legends. Isolation of PBMCs was performed using standard Ficoll-Paque density centrifugation, as described in Supplemental Materials and Methods (SDC, <http://links.lww.com/TXD/A374>).

ELISpot Assay

IFN- γ ELISpot assays were performed as previously described⁵²⁻⁵⁶ and as detailed in Supplemental Materials and Methods (SDC, <http://links.lww.com/TXD/A374>). IFN- γ -specific spot-forming cells were counted on a Bioreader 5000 Pro-Ea (BIO-SYS GmbH, Karben, Germany). Wells exhibiting 0 spots were assigned the value 0.5 for the calculation of geometric means. ELISpot data were expressed as the geometric mean of 4 replicate spot-forming cell counts per 2×10^5 PBMCs and were represented as Tukey box plots (when $n > 3$) or as scatter plots (when $n = 3$).

Intracellular Cytokine Staining

Freshly isolated PBMCs (1×10^6 cells) were incubated with the indicated stimulants (1 μ g/mL CMV pp65 antigen, 0.33 μ g/mL CMV lysate, 80 μ L [8 U]/mL CMVIG) in the presence of costimulatory anti-CD28 (1 μ g/mL) and anti-CD49d (1 μ g/mL) antibodies (BD, Heidelberg, Germany) for 6 h (T-cell

protocol; all cytokines except IL-10 and IL-12) or 16h (APC protocol; IL-10 and IL-12 staining) at 37 °C. Samples incubated with AIM-V medium with the same amount of costimulatory anti-CD28 and anti-CD49d antibodies served as negative control. After incubating for 2h (T-cell protocol) or 12h (APC protocol) at 37 °C in a humidified atmosphere under 5% CO₂, 10mg/mL brefeldin A (Sigma-Aldrich, Munich, Germany) was added to prevent cytokine secretion and incubated for an additional 4h. Stimulated cells were washed twice with phosphate-buffered saline before further cell surface and intracellular cytokine staining, as detailed in Supplemental Materials and Methods (SDC, <http://links.lww.com/TXD/A374>). The cytokine-producing cells were detected using a Canto II flow cytometer (BD) and the data were analyzed using Flow Jo Data Analysis Software (version 10). The gating strategy is shown in Figure S2 (SDC, <http://links.lww.com/TXD/A374>). Results are reported as percentage of the gated population producing the indicated cytokines.

Statistics

Statistical analysis was performed using GraphPad Prism 5.04. Two-group comparisons of quantitative results were performed using the 2-sided nonparametric Mann-Whitney U (MWU) test. *P* values <0.05 were considered statistically significant.

RESULTS

CMVIG Stimulates IFN- γ Secretion by PBMCs From CMV-Seropositive and -Negative Individuals

We investigated the immunomodulatory capacity of CMVIG on freshly isolated PBMCs of 15 healthy individuals (10 CMV-seropositive and 5 CMV-seronegative). PBMCs were incubated for 19h at 37 °C in the absence or presence of CMVIG (80 μ L/mL, corresponding to 8 U/mL). The number of IFN- γ -secreting cells was quantified by IFN- γ ELISpot. The number of activated immune cells secreting IFN- γ was significantly increased following treatment with CMVIG (Figure 1A; MWU *P*<0.001). The stimulatory effect of CMVIG was equally observed in CMV-seronegative and -seropositive subjects (Figure 1B; MWU *P*=0.012 and *P*<0.001, respectively). As expected, CMV antigens (pp65 or CMV lysate; 1 μ g/mL) had no stimulatory activity on PBMCs from CMV-seronegative donors (Figure 1C). Interestingly, however, incubation of PBMCs from CMV-seronegative donors with CMV antigens opsonized by preincubation with the same amount of CMVIG resulted in a weaker increase of IFN- γ -secreting cells compared with PBMCs treated with CMVIG alone (Figure 1C). Although this reduced response to CMVIG in the presence of antigen was not statistically significant, it suggests that CMV antigens partly dampened the stimulatory effect of CMVIG.

Next, we compared the stimulatory activity of CMVIG with that of a normal IVIG. IVIG preparations are characterized by a lower and nonstandardized proportion of anti-CMV immunoglobulin G (IgG) (varying between 4- and 8-fold less, depending on the product batch) compared with CMVIG preparations.^{21,22} Incubation of PBMCs from CMV-seropositive donors with the same amount of CMVIG or IVIG (80 μ L/mL) resulted in an increase of IFN- γ -secreting cells by ELISpot in both cases, albeit weaker and not statistically significant for IVIG (MWU *P*=0.085), compared with CMVIG (MWU *P*=0.034) (Figure 1D).

We next evaluated the stimulatory activity of CMVIG on CMV antigen-specific cell-mediated immunity. Freshly isolated PBMCs from 3 CMV-seropositive donors were stimulated with increasing amounts of nonopsonized and opsonized CMV antigens (pp65 or CMV lysate; up to 1 μ g/mL) and with CMVIG alone or medium as controls (Figure 2). We observed a dose-dependent increase in antigen-reactive effector cells measured by ELISpot that was further increased after antigen opsonization, for each of the 3 donors and at each antigen concentration tested (Figure 2A and B).

Altogether, these observations suggest that CMVIG exerts stimulatory effects on cell-mediated innate (antigen-independent) and adaptive (CMV-specific) immune responses.

Modulatory Effect of CMVIG on Effector Cells of Innate and Adaptive Immunities

To better characterize CMVIG-mediated immunostimulatory effects, intracellular cytokine staining and flow cytometry analyses were conducted on PBMCs from 5 CMV-seropositive donors, stimulated with CMV antigens (1 μ g/mL pp65 or 0.33 μ g/mL CMV lysate), CMVIG (80 μ L/mL) or opsonized CMV antigens. Expression of Th-1 polarizing (TNF, IFN- γ , IL-2, IL-12) and Th-2 polarizing (IL-4, IL-10) cytokines was investigated in cells of the innate (NK, monocytes) and adaptive (Th, CTL) immune responses.

The percentage of TNF- and IFN- γ -expressing NK cells was strongly increased upon stimulation with CMVIG alone (Figure 3A and B; MWU *P* = 0.008 versus medium control). The median (range) percentage of TNF⁺ NK cells increased from 0.2% (0.05%–0.7%) in the unstimulated (medium) condition to 8.3% (4.7%–15.6%) and that of IFN- γ ⁺ NK cells increased from 0.5% (0.2%–2.4%) to 25.7% (19.3%–38.7%). CMV antigens did not stimulate the production of TNF and IFN- γ in NK cells (Figure 3A and B). Interestingly, however, the presence of antigens slightly reduced the stimulatory effect of CMVIG. The median percentage of TNF⁺ NK cells decreased from 8.3% to 6.0% (opsonized [ops.] pp65) and to 6.9% (ops. CMV lysate), whereas the median percentage of IFN- γ ⁺ NK cells decreased from 25.7% to 21.4% (ops. pp65) and to 21.0% (ops. CMV lysate) (Figure 3A and B). Although the differences in distribution were not statistically significant (MWU *P* > 0.05), these reductions in NK cell populations were consistently observed at the individual level. Indeed, when analyzing the difference in stimulatory capacity per donor after subtracting the background signal (ie, medium background subtracted from response to CMV antigens, and CMVIG-mediated response subtracted from that to opsonized CMV antigens), it appeared clearly that treatment with opsonized CMV antigens reduced the proportion of TNF- and IFN- γ -producing NK cells in all donors (with the exception of donor 3 for TNF), with a maximum reduction of 4.1 percentage points (TNF in donor 4 for ops. pp65) and of 6.7 percentage points (IFN- γ in donor 5 for ops. pp65) (Figure S3A and B, SDC, <http://links.lww.com/TXD/A374>).

In contrast to its positive effect on the production of TNF and IFN- γ by NK cells, CMVIG treatment did not stimulate the production of IL-2 and IL-4 by NK cells (MWU *P* > 0.05), and IL-2⁺ and IL-4⁺ NK cells remained very low in all conditions (Figure 3C and D).

On the other hand, CMVIG treatment of PBMCs stimulated the production of the Th-1 polarizing cytokine IL-12 by monocytes (MWU *P*=0.008), as did the CMV antigens

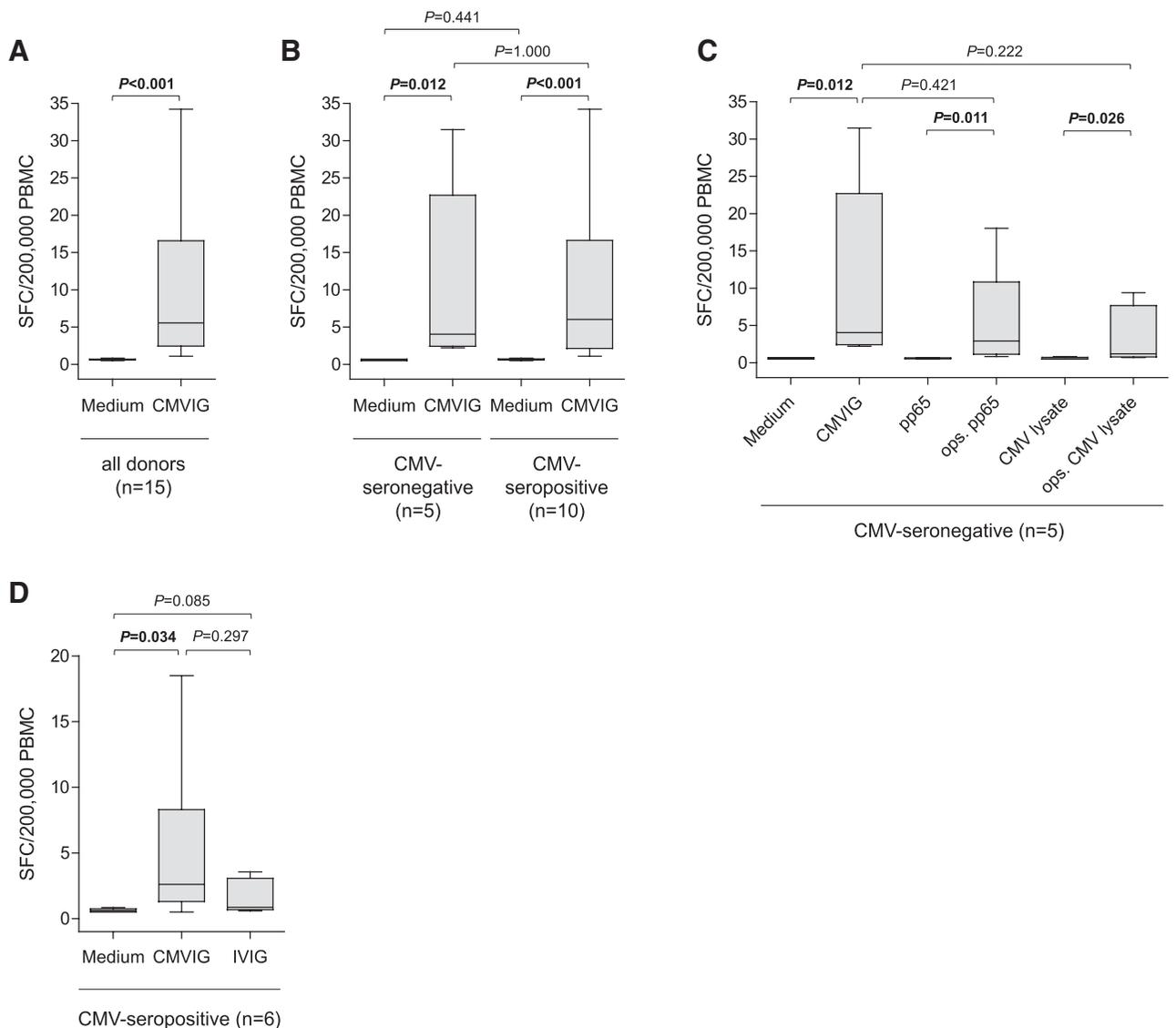


FIGURE 1. Stimulatory effect of CMVIG and IVIG on PBMCs from CMV-seronegative and -seropositive donors. Freshly isolated PBMCs from healthy individuals were incubated for 19h with the indicated stimulants, and the number of IFN- γ -producing cells was measured by IFN- γ ELISpot. Differences between groups were assessed using the 2-sided nonparametric Mann-Whitney U test. Statistically significant *P* values are shown in bold. A, The response of PBMCs from 15 donors (5 CMV-seronegative and 10 CMV-seropositive [donors 1–10]) was significantly increased in the presence of CMV immunoglobulin (CMVIG; 80 μ L/mL or 8 U/mL) compared with the unstimulated control (Medium). B, ELISpot results from (A), depicted according to the donors' CMV serostatus, showed a comparable stimulatory effect of CMVIG (80 μ L/mL) in CMV-seronegative and -seropositive subjects. C, PBMCs from the 5 CMV-seronegative donors shown in (A) and (B) were stimulated with CMVIG (80 μ L/mL), CMV antigens (1 μ g/mL pp65 or CMV lysate), or opsonized CMV antigens (ops. pp65 or ops. CMV lysate, that is, 1 μ g/mL antigens preincubated with 80 μ L/mL CMVIG). The stimulatory effect of CMVIG was slightly reduced in the presence of CMV antigens. D, The response of PBMCs from 6 CMV-seropositive individuals (donors 11–16) was increased in the presence of both CMVIG (80 μ L/mL) and normal IVIG (80 μ L/mL), compared with the unstimulated control (medium), albeit not statistically significantly for IVIG. CMV, cytomegalovirus; CMVIG, CMV immunoglobulin; ELISpot, enzyme-linked immunospot; IFN, interferon; ops., opsonized; PBMC, peripheral blood mononuclear cell; pp65, phosphoprotein 65; SFC, spot-forming cell.

pp65 (MWU $P=0.016$) and CMV lysate (MWU $P=0.008$) (Figure 4A). The stimulatory activity of opsonized CMV antigens, though, was comparable with that of the nonopsonized antigens (Figure 4A). As opposed to the CMVIG-mediated increase in IL-12, the production of the Th-2 polarizing cytokine IL-10 by monocytes was neither affected by CMVIG nor by CMV antigens (nonopsonized or opsonized) (Figure 4B).

Next, we analyzed the cytokine profile of effector Th cells and CTLs in PBMCs treated with CMVIG and with nonopsonized and opsonized CMV antigens. As expected, CMV antigens significantly stimulated Th cells to produce TNF and

IFN- γ (Figure 5A and B; MWU $P=0.008$ versus medium, in all cases). CMVIG treatment also activated Th cells to produce TNF and IFN- γ , albeit not significantly (Figure 5A and B; MWU $P=0.421$ for TNF $^+$ Th cells and $P=0.056$ for IFN- γ^+ Th cells, compared with medium). Interestingly, the stimulatory activity of CMV antigens on TNF and IFN- γ production by Th cells was slightly increased upon antigen opsonization, although not quite significantly (Figure 5A and B; MWU P values between 0.056 and 0.222). The same trend of induction of TNF $^+$ and IFN- γ^+ Th cells by CMV antigens and a further increase in the presence of CMVIG was consistently observed at the level of individual donors (Figure S3C and D,

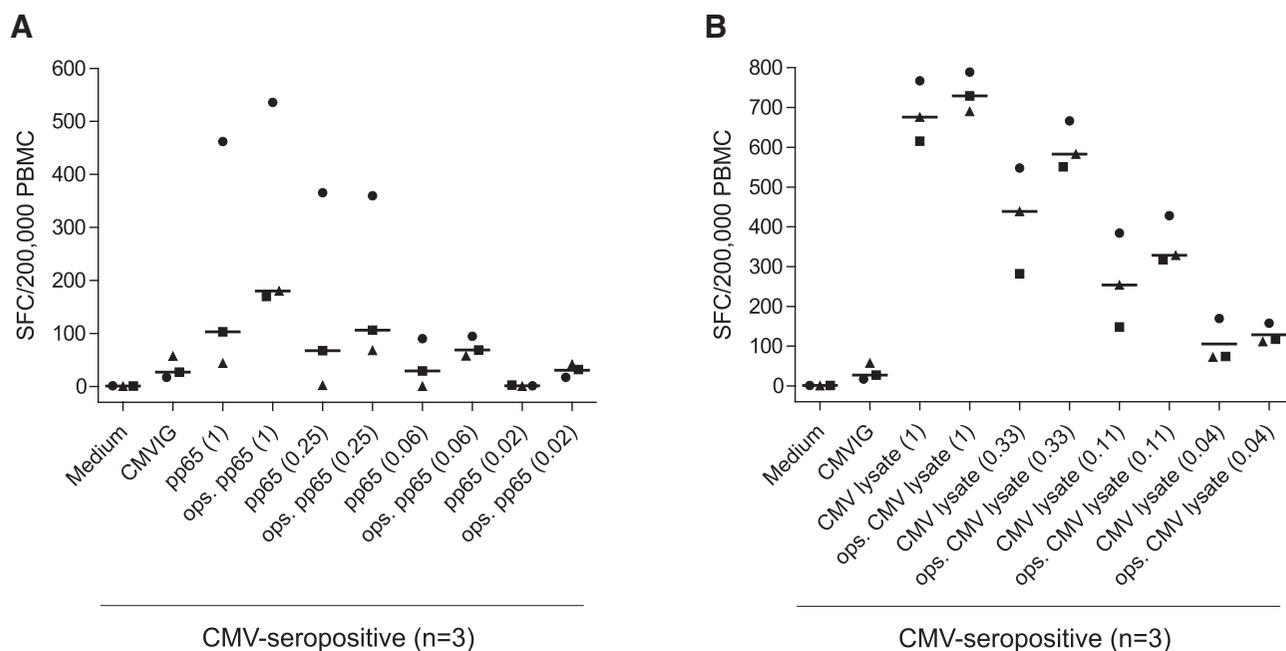


FIGURE 2. Stimulatory activity of CMV antigens opsonized with CMVIG on PBMCs from CMV-seropositive donors. Freshly isolated PBMCs from 3 healthy CMV-seropositive individuals (donors 1–3 shown as circle, triangle, and square, respectively) were incubated for 19h with increasing amounts of CMV antigens (A, 0.02–1 $\mu\text{g/mL}$ pp65; B, 0.04–1 $\mu\text{g/mL}$ CMV lysate) or of opsonized CMV antigens (ie, antigens preincubated with CMVIG; [A] ops. pp65; [B] ops. CMV lysate). Medium or CMVIG served as controls. CMVIG was adjusted to a final concentration of 80 $\mu\text{L/mL}$ (in CMVIG alone and opsonized antigens conditions). The number of IFN- γ -producing cells was measured by IFN- γ ELISpot. A and B, The dose-dependent response of PBMCs to CMV antigens was slightly increased in the presence of CMVIG (ops. pp65 and ops. CMV lysate). Differences between groups (medium vs CMVIG and nonopsonized vs opsonized antigens for each antigen concentration) using the Mann-Whitney U test were not statistically significant, most likely because of the low number of measurements (n=3). CMV, cytomegalovirus; CMVIG, CMV immunoglobulin; ELISpot, enzyme-linked immunospot; IFN, interferon; ops., opsonized; PBMC, peripheral blood mononuclear cell; pp65, phosphoprotein 65; SFC, spot-forming cell.

SDC, <http://links.lww.com/TXD/A374>). By contrast, the number of IL-2-, IL-4-, and IL10-producing Th cells remained low and unaffected in all conditions (Figure 5C–E).

As opposed to their stimulatory effect on Th cells, CMV antigens did not stimulate CTL to produce TNF and IFN- γ (Figure 6A and B). By contrast, CMVIG treatment significantly increased TNF⁺ and IFN- γ ⁺ CTL (Figure 6A and B; MWU both P values=0.008 versus medium). The median (range) percentage of TNF⁺ CTL increased from 0.03% (0.01%–0.2%) in the unstimulated (medium) condition to 1.3% (0.9%–6.1%) and that of IFN- γ ⁺ CTL increased from 0.14% (0.1%–0.2%) to 2.5% (2.2%–12.2%). Interestingly, although CMV antigens alone showed no stimulatory activity on cytokine production in CTL, opsonized antigens slightly increased the CMVIG-mediated stimulatory effect on TNF⁺ and IFN- γ ⁺ CTL, albeit nonsignificantly (Figure 6A and B; MWU P =0.310 in all cases). The stimulatory effect mediated by opsonized CMV antigens was also clearly observed in all 5 individual donors, with a maximum increase in TNF⁺ CTL of 3.6 percentage points (donor 3, ops. pp65) and a maximum increase in IFN- γ ⁺ CTL of 3.0 percentage points (donor 3, ops. pp65) versus CMVIG (Figure S3E and F, SDC, <http://links.lww.com/TXD/A374>). Finally, as observed for Th cells, the number of IL-2- and IL-4-producing CTL remained low and unaffected in all conditions (Figure 6C and D).

Altogether, the intracellular staining studies identified differential effects of CMVIG and CMVIG-opsonized CMV antigens on the cytokine secretion profile of NK cells, monocytes, Th cells, and CTL of CMV-seropositive healthy donors. Notably, the production of the Th1-type cytokines TNF and IFN- γ was strongly increased in NK cells and CTL and to

some extent in Th cells. Opsonized CMV antigens enhanced slightly (Th) or strongly (CTL) CMVIG-mediated stimulation, whereas they partly suppressed CMVIG-mediated stimulation on NK cells.

Sensitivity of CMVIG-treated PBMCs to Immunosuppression

Cyclosporine A and tacrolimus are immunosuppressive agents commonly used posttransplant for prophylaxis of organ rejection. They both act as calcineurin inhibitors to block *IL-2* gene expression and thus T-cell activation.⁵⁷ Given the immunomodulatory effect of CMVIG on cellular immunity, we then asked whether treatment with cyclosporine A or tacrolimus would alter the sensitivity of PBMCs to CMVIG or opsonized CMV antigens.

PBMCs freshly isolated from 3 CMV-seropositive healthy donors were stimulated for 19h with CMVIG (8.8 $\mu\text{L/mL}$), CMV lysate (0.11 $\mu\text{g/mL}$), or opsonized CMV lysate in the presence of increasing amounts of cyclosporine A (up to 10 μM ; Figure 7) or tacrolimus (up to 125 ng/mL; Figure S4, SDC, <http://links.lww.com/TXD/A374>). The stimulatory effects of CMVIG, CMV lysate, and CMVIG-opsonized CMV lysate were observed at low concentrations of cyclosporine A (Figure 7) and tacrolimus (Figure S4, SDC, <http://links.lww.com/TXD/A374>) and were comparably inhibited by higher concentrations of calcineurin inhibitors (Figures 7 and Figure S4, SDC, <http://links.lww.com/TXD/A374>). Of note, the target blood level of cyclosporine A in transplant recipients usually ranges from 100 to 800 ng/mL,^{58–61} which corresponds to molar concentrations ranging from 0.083 to 0.664 μM . This precisely corresponds to the range of cyclosporine A

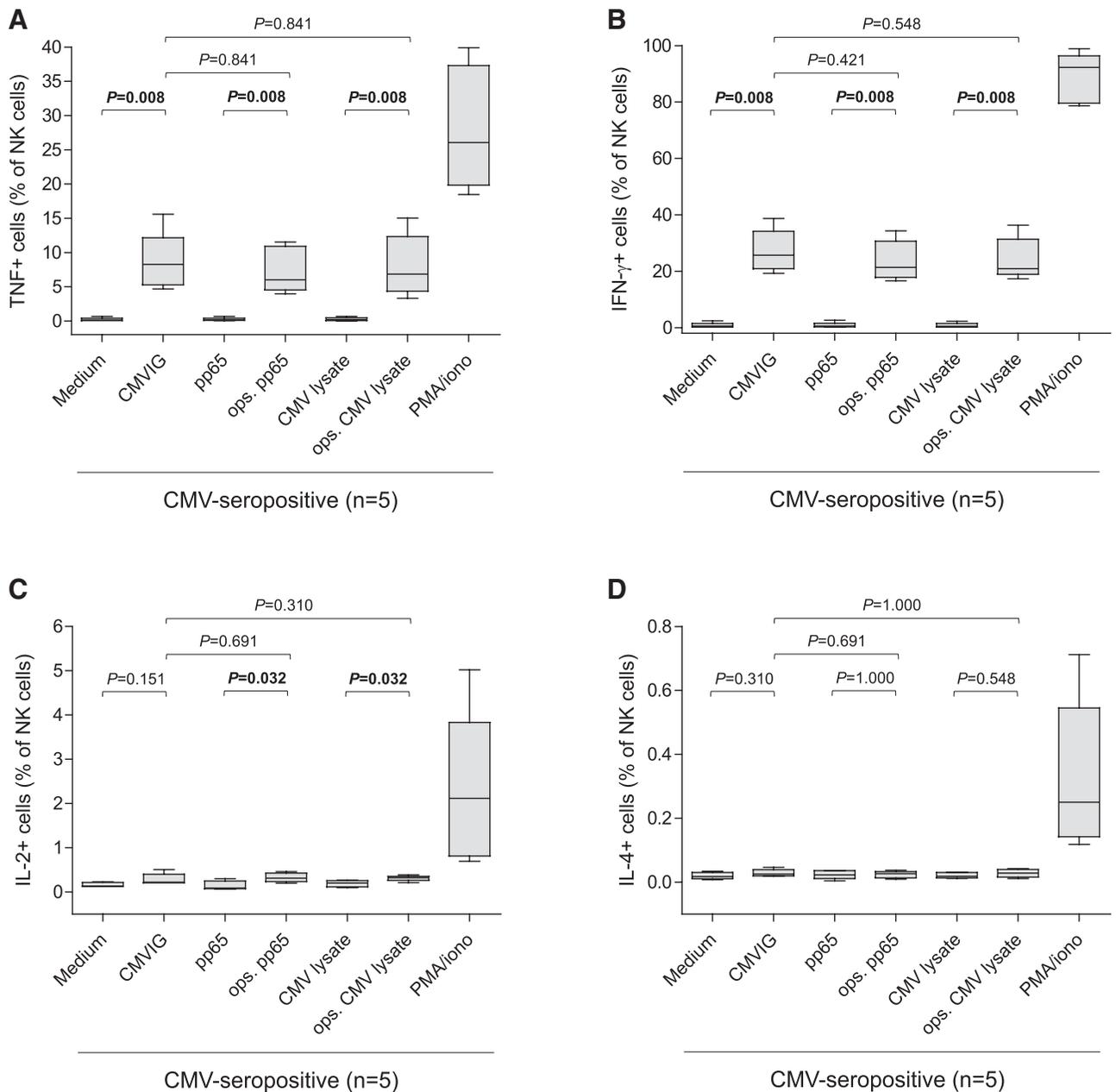


FIGURE 3. Immunomodulatory effect of CMVIG on NK cells. Freshly isolated PBMCs from 5 healthy CMV-seropositive individuals (donors 1–5) were stimulated, stained, and analyzed by flow cytometry as described in Materials and Methods. CMV pp65 antigen was used at 1 $\mu\text{g}/\text{mL}$, CMV lysate at 0.33 $\mu\text{g}/\text{mL}$, and CMVIG at 80 $\mu\text{L}/\text{mL}$. PMA/iono served as positive control. The number of CD56 $^+$ CD3 $^-$ NK cells expressing the cytokines TNF (A), IFN- γ (B), IL-2 (C), or IL-4 (D) was quantified and expressed as percentage of gated NK cells. Two-group comparisons (medium vs CMVIG, nonopsonized vs opsonized antigens, and CMVIG vs opsonized antigens) were assessed using the Mann-Whitney U test. Statistically significant *P* values are shown in bold. The frequency of TNF- and IFN- γ -producing NK cells was significantly increased in the presence of CMVIG. CD, cluster of differentiation; CMV, cytomegalovirus; CMVIG, CMV immunoglobulin; IFN, interferon; IL, interleukin; ops., opsonized; NK, natural killer; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin; PBMC, peripheral blood mononuclear cells; pp65, phosphoprotein 65; TNF, tumor necrosis factor.

concentrations showing weak to strong inhibition of PBMC response in our ELISpot assay (Figure 7).

DISCUSSION

This study investigated the immunomodulatory properties of CMVIG and CMVIG-opsonized CMV antigens on isolated human PBMCs *in vitro*.

We showed that CMVIG can increase the number of IFN- γ -secreting PBMCs of both CMV-seronegative and -seropositive individuals. In CMV-seropositive donors, the frequency

of NK cells and CTLs (and to a lesser extent of Th cells) producing the Th1-type cytokines TNF and IFN- γ was increased in the presence of CMVIG, as was the frequency of monocytes producing the Th1-polarizing cytokine IL-12. The investigated cells did not express Th2-polarizing cytokines (IL-4, IL-10). CMVIG-opsonized CMV antigens exerted varying effects on the different immune cell populations. They slightly reduced the CMVIG-mediated stimulation of NK cells, whereas they enhanced to some extent the CMV antigen-induced Th-cell activation, as measured by the frequency of TNF- and IFN- γ -producing cells. Importantly, in CMV-seropositive individuals,

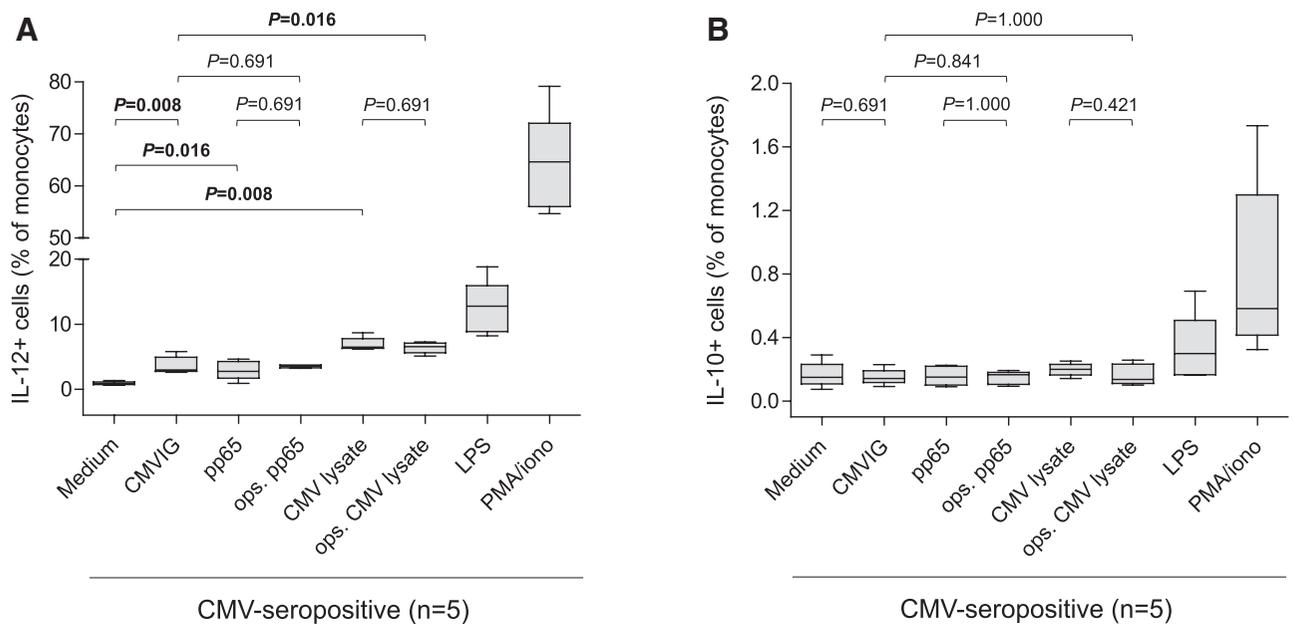


FIGURE 4. Immunomodulatory effect of CMVIG on monocytes. Freshly isolated PBMCs from 5 healthy CMV-seropositive individuals (donors 2–6) were stimulated, stained, and analyzed by flow cytometry as described in Materials and Methods. CMV pp65 antigen was used at 1 $\mu\text{g}/\text{mL}$, CMV lysate at 0.33 $\mu\text{g}/\text{mL}$, and CMVIG at 80 $\mu\text{L}/\text{mL}$. LPS and PMA/iono served as positive controls. The number of CD14⁺ cells (monocytes) expressing the cytokines IL-12 (A) and IL-10 (B) was quantified and expressed as percentage of gated monocytes. Two-group comparisons (medium vs CMVIG, nonopsonized vs opsonized antigens, and CMVIG vs opsonized antigens) were assessed using the Mann-Whitney U test. Statistically significant *P* values are shown in bold. The proportion of IL-12-producing monocytes was slightly but significantly increased in the presence of CMVIG. In contrast, the proportion of IL-10-producing monocytes did not increase in the presence of CMVIG or CMV antigens (nonopsonized or opsonized). CD, cluster of differentiation; CMV, cytomegalovirus; CMVIG, CMV immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; ops., opsonized; PBMC, peripheral blood mononuclear cells; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin; pp65, phosphoprotein 65.

opsonized CMV antigens enhanced CMVIG-mediated stimulation of CTL (expressing TNF and IFN- γ), whereas CMV antigens alone showed no stimulatory activity.

The CMVIG-mediated increase in the number of IFN- γ -secreting cells by ELISpot in both CMV-seronegative and -seropositive healthy donors indicates a global stimulatory effect on innate immune cells. This is in line with the increase in the number of TNF- and IFN- γ -producing NK cells identified by flow cytometry. Moreover, the dampening of CMVIG-mediated stimulation by opsonized CMV antigens noted in the IFN- γ ELISpot assay mirrored that observed on (TNF- and) IFN- γ -producing NK cells by flow cytometry, further suggesting that CMVIG can stimulate innate immune cells like NK cells to produce the immunostimulatory cytokines TNF and IFN- γ . The mechanism of NK cell activation by CMVIG might be similar to that described by Tha-In et al³⁸ in response to IVIG.³⁸ They showed that multimers of IgG bind to the surface of maturing DCs, allowing their recognition by NK cells via Fc γ RIIIA (CD16) on NK cells. This DC–NK cell interaction activates NK cells, which secrete IFN- γ .³⁸ In turn, secretion of IFN- γ by NK cells is expected to stimulate the maturation and activation of T cells, DCs, and monocytes/macrophages.⁶² Mature DCs and monocytes/macrophages are known to secrete the Th1-polarizing cytokine IL-12, which can further boost NK cell activation.^{8,62} This possible scenario is in agreement with the cytokine profile of NK, monocytes, and T cells measured in our study. In contrast, IgG-bound Fc γ RIIIA (CD16) on resting NK cells is known to synergize with other surface receptors to stimulate NK cells to produce TNF and IFN- γ .⁶³ Given that this synergy can be induced by monoclonal antibodies directed against these coactivation receptors,⁶³ it is possible that some antibodies of the CMVIG preparation

reproduce this synergistic activation of resting NK cells in our isolated PBMCs. In that regard, it is conceivable that the anti-CD49d antibody used for costimulation in the intracellular staining assay might play a role in this coactivation pathway. Indeed, engagement of CD49d ($\alpha_4\beta_1$ integrin) at the surface of NK cells was also shown to contribute to NK cell activation.⁶⁴

As mentioned above, secretion of IL-12 by APCs (such as monocytes, as observed in our study) and of IFN- γ by NK cells might contribute to the CMVIG-mediated stimulation of CD4⁺ Th and CD8⁺ CTL. In addition, according to the acknowledged costimulatory threshold model,^{65–68} cross-linking of T-cell surface receptors by antibodies of the CMVIG preparation might provide a strong activation signal. As noted above for NK cells, it is likely that the anti-CD28 and anti-CD49d antibodies used as costimulatory signals for intracellular cytokine staining^{68–72} further contributed to T-cell activation by CMVIG in our assay.

We found that the impact of CMV antigens on CMVIG-mediated stimulation differed with the immune cell subpopulation considered. According to the above-proposed models of CMVIG-mediated activation, the partial inhibition of CMVIG-mediated activation of NK cells by CMV antigens might be explained by a “titration” effect. In particular, opsonized CMV antigens might not stimulate NK cells as immunocomplex but might instead interfere with or prevent the DC–NK cell crosstalk or the synergy mediated by free immunoglobulins on NK surface receptors. In contrast, CMV antigen opsonization by CMVIG is expected to promote antigen uptake and processing by APCs, and the presentation of peptides in the context of major histocompatibility complex class II and class I (cross-presentation) molecules.^{27–30} Indeed, CMV antigen-induced stimulation of CD4⁺ T cells was slightly increased by opsonized antigens, and despite a nondetectable

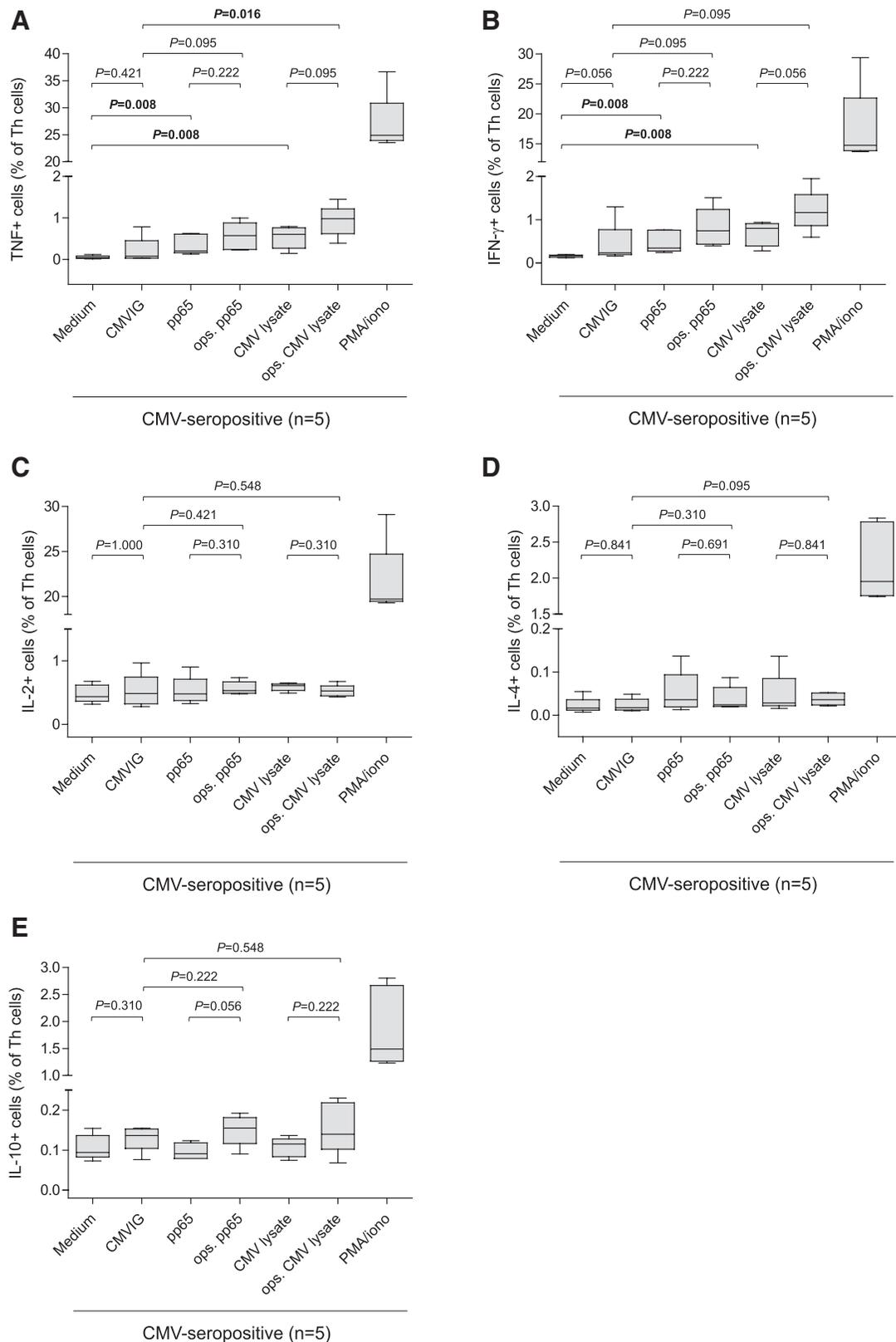


FIGURE 5. Immunomodulatory effect of CMVIG on Th cells. Freshly isolated PBMCs from 5 healthy CMV-seropositive individuals ([A–D] donors 1–5: T-cell protocol; [E] donors 2–6: APC protocol) were stimulated, stained, and analyzed by flow cytometry as described in Materials and Methods. CMV pp65 antigen was used at 1 $\mu\text{g}/\text{mL}$, CMV lysate at 0.33 $\mu\text{g}/\text{mL}$, and CMVIG at 80 $\mu\text{L}/\text{mL}$. PMA/iono served as positive control. The number of CD4⁺CD3⁺ T helper (Th) cells expressing the cytokines TNF (A), IFN- γ (B), IL-2 (C), IL-4 (D), or IL-10 (E) was quantified and expressed as percentage of gated Th cells. Two-group comparisons (medium vs CMVIG, nonopsonized vs opsonized antigens, and CMVIG vs opsonized antigens) were assessed using the Mann-Whitney U test. Statistically significant *P* values are shown in bold. The frequency of TNF- and IFN- γ -producing Th cells was slightly but not significantly increased in the presence of CMVIG. APC, antigen-presenting cells; CD, cluster of differentiation; CMV, cytomegalovirus; CMVIG, CMV immunoglobulin; IFN, interferon; IL, interleukin; ops., opsonized; PBMC, peripheral blood mononuclear cell; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin; pp65, phosphoprotein 65; Th, T helper; TNF, tumor necrosis factor.

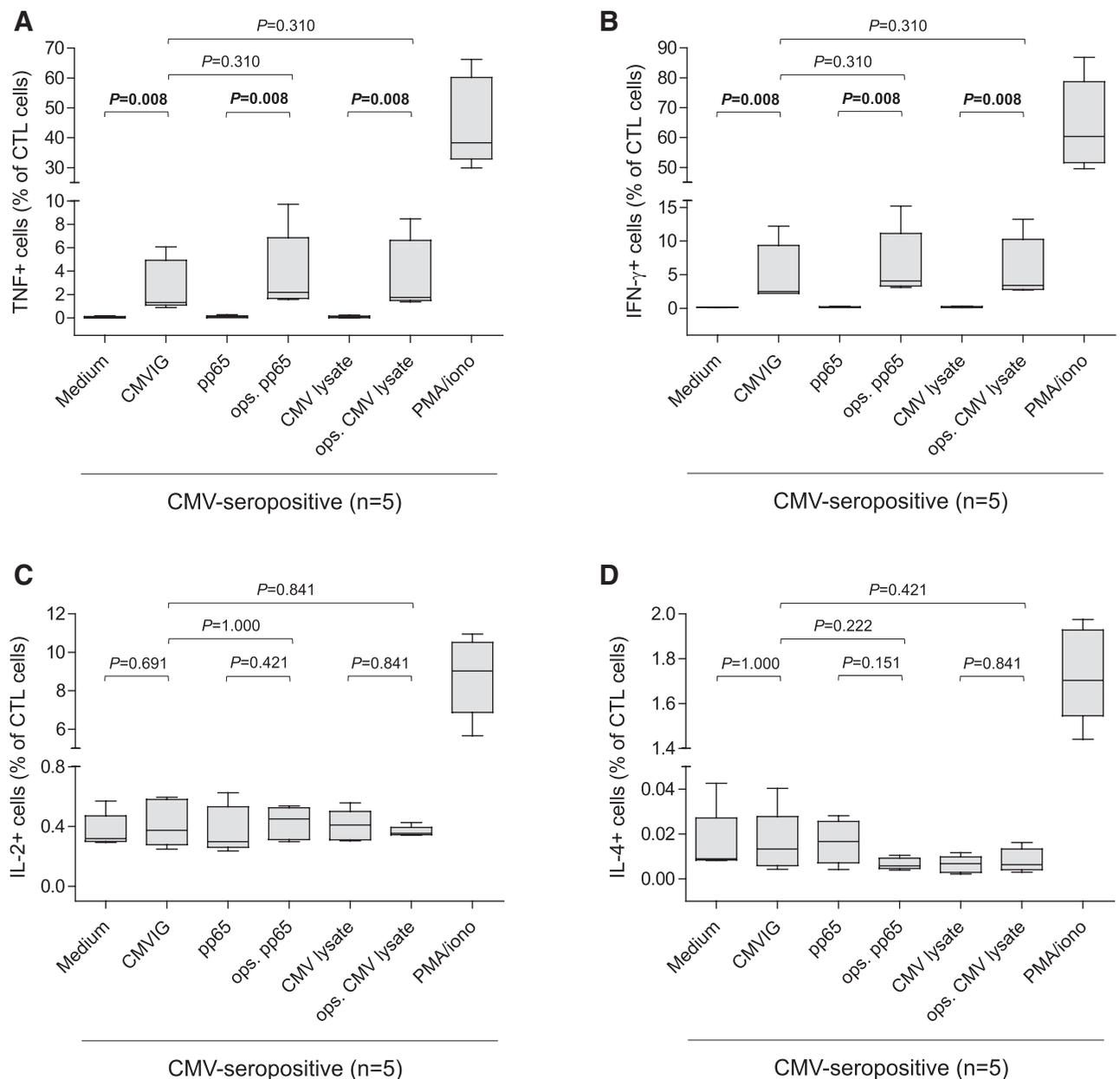


FIGURE 6. Immunomodulatory effect of CMVIG on CTL. Freshly isolated PBMCs from 5 healthy CMV-seropositive individuals (donors 1–5) were stimulated, stained, and analyzed by flow cytometry as described in Materials and Methods. CMV pp65 antigen was used at 1 $\mu\text{g}/\text{mL}$, CMV lysate at 0.33 $\mu\text{g}/\text{mL}$, and CMVIG at 80 $\mu\text{L}/\text{mL}$. PMA/iono served as positive control. The number of CD8 $^+$ CD3 $^+$ CTL expressing the cytokines TNF (A), IFN- γ (B), IL-2 (C), or IL-4 (D) was quantified and expressed as percentage of gated CTL. Two-group comparisons (medium vs CMVIG, nonopsonized vs opsonized antigens, and CMVIG vs opsonized antigens) were assessed using the Mann-Whitney U test. Statistically significant *P* values are shown in bold. The frequency of TNF- and IFN- γ -producing CTL was significantly increased in the presence of CMVIG. CD, cluster of differentiation; CMV, cytomegalovirus; CMVIG, CMV immunoglobulin; CTL, cytotoxic T lymphocyte; IFN, interferon; IL, interleukin; ops., opsonized; PBMC, peripheral blood mononuclear cell; PMA/iono, phorbol 12-myristate 13-acetate/ionomycin; pp65, phosphoprotein 65; TNF, tumor necrosis factor.

stimulation of CD8 $^+$ T cells by CMV antigens alone, CMVIG-mediated stimulation of CD8 $^+$ T cells was enhanced by opsonized CMV antigens. Altogether, our results suggest that CMVIG can increase the immunogenicity of CMV antigens, probably by promoting the uptake of immunocomplexes by APCs and their presentation both on major histocompatibility complex class II and class I (cross-presentation) to activate CD4 $^+$ and CD8 $^+$ T-cell responses, respectively. Thus, our results indicate that in addition to its antiviral effects, such as virus neutralization, CMVIG can stimulate CMV-specific immunity. The latter may be particularly relevant in immunosuppressed transplant recipients, who count as one of the

most vulnerable population in terms of development of CMV infection and disease.

Finally, our study showed that, although to a possibly lower extent, the stimulatory effects of CMVIG were also observed in PBMCs treated with clinically relevant concentrations of the immunosuppressive agents cyclosporine A or tacrolimus. We thus expect that CMVIG is able to promote cell-mediated immunity to CMV even in immunosuppressed transplant recipients.

Our study has several limitations. First, because of the exploratory nature of this research project, only a limited number of individuals could be included in each analysis. This

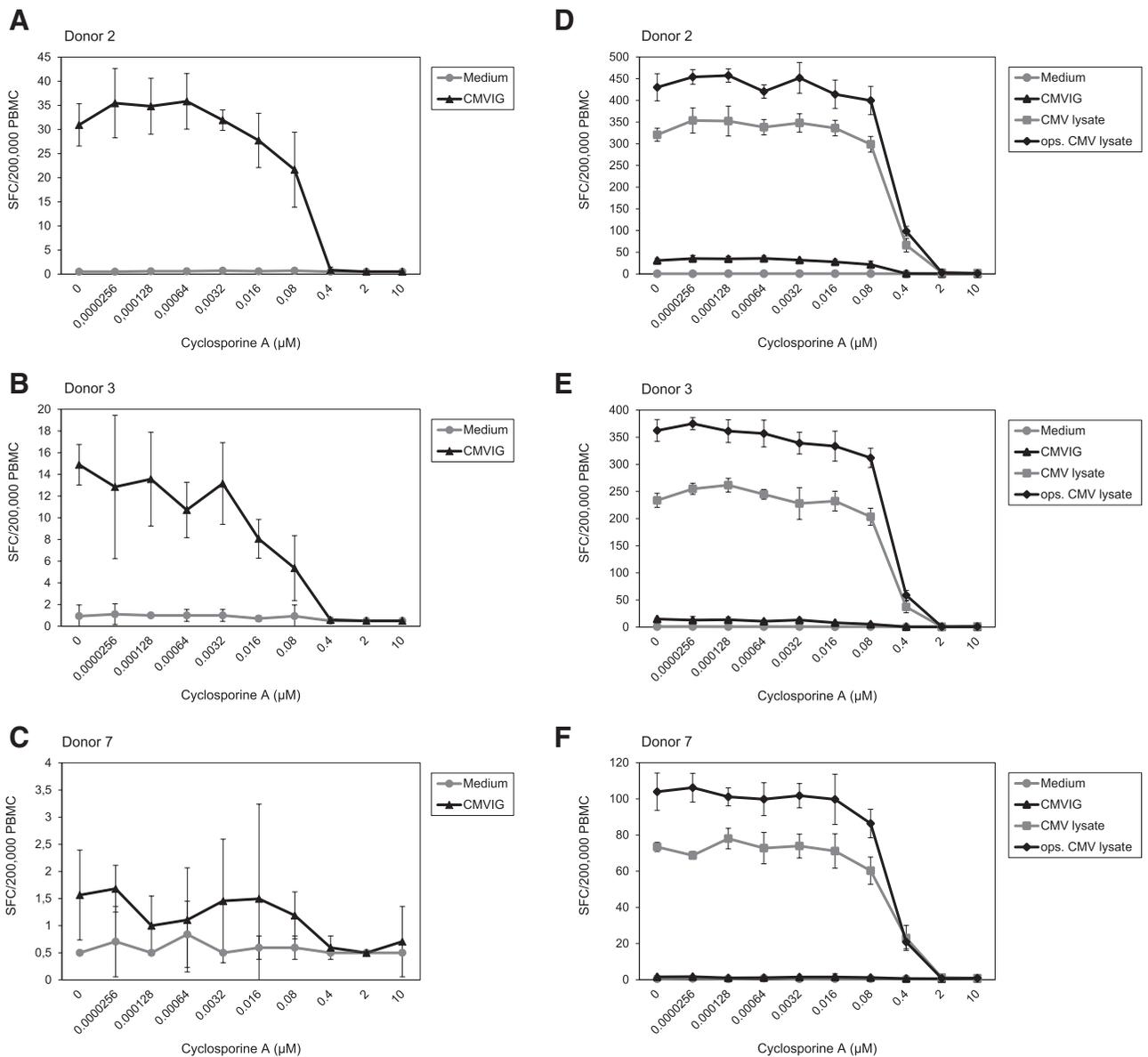


FIGURE 7. Sensitivity of CMVIG-treated PBMCs to the immunosuppressive agent cyclosporine A. PBMCs freshly isolated from 3 healthy CMV-seropositive individuals ([A and D] donor 2; [B and E] donor 3; [C and F] donor 7) were incubated for 19h with the indicated stimulants (8.8 $\mu\text{L/mL}$ CMVIG, 0.11 $\mu\text{g/mL}$ CMV lysate, opsonized CMV lysate, or medium as control) in the presence of increasing amounts of cyclosporine A. The number of IFN- γ -producing cells was measured by IFN- γ ELISpot. Geometric means of SFC/ 2×10^5 PBMCs (\pm SD) are shown. To better visualize the stimulating effect of CMVIG alone, the results of medium and CMVIG conditions are shown separately at a different scale (A–C), in addition to all conditions (D–F). The beneficial anti-CMV effects mediated by CMVIG were also observed in PBMCs treated with clinically relevant concentrations of cyclosporine A. CMV, cytomegalovirus; CMVIG, CMV immunoglobulin; ELISpot, enzyme-linked immunospot; IFN, interferon; ops., opsonized; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell.

prevented relevant statistical analyses and is probably the main cause for the lack of statistical significance observed in many group comparisons. However, the consistency of the effects observed at the individual level in our flow cytometry analyses strongly argues in favor of their biological significance. Second, this study aimed at investigating early events following treatment with CMVIG and thus did not consider the late anti-inflammatory effects associated with CMVIG that contribute to reduction of organ rejection after transplantation.^{18,48–51,73} Finally, the characterization of the stimulatory effect of CMVIG on innate immunity conducted in CMV-seropositive individuals by intracellular staining and flow cytometry could not be compared with that in CMV-seronegative subjects.

In conclusion, we showed that CMVIG can stimulate effector cells of both the innate and adaptive immune systems and promote the immunogenicity of CMV antigens. The Th1-polarized environment of preactivated NK cells might potentially respond more rapidly and effectively to CMV infection in posttransplant patients treated with CMVIG, including in CMV-seronegative patients. This is particularly relevant, because NK cells play an essential role in the early control of CMV infection.⁷⁴ Altogether, this study demonstrates that, in addition to the transfer of passive humoral immunity, the use of CMVIG in transplantation might promote a stronger and faster cellular response to CMV infection and thus contribute to CMV prophylaxis.

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