Simultaneous Activation of Viral Antigen-specific Memory CD4$^+$ and CD8$^+$ T-cells Using mRNA-electroporated CD40-activated Autologous B-cells

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Summary: Recently, it has become obvious that not only CD8$^+$ T-cells, but also CD4$^+$ T-helper cells are required for the induction of an effective, long-lasting cellular immune response. In view of the clinical importance of cytomegalovirus (CMV) and human immunodeficiency virus (HIV) infection, we developed 2 strategies to simultaneously reactivate viral antigen-specific memory CD4$^+$ and CD8$^+$ T-cells of CMV-seropositive and HIV-seropositive subjects using mRNA-electroporated autologous CD40-activated B cells. In the setting of HIV, we provide evidence that CD40-activated B cells can be cultured from HAART-naive HIV-1 seropositive patients. These cells not only express and secrete the HIV p24 antigen after electroporation with codon-optimized HIV-1 *gag* mRNA, but can also be used to in vitro reactivate Gag antigen-specific interferon-γ–producing CD4$^+$ and CD8$^+$ autologous T-cells. For the CMV-specific approach, we applied mRNA coding for the pp65 protein coupled to the lysosomal-associated membrane protein-1 to transfect CD40-activated B cells to induce CMV antigen-specific CD4$^+$ and CD8$^+$ T-cells. More detailed analysis of the activated interferon-γ–producing CMV pp65 tetramer positive CD8$^+$ T-cells revealed an effector memory phenotype with the capacity to produce interleukin-2. Our findings clearly show that the concomitant activation of both CD4$^+$ and CD8$^+$ (memory) T-cells using mRNA-electroporated CD40-B cells is feasible in CMV and HIV-1–seropositive persons, which indicates the potential value of this approach for application in cellular immunotherapy of infectious diseases.

Key Words: B cells, CMV infection, HIV-1 infection, antigen-presenting cell-based immunotherapy, mRNA transfection

(*J Immunother* 2006;29:512–523)

Cellular immunotherapy using antigen-loaded antigen-presenting cells (APCs) such as dendritic cells (DCs) has recently been the subject of several clinical trials, because it is now generally accepted that exploiting the immune regulatory capacities of these APCs holds great promise for the treatment of cancer, autoimmune, and infectious diseases.[1] Especially infections with viruses that have a latent reservoir, like cytomegalovirus (CMV) and human immunodeficiency virus (HIV), have become interesting immunotherapy targets. In the setting of HIV-1 immunotherapy, antigen-loaded DCs have already been applied successfully to stimulate HIV-specific interferon (IFN)-γ–producing CD8$^+$ T-cells in vitro.[2,3] Convincing in vivo results, using DCs loaded with inactivated autologous virus have been obtained by Lu et al[4] in untreated HIV-seropositive patients. Immunotherapy to fight CMV reactivation in immunosuppressed patients after hematopoietic stem cell transplantation has also become a major field of investigation in cellular immunotherapy. For CMV adoptive T-cell therapy, Trivedi et al[5] for example have shown that DCs loaded with CMV antigens can be used to generate CMV-specific T-lymphocytes.

DC-based immunotherapy suffers from some drawbacks, such as the need for multiple phlebotomies to obtain sufficient DCs for immunotherapy protocols and the fact that monocyte-derived DCs cannot be expanded.[6] Therefore, the use of antigen-loaded CD40-activated B-cells (CD40-B cells) to induce antigen-specific T-cell responses has been explored by us and others.[7–12] mRNA electroporation is a gene transfer method initially developed for DCs, T cells, and stem cells.[13–16] mRNA-based loading of CD40-B cells was demonstrated

Received for publication September 8, 2005; accepted January 12, 2006.
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Supported by Grants No. G.0456.03, G.0313.01, and WO.012.02N of the Fund for Scientific Research—Flanders, Belgium (FWO-Vlaanderen), agreement 2002/210 with the Agence de Recherche sur le SIDA (ANRS) of France and a Concerted Research Action Grant No. 802 of the University of Antwerp (GOA BOF UA).

Glenn A. Van den Bosch was holder of a PhD fellowship from the FWO-Vlaanderen. Ellen Van Gulck holds a PhD fellowship from the Flemish Institute for Science and Technology (IWT). Viggo F. I. Van Tendeloo is a postdoctoral fellow of the FWO-Vlaanderen.
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to be as efficient as that in monocyte-derived DCs, suggesting that the CD40-B cell system constitutes an excellent alternative APC source with proliferative capacity, thereby circumventing the disadvantages inherent in DC-based immunotherapy related to their nondividing state.[11–12]

The benefits of applying mRNA electroporation over traditional antigen loading methods such as viral transduction or peptide pulsing have been shown in detail by several investigators and make this approach a safe and feasible option for clinical immunotherapy protocols.[17–18] Because it has been demonstrated that the expansion of both $CD4^+$ and $CD8^+$ T-cell responses is beneficial for the induction or reactivation of a robust memory cellular immune response, the simultaneous induction of $CD4^+$ and $CD8^+$ T-cell responses using the mRNA strategy would combine the advantages of both concepts.[19] CD40-B cells are known to process exogenous antigens and load them into the major histocompatibility (MHC) class II molecules after CD40-CD40 ligand interaction and tumor lysate pulsing.[10,20] However, because mRNA is mainly processed through the MHC class I pathway, leading to $CD8^+$ T-cell activation, the induction of $CD4^+$ T-cell responses through mRNA requires either the use of mRNA coding for a secreted antigenic protein that can be taken up by the APC for MHC class II processing or the artificial linking of the mRNA antigen sequence to endosomal targeting sequences.

To our knowledge, this is the first report that RNA electroporation of CD40-B cells leads to simultaneous activation of antigen-specific $CD4^+$ and $CD8^+$ T-cells. Furthermore, we describe the feasibility of using CD40-B cells in HIV-seropositive patients for reactivation of both $CD4^+$ and $CD8^+$ Gag-specific T-cells. Considering the clinical importance of CMV and HIV, we developed 2 strategies to simultaneously reactivate in vitro viral antigen-specific memory $CD4^+$ and $CD8^+$ T-cells of CMV-seropositive and HIV-seropositive subjects using Sig-pp65 CMV–lysosomal-associated membrane protein-1 (LAMP1) and codon-optimized HIV-1 *gag* mRNA-electroporated autologous CD40-B cells respectively. Further phenotypical analysis and cytokine profiling was performed to demonstrate that this immunotherapy approach is feasible in CMV and HIV-1–seropositive persons.

MATERIALS AND METHODS

Study Population

Peripheral blood samples (100 mL) were obtained from 11 HIV-1–seropositive patients recruited at the Clinical Department of the Institute of Tropical Medicine (ITM, Antwerp, Belgium), according to institutional guidelines and after obtaining informed consent. Inclusion criteria were: antiviral therapy-naive individuals having a $CD4^+$ T-cell count above 400/μL at the time of the previous blood donation. Characteristics of the HIV(+) subjects are summarized in Table 1. Peripheral blood mononuclear cells (PBMCs) of HIV-1–seronegative, CMV-seropositive individuals were obtained from healthy blood donors who were serologically typed for CMV seropositivity. Mononuclear cells from all subjects were isolated by Ficoll-Hypaque gradient separation (LSM, ICN Biomedicals, Costa Mesa, CA).

Human Leukocyte Antigen Typing of PBMCs

To determine human leukocyte antigen-A2 (HLA-A2) expression, PBMCs were at first incubated with the supernatant of the BB7-2 hybridoma (anti-HLA-A2, ATCC, Manassas, VA), followed by staining with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins (Dako, Heverlee, Belgium). HLA-A2 presence or absence was analyzed by flow cytometry using a FACScan analytical flow cytometer (Becton Dickinson, Erembodegem, Belgium).

Cell Lines

T2 cells (TAP-deficient, HLA-$A2^+$, T × B hybrid) were kindly provided by Dr Pierre Van der Bruggen (Ludwig Institute for Cancer Research, Brussels, Belgium) and were cultured in complete medium consisting of Iscove modified Dulbecco medium (IMDM) supplemented with 2 mM L-glutamine (Invitrogen, Merelbeke, Belgium), 10 μg/mL gentamicin (Invitrogen, Merelbeke, Belgium), 1.25 μg/mL amphotericin B (Fungizone, Invitrogen, Merelbeke, Belgium), and 10% fetal calf serum (FCS; Sera Laboratory, Sussex, UK). 3T3 feeder cells stably transfected with CD40 ligand (t-CD40L 3T3 feeder cells) were kindly provided by Dr K. Thielemans (Medical School of the Vrije Universiteit Brussel, Brussels, Belgium) and were cultured in IMDM supplemented with 2 mM L-glutamine, 10 μg/mL gentamicin, and 500 μg/mL G418 (Invitrogen). All cells were maintained in logarithmic phase growth at 37°C in a humidified atmosphere supplemented with 5% CO_2.

In Vitro Culture of CD40-B cells

CD40-B cells were generated from PBMCs by coculturing whole PBMC at 2×10^6 cells/mL with irradiated (96 Gy) t-CD40L 3T3 feeder cells in IMDM supplemented with 10% FCS, 5 μg/mL insulin (Sigma, St Louis, MO), 10 μg/mL gentamicin, 2 ng/mL interleukin (IL)-4, and 1.9×10^{-7} M cyclosporin A (Novartis Pharma, Vilvoorde, Belgium).[7] Cultured cells were transferred to irradiated new t-CD40L 3T3 feeder cells every 3 days. Before use in cocultures, CD40-B cells were Ficoll-Hypaque density centrifuged and cryopreserved to remove nonviable cells and remaining t-CD40L 3T3 feeder cells.

Freezing and Thawing Procedure

PBMCs or CD40-B cells were frozen in cryotubes (Nunc CryoTube Vials, Nalgene Nunc International, Roskilde, Denmark) at a concentration of 1 to 10×10^6/mL in 90% FCS and 10% dimethyl sulfoxide (Sigma). Cell suspensions were slowly frozen (−1°C/min) to −80°C by using a cryo-freezing container (Nalgene Nunc International, Rochester, NY). Frozen cells were

thawed quickly in a 37°C water bath, followed by the addition of 100 μg/mL DNaseI (Roche Diagnostics, Vilvoorde, Belgium) and 50 μL/mL of a 3.79% $MgSO_4$ solution for 10 minutes. Next, cells were centrifuged and resuspended in IMDM for 15 minutes to remove residual dimethyl sulfoxide. Finally, cells were washed once and resuspended in culture medium.

Endocytosis Experiments

To evaluate the mannose-receptor–mediated endocytosis capacity of CD40-B cells, 500,000 cultured CD40-B cells were incubated at 4°C and at 37°C for 1 hour with FITC-dextran (MW 40000, Sigma-Aldrich, Bornem, Belgium) at a concentration of 10 μg/mL in IMDM. Endocytosis was stopped by washing 2 times with ice-cold phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Afterwards, cells were stained with phycoerythrin (PE)-conjugated anti-CD19 and peridinin chlorophyll protein (PerCP)-conjugated anti-CD45 antibodies to gate exclusively on the CD40-B cells. To evaluate macropinocytosis, similar experiments were performed with FITC-albumin (Sigma-Aldrich) at a concentration of 500 μg/mL IMDM. All monoclonal antibodies used in this study were from Becton Dickinson (Erembodegem, Belgium).

Plasmid DNA Constructs

mRNA encoding CMV pp65 protein was purchased from Curevac (Tübingen, Germany). The pGEM4Z/influenza M1/A64 plasmid was kindly provided by Dr A. Steinkasserer (University of Erlangen, Erlangen, Germany). The pGEM4Z/(h)gag/A64 plasmid, used to prepare a humanized (codon-optimized) mRNA encoding the H × B2 HIV-1 Gag protein, was provided by Dr K. Thielemans (Medical School of the Vrije Universiteit Brussel, Brussels, Belgium). The original humanized *gag* cDNA was provided by Dr B. Verrier (BioMérieux, Paris, France). The pGEM4Z/Sig-pp65-LAMP1 plasmid was provided by Dr C. Bonini (Cancer Immunotherapy and Gene Therapy Program, S. Raffaele Scientific Institute, Milano, Italy). These plasmids were propagated in *Escherichia coli* supercompetent cells (Stratagene, La Jolla, CA) and purified on endotoxin-free Qiagen-tip 100 columns (Westburg, Leusden, The Netherlands). Next, the plasmids were linearized with SpeI (MBI Fermentas, St Leon-Rot, Germany), purified using a polymerase chain reaction purification kit (Westburg) and used as DNA template for in vitro transcription. Transcription was carried out in a final 20 μL reaction mix at 37°C using a T7 Opti mRNA transcription kit (CureVac) to generate 5′ capped in vitro transcribed mRNA. Purification of mRNA was performed by DNaseI digestion, followed by LiCl precipitation, according to the manufacturer's instructions.

mRNA Electroporation

Electroporation of mRNA was carried out as described previously with minor modifications.[21] Briefly, before electroporation, 10×10^6 CD40-B cells were washed twice with Optimix washing solution (EquiBio, Ashford, Middlesex, UK) and resuspended in Optimix electroporation buffer (EquiBio). Subsequently, 0.2 mL of the cell suspension was mixed with 20 μg of in vitro transcription mRNA and electroporated in a standard 0.4 cm cuvette at 300 V and 150 μF using an Easyject Plus device (EquiBio). After electroporation, fresh complete medium was added to the cell suspension and cells were further incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2. All mock electroporations were performed according to this protocol but without the use of mRNA.

Intracellular Staining for HIV Gag Protein

CD40-B cells were electroporated with humanized (h)*gag* mRNA and analyzed for intracellular Gag expression 24 hours after electroporation. To this end, mRNA-electroporated and mock-electroporated (control) cells were stained with [FITC]-conjugated anti-CD19 and [PerCP]-conjugated anti-CD45 antibodies, after washing with PBS containing 0.5% bovine serum albumin. To fixate, the cells were washed and fixed for 10 minutes with Fix and Lyse solutions (Becton Dickinson, Erembodegem, Belgium) at room temperature. Next, cells were permeabilized with Perm2 solution (Becton Dickinson) for 30 minutes at room temperature, stained for 15 minutes at 4°C with 0.5 μL of anti-GAG HIV-1 RD-1 conjugated monoclonal antibody (Beckman Coulter, Paris, France) and analyzed by flow cytometry.

p24 Enzyme-linked Immunosorbent Assay

CD40-B cells, mock-electroporated or (h)*gag* mRNA-electroporated, were cultured in complete culture medium at 2×10^5 cells/200 μL in 96-well plates. After 24 hours, supernatant samples were collected and p24 Gag secretion was measured by the Innotest HIV Antigen mAb enzyme-linked immunosorbent assay system (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions.

IFN-γ ELISPOT Assay

Multiscreen mixed cellulose esters (HA) plates (Millipore, Bedford, MA) were used in combination with the human IFN-γ enzyme-linked immunospot (ELISPOT) kit (Diaclone, Besançon, France). Coating of the plates was carried out according to the manufacturer's instructions. After overnight coating, the plates were washed with PBS and blocked with serum-free lymphocyte medium (AIM-V) medium containing 10% human AB (hAB) serum (Sigma-Aldrich, Belgium). For some experiments, $CD4^+$ T-cells were positively selected using EasySep $CD4^+$ T-cell enrichment cocktail (StemCell Technologies, Meylan, France) according to the manufacturer's instructions. One hundred micro liters of PBMCs, $CD4^+$ T-cells, or $CD4^+$ depleted PBMCs (effector cells) at a concentration of 5×10^6 cells/mL, and 100 μL of mock or (h)*gag* mRNA-electroporated autologous CD40-B cells (stimulator cells) at a concentration of 5×10^5 cells/mL were dispensed

per well. For all cell suspensions, IMDM supplemented with 5% hAB serum was used as medium. Conditions with PBMC, $CD4^+$ T-cells, or $CD4^+$ T-cell–depleted PBMC alone, mock-electroporated CD40-B cells alone, and (h)*gag* mRNA-electroporated CD40-B cells alone were used as negative controls. Effector cells stimulated with 0.5 μg/well phytohaemagglutinin (PHA) were used as a positive control. After a coculture period of 24 hours, the plates were further developed as described in the manufacturer's protocol. Spot numbers were counted using an automated ELISPOT reader (AID, Strassberg, Germany).

Induction of CMV pp65 Antigen-specific T-cells With mRNA-electroporated CD40-B cells

CD40-B cells were electroporated with CMV pp65 or Sig-CMV pp65-LAMP1 mRNA and resuspended in IMDM supplemented with 5% hAB serum. Four to 12 hours after electroporation, antigen-loaded cells were used for stimulation of autologous PBMCs. Briefly, 5×10^6 CD40-B cells were cocultured with 20×10^6 autologous PBMCs in IMDM supplemented with 5% hAB serum, 2 ng/mL IL-4, and 10 ng/mL IL-7. On days 2 and 4 of the cocultures, 20 U/mL IL-2 was added. After 7 days of culture, cells were restimulated for 3 to 6 hours with peptide-loaded or unloaded T2 cells, with recombinant pp65 protein or with complete medium depending only on the experiment performed [in the presence of 10 μL Golgi-Plug (Becton Dickinson) per milliliter medium]. Subsequently, the cells were washed with PBS, stained for 20 minutes at room temperature with 1 μg/mL of CMV pp65/HLA*0201 tetramer (Orpegen Pharma, Heidelberg, Germany). Next, membrane staining was accomplished by incubating for 15 minutes at 4°C with the following monoclonal antibodies: [APC-Cy7]-conjugated CD3, [APC]-conjugated CD45RA, [Pacific Blue]-conjugated CD4, [PerCP]-conjugated CD8, and [PE-Cy7]-conjugated CCR7. After a wash step, cells were fixed for 10 minutes with Fix and Lyse solutions (Becton Dickinson, Erembodegem, Belgium) at room temperature, washed again, and permeabilized with Perm2 solution (Becton Dickinson) for 10 minutes at room temperature. Next, cells were washed and intracellularly stained overnight at 4°C with [FITC]-conjugated anti–IFN-γ and/or [PE]-conjugated anti–IL-2. Before measurement, cells were washed again and resuspended in 0.5 mL PBS. All flow cytometric analyses of intracellular staining were carried out on the BD LSR II System (Becton Dickinson).

Statistical Analysis

Results are expressed as mean ± SD or ± SEM as indicated. Comparisons were validated using a 2-sided Student *t* test. A P value ≤ 0.01 was considered to be statistically significant.

RESULTS

CD40-B cells can be Cultured From PBMCs of HIV-1–Seropositive Donors and Have the Capacity to Capture Antigens Through Mannose Receptor-mediated Endocytosis and Macropinocytosis

From all HIV-1–infected subjects included in the study (Table 1), CD40-B cells could be cultured out of isolated PBMCs as described in the Materials and Methods section. After 11 to 19 days (mean ± SD: 15 ± 4 days, n = 11) of culture, at least 75% of the cells had the $CD45^+CD19^+$ phenotype of CD40-B cells (range: 75% to 89%, mean ± SD: 83 ± 4%, n = 11), whereas the percentage of T-lymphocytes was below 15% of the viable cells in all cultures. No more than 1% of irradiated tCD40L-3T3 cells were present in the CD40-B cell populations used for the experiments. Because the uptake of antigens is necessary for the presentation of antigens through the MHC class II pathway, we examined the antigen uptake capacities of the cultured CD40-B cells. Classically, DCs take up antigen through 2 mechanisms: mannose receptor-mediated endocytosis and macropinocytosis.[22] To evaluate the mannose receptor-mediated endocytosis of CD40-B cells, the uptake of FITC-labeled dextran was measured flow cytometrically. The dextran uptake by CD40-B cells at 37°C was significantly higher than the incubation at 4°C, which clearly shows that these cells are capable of taking up antigen through their mannose receptors (data not shown). To evaluate the macropinocytosis capacity of CD40-B cells, the uptake of FITC-labeled albumin was measured by flow cytometry. The albumin uptake by CD40-B cells at 37°C was significantly higher than the incubation at 4°C, demonstrating that these cells are also capable of taking up antigen through continuous internalization of large volumes of fluid (data not shown). No differences in antigen uptake capacities of CD40-B cells of HIV-1–seronegative or seropositive subjects were observed.

CD40-B cells of HIV-1–Seropositive Donors Express and Secrete Gag Protein After Electroporation With Codon-optimized *gag* mRNA

To demonstrate that the electroporation of CD40-B cells with codon-optimized HIV-1 *gag* mRNA encoding a consensus H × B2 HIV-1 Gag protein leads to efficient expression of the protein, we performed an intracellular staining with an antibody directed against the HIV-1 55, 39, 33 and 24 kd core proteins. Twenty-four hours after electroporation of CD40-B cells with (h)*gag* mRNA, Gag expression in these cells was significantly higher than that in mock-electroporated CD40-B cells, which served as a negative control (Fig. 1A). To activate $CD4^+$ T-cells, the translated protein needs to be secreted, to be taken up (again) and to be presented by MHC class II molecules. Therefore, we measured the p24 antigen concentration in the supernatant of mock and (h)*gag* mRNA-electroporated

TABLE 1. Demographic and Clinical Information for HIV-1–Seropositive Patients

Patient	Number	Age (y)	Sex	HIV + (y)	CD4 Count (cells/μL)	Viral Load (copies/mL)
1	239472	53	M	10	739	50800
2	36799	44	F	9	1996	< 50
3	427443	35	F	2	600	1480
4	511049	28	M	1	520	182000
5	342473	33	M	5	481	320000
6	329541	62	M	6	796	744000
7	240684	57	M	> 10	321	327000
8	177855	54	M	1	631	105000
9	336272	37	M	3	758	2530
10	156281	48	M	1	906	14500
11	408632	35	M	3	854	19200

All included HIV-1–seropositive patients were HAART-naive or off treatment for more than 6 months. For each patient age, sex, number of years of HIV-1 seropositivity, $CD4^+$ T-cell count, and viral load at the time of blood donation are indicated in the table.

CD40-B cells. As shown in Figure 2B, p24 antigen was strongly secreted by the (h)*gag*-electroporated CD40-B cells, whereas the mock-electroporated CD40-B cells did not produce any p24 protein. No p24 secretion was observed in the mock-electroporated CD40-B cells of HIV-1–seropositive subjects included in the study. The experiments above demonstrate that humanized *gag* mRNA is efficiently translated after electroporation and can be used to load the CD40-B cells with the Gag antigen.

Codon-optimized *gag* mRNA-electroporated Autologous CD40-B cells can Reactivate Both Gag-specific $CD4^+$ and $CD8^+$ T-cells in HIV-1–Seropositive Subjects

To demonstrate the ability of (h)*gag* mRNA-electroporated CD40-B cells of reactivating Gag-specific T-cells in HIV-1–seropositive subjects, we directly restimulated ex vivo PBMCs of these subjects with autologous mock-electroporated or (h)*gag* mRNA-electroporated

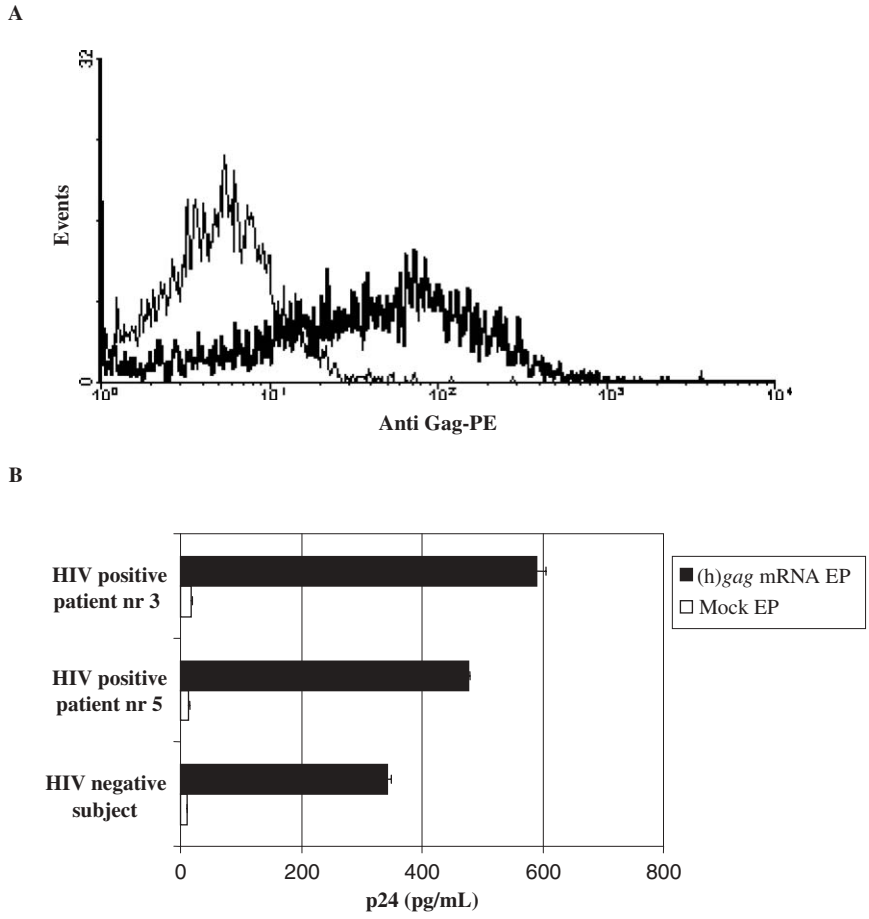

FIGURE 1. CD40-B cells that were electroporated with (h)*gag* mRNA express the Gag protein and secrete the p24 antigen. A, Histogram showing the intracellular Gag expression for (h)*gag* mRNA-electroporated CD40-B cells (bold line) and mock-electroporated CD40-B cells (thin line). Gating was performed on viable $CD45^+CD19^+$ cells. Results shown are representative of 3 individual experiments. B, p24 secretion of CD40-B cells 24 hours after mock or (h)*gag* mRNA electroporation as measured by p24 enzyme-linked immunosorbent assay. Error bars indicate SD.

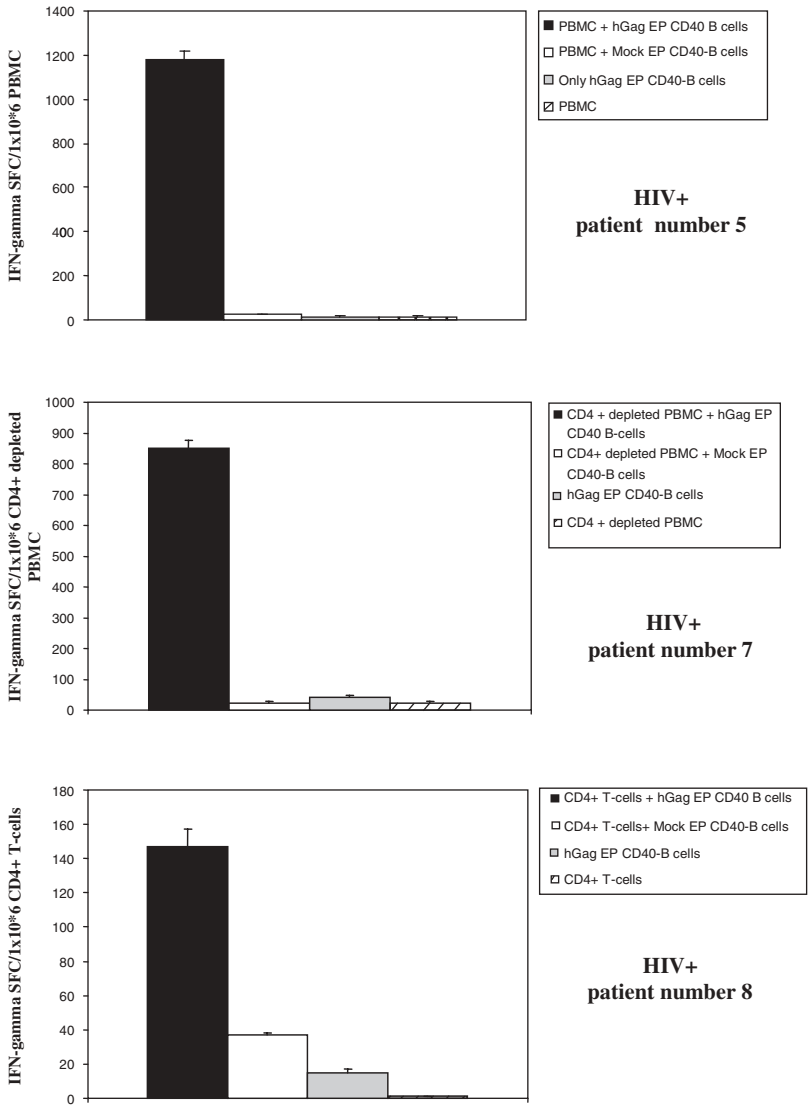

FIGURE 2. (h)*gag* mRNA-electroporated CD40-B cells can reactivate antigen-specific memory T-cells in HIV-1–infected individuals. Graphs represent the number of IFN-γ SFCs per million effector cells after stimulation with mock or (h)*gag* mRNA-electroporated (EP) CD40-B cells. Representative graphs are shown for PBMCs (patient number 5), $CD4^+$ T-cell depleted PBMCs (patient number 7), and $CD4^+$ T-cells (patient number 8) used as effector cells. Negative controls included (h)*gag* mRNA-electroporated CD40-B cells only, PBMCs only, $CD4^+$ T-cell–depleted PBMCs only, or $CD4^+$ T-cells only. All results shown were obtained by a direct IFN-γ ELISPOT assay (error bars indicate SEM).

CD40-B cells during a 24-hour IFN-γ ELISPOT assay. For all subjects tested, a statistically significant ($P < 0.01$) higher number of IFN-γ–producing spot-forming cells (SFCs) per million PBMCs were found after stimulation of PBMCs with the (h)*gag* mRNA-electroporated CD40-B cells than after stimulation with mock-electroporated CD40-B cells, which demonstrates the antigen specificity of the observed ex vivo responses (Table 2 and Fig. 2). Conditions with mock-electroporated or (h)*gag* mRNA-electroporated CD40-B cells without PBMCs did not give rise to any significant number of IFN-γ–producing SFCs. The number of Gag-specific SFCs per million PBMCs varied between patients, but no significant correlation was observed with the viral load ($r = -0.24$, $P = 0.124$) or the $CD4^+$ count ($r = 0.11$, $P = 0.307$) as measured at the time of blood donation. The same observations were made for the correlations of viral load with the number of Gag-specific SFCs per million $CD4^+$ T-cells ($r = -0.02$, $P = 0.200$) and per million $CD4^+$ T-cell–depleted PBMCs ($r = 0.26$, $P = 0.203$). To evaluate the nature of the IFN-γ–producing T-cells, $CD4^+$ T-cells from PBMC of HIV-1–seropositive subjects were positively isolated. $CD4^+$ T-cells and the $CD4^+$ T-cell–depleted PBMC population containing the $CD8^+$ T-cells were stimulated with either mock-electroporated or (h)*gag* mRNA-electroporated autologous CD40-B cells to determine the contribution of both T-cell phenotypes to the overall cellular anti-Gag response. In all patients tested, the observed $CD4^+$ and $CD8^+$ T-cell responses against the (h)*gag* mRNA-electroporated CD40-B cells were significantly higher than the responses observed against the mock-electroporated CD40-B cells (all $P < 0.01$) (Table 2 and Fig. 2). The Gag-specific $CD4^+$ T-cell responses were lower than the $CD8^+$ T-cell responses, but were clearly present, which demonstrates the ability of CD40-B cells to present the Gag antigen also through the MHC class II pathway.

TABLE 2. (h)*gag* mRNA-electroporated CD40-B cells can Reactivate Antigen-specific Memory T-cells in HIV-1-Infected Individuals

		PBMCs		$CD4^+$T-cells		$CD4^+$T-cell Depleted PBMCs	
No.	Reference Number	Gag-specific Response (SFC/10*6 PBMC)	Mock Response (SFC/10*6 PBMC)	Gag-specific Response (SFC/10*6 CD4+)	Mock Response (SFC/10*6 CD4+)	Gag-specific Response (SFC/10*6 cells)	Mock Response (SFC/10*6 cells)
1	239472	80 ± 10	8 ± 3				
2	36799	762 ± 11	346 ± 43				
3	427443	892 ± 67	87 ± 5				
4	511049	87 ± 6	48 ± 5				
5	342473	1182 ± 20	23 ± 3				
6	329541	160 ± 12	34 ± 6				
7	240684			145 ± 4	58 ± 1	851 ± 26	23 ± 4
8	177855			147 ± 10	37 ± 1	940 ± 56	164 ± 22
9	336272			63 ± 9	15 ± 2	537 ± 25	96 ± 3
10	156281			36 ± 10	3 ± 2	494 ± 39	56 ± 20
11	408632			385 ± 18	204 ± 4	1116 ± 18	248 ± 15

IFN-γ ELISPOT results after stimulation with (h)*gag* mRNA-electroporated CD40-B cells or mock-electroporated CD40-B cells are shown for all HIV-1–seropositive donors using 3 different effector populations: whole PBMCs, positively isolated $CD4^+$ T-cells, and $CD4^+$ T-cell–depleted PBMCs. The number of counted spots is presented as mean ± SEM (analysis in quadruplicate) of IFN-γ SFCs per million effector cells.

CMV pp65 mRNA-electroporated CD40-B cells can Activate CMV pp65-specific Effector Memory $CD8^+$ T-cells in CMV-seropositive Donors

To show that CMV pp65 mRNA-electroporated CD40-B cells are able to activate CMV pp65-specific memory $CD8^+$ T-cells in CMV-seropositive donors, these cells were cocultured with autologous PBMC in an effector : CD40-B cell ratio of 4:1. After 1 week of coculture, the primed PBMCs were either left unstimulated or were restimulated for 5 hours with CMV pp65 peptide-pulsed T2 cells or with unloaded T2 cells as control stimulators. PBMCs cocultured for 1 week with mock-electroporated CD40-B cells were used to evaluate the CMV pp65-specific cellular immune response in unprimed PBMCs (ie, without specific stimulation). This control was also meant to exclude any influence of added cytokines (IL-2, IL-4, and IL-7) on the percentage of CMV pp65 tetramer-positive $CD8^+$ T-cells. After 1 week of stimulation with CMV pp65 mRNA-electroporated CD40-B cells, a significantly higher percentage of CMV pp65 tetramer positive cells was observed than the PBMCs, that had been cocultured with mock-electroporated CD40-B cells ($P < 0.01$) (Fig. 3A: lower vs. upper panels). Stimulation of PBMC from CMV-seropositive donors with influenza matrix protein (M1) mRNA-electroporated autologous CD40-B cells for 1 week did not result in expansion of CMV pp65-tetramer positive cells, which demonstrates the antigen specificity once again (data not shown). A comparison between the lower panels of Figure 3A indicates that the percentage of CMV pp65 tetramer-positive $CD8^+$ T-cells was not different according to the type of restimulation. Restimulation with the CMV pp65 peptide-loaded T2 cells, however, resulted in a significantly higher percentage of IFN-γ–producing cells than restimulation with unloaded T2 cells or with medium only, which confirms the antigen specificity of the immune response induced. A fraction of these IFN-γ–producing CMV pp65-specific $CD8^+$ T-cells were also expressing IL-2 (Fig. 3B). A significant lower number of IL-2–expressing $CD8^+$ or $CD4^+$ T-cells was observed without restimulation or in the unprimed PBMC population. Restimulation of unprimed PBMC with CMV pp65 peptide-loaded T2 cells did not give rise to any IFN-γ–producing $CD8^+$ T-cells. The majority of the CMV tetramer-positive cells were also producing IFN-γ, demonstrating the effector function of the reactivated CMV pp65-specific $CD8^+$ T-cells (Fig. 3A). In all donors tested, the induced antigen-specific, IFN-γ and IL-2–producing $CD8^+$ T-cells seemed to have a phenotype corresponding to effector memory cells ($CD45RA^-CCR7^-$) (Fig. 3C).

Sig-CMV pp65-LAMP1 mRNA-electroporated CD40-B cells can Reactivate CMV pp65-specific $CD4^+$ and $CD8^+$ Simultaneously in CMV-seropositive Subjects

To simultaneously reactivate CMV pp65-specific $CD8^+$ and $CD4^+$ T-cells using mRNA-electroporated CD40-B cells, we used a Sig-CMV pp65-LAMP1 mRNA construct containing a lysosomal membrane protein sequence that is able to target the mRNA to the endosomal compartment, thus promoting antigen presentation on MHC class II molecules. Mock-electroporated or Sig-CMV pp65–LAMP1 mRNA-electroporated CD40-B cells were used for stimulation of autologous PBMCs of CMV-seropositive donors in an effector:CD40-B cell ratio of 4:1. After 1 week, the PBMCs were either left unstimulated or were restimulated with CMV pp65 peptide-pulsed T2 cells or with recombinant pp65 protein. Upon restimulation of the primed PBMC with the CMV peptide-loaded T2 cells, a higher percentage of IFN-γ–producing $CD8^+$ T-cells was observed than on restimulation with unloaded T2 cells or with medium only (Fig. 4A). PBMCs, that were initially stimulated with mock-electroporated CD40-B cells did

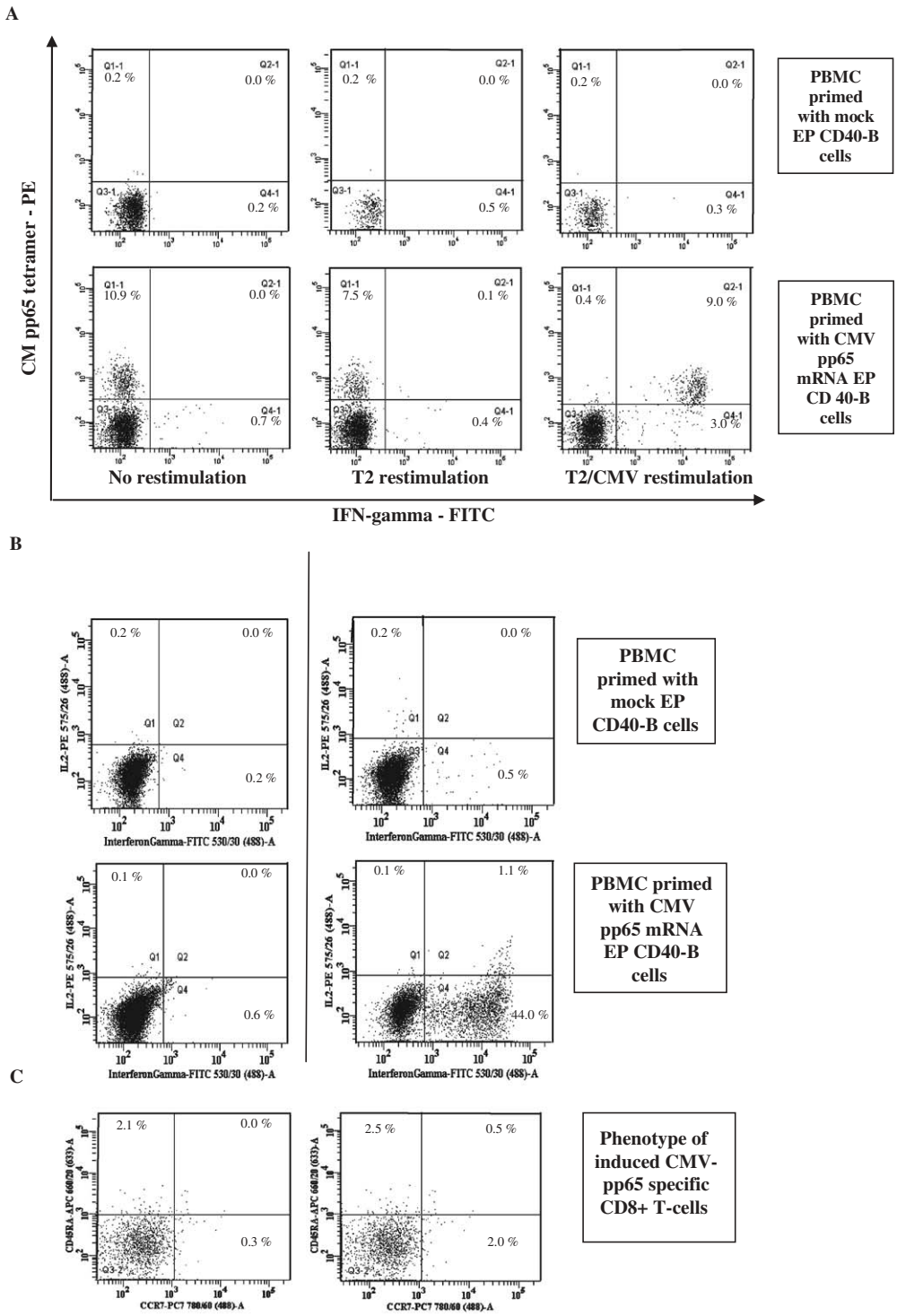

FIGURE 3. CMV pp65 mRNA-electroporated CD40-B cells can reactivate CMV pp65-specific effector memory $CD8^+$ T-cells in CMV-seropositive individuals. A, CMV pp65 tetramer and intracellular IFN-γ staining performed on PBMCs that were primed with mock-electroporated (EP) or CMV pp65 mRNA-electroporated CD40-B cells and subsequently restimulated with unloaded or CMV pp65 peptide-pulsed T2 cells. Gating was performed on viable $CD3^+$ $CD8^+$ T-lymphocytes. Percentages within the total $CD8^+$ T-cell population are shown. B, Intracellular IFN-γ and IL-2 staining performed on PBMCs that were primed with mock-electroporated or CMV pp65 mRNA-electroporated CD40-B cells and subsequently restimulated with unloaded or CMV pp65 peptide-pulsed T2 cells. Gating was performed on viable $CD3^+$ $CD8^+$ T-lymphocytes. Percentages within the total $CD8^+$ T-cell population are shown. C, Phenotype of CMV pp65 tetramer-positive $CD8^+$ T-cells (left dot plot) and IFN-γ+IL-2+CMV pp65-specific $CD8^+$ T-cells (right dot plot). Results shown are representative of 5 individual experiments.

not give rise to antigen-specific $CD8^+$ T-cell expansion upon restimulation. In contrast, IFN-γ expression was induced in $CD4^+$ T-cells upon restimulation of the primed PBMCs with the CMV pp65 recombinant protein, but not upon restimulation with CMV pp65 peptide-loaded T2 cells (Fig. 4A, C). Furthermore, PBMCs that were initially stimulated with mock-electroporated CD40-B cells did not give rise to any significant antigen-specific $CD4^+$ T-cell activation upon restimulation with recombinant pp65 protein, which demonstrates the antigen specificity of the $CD4^+$ T-cell response induced.

Recombinant CMV pp65 protein was added to some of the cocultures for processing and subsequent MHC class II presentation by the APC in the coculture,

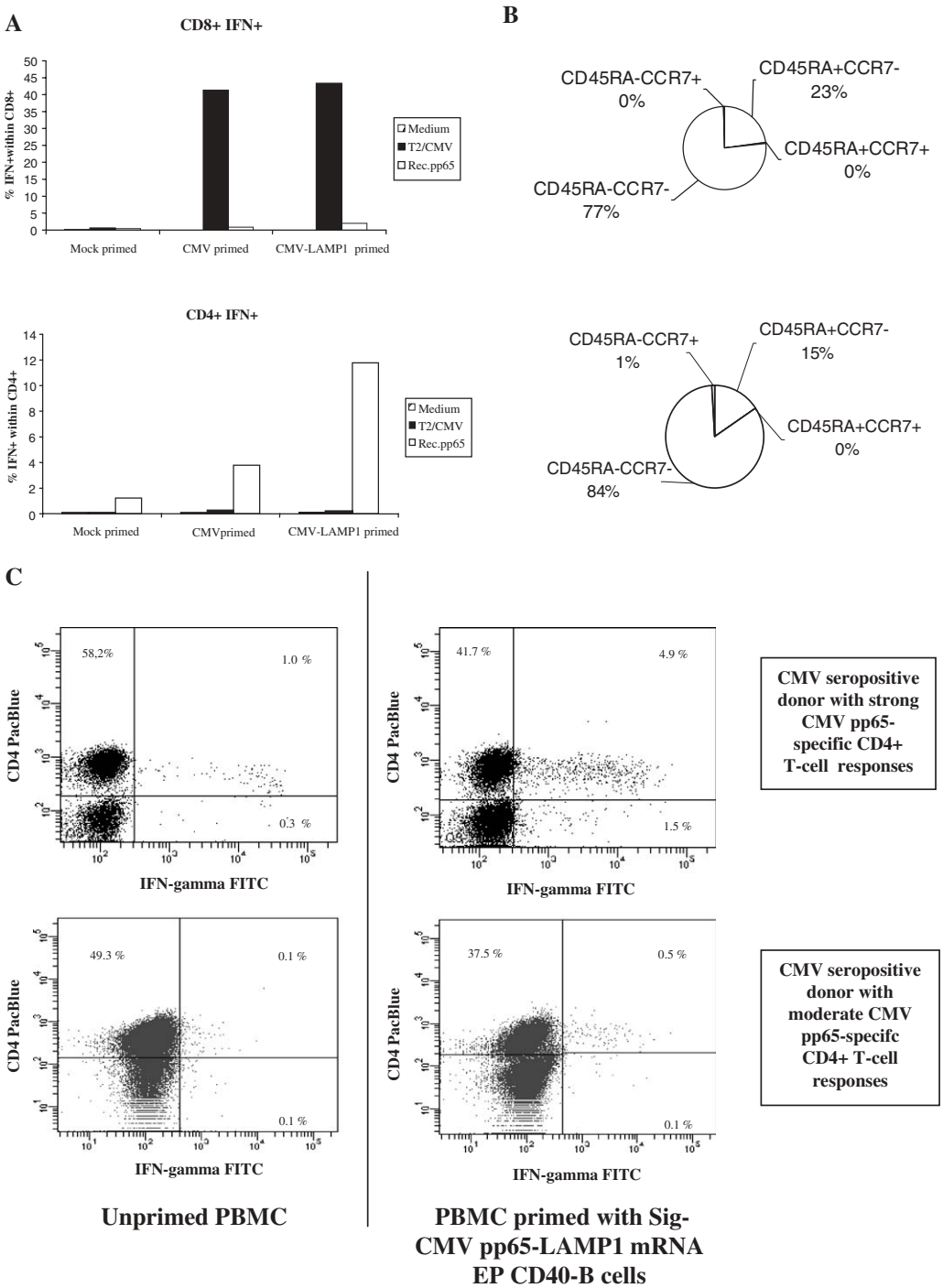

FIGURE 4. Sig-CMV pp65-LAMP1 mRNA-electroporated CD40-B cells can reactivate CMV pp65-specific, IFN-γ–producing memory $CD4^+$ T-cells in CMV-seropositive individuals. A, Bars in graphs represent percentage of IFN-γ–producing cells for mock, CMV pp65, and Sig-CMV pp65-LAMP1 primed PBMCs without restimulation (medium), with CMV pp65 peptide-pulsed T2 restimulation (T2/CMV) and with recombinant pp65 protein restimulation (Rec. pp65). Percentages within the $CD8^+$ T-cell population (upper graph) or within the $CD4^+$ T-cell population (lower graph) are shown. B, Phenotype distribution of induced CMV pp65-specific IFN-γ–producing $CD8^+$ T-cells (upper distribution diagram) and $CD4^+$ T-cells (lower distribution diagram). A representative result of 5 individual experiments is shown. C, Intracellular IFN-γ staining performed on PBMCs that were primed with mock-electroporated or Sig-CMV pp65-LAMP1 mRNA-electroporated CD40-B cells and subsequently restimulated with recombinant pp65 protein. Gating was performed on viable $CD3^+$ T-lymphocytes. Percentages within total $CD3^+$ T-cell population are shown. Representative results of a CMV-seropositive donor with a strong (upper dot plots) and a moderate (lower dot plots) CMV pp65-specific $CD4^+$ T-cell response are shown.

to compare the level of CMV pp65-specific $CD4^+$ T-cell stimulation by Sig-CMV pp65-LAMP mRNA-electroporated CD40-B cells with CMV pp65 mRNA-electroporated and mock-electroporated CD40-B cells. The evidence that CMV pp65 protein was processed through the MHC class II pathway by the APC in the coculture can be deduced from the observation that only $CD4^+$ T-cells, but not $CD8^+$ T-cells (actually originating from the same coculture), were restimulated to a higher IFN-γ response (Fig. 4A). This allowed one to compare the priming capacity on $CD4^+$ T-cells by the different conditions tested. Sig-CMV pp65-LAMP mRNA-electroporated CD40-B cells induced a higher level of IFN-γ–producing $CD4^+$ T-cells than CMV pp65 mRNA-electroporated or mock-electroporated CD40-B cells (Fig. 4A).

In a minority of CMV-seropositive donors, CMV pp65-specific IFN-γ–producing $CD4^+$ T-cells could be observed after restimulation with recombinant pp65 protein of PBMCs that were primed for 1 week with CMV pp65 mRNA-electroporated CD40-B cells, suggesting cross-presentation and/or cross-priming by these CD40-B cells. After priming of the PBMCs with CD40-B cells that were antigen-loaded with the Sig-CMV pp65-LAMP1 mRNA, the induced CMV pp65-specific T-cells had a $CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$ phenotype, which corresponds with effector memory and effector cells, respectively (Fig. 4B).

DISCUSSION

In this paper, we demonstrate that mRNA-transfected autologous CD40-B cells can be used to reactivate CMV pp65 and HIV Gag-specific memory $CD8^+$ and $CD4^+$ T-cells in CMV and HIV-1–seropositive individuals, respectively. Autologous CD40-B cells constitute an attractive alternative to DCs, because they can be generated ex vivo in large quantities for immunotherapeutic purposes starting from a limited amount of peripheral blood.[7] Moreover, several studies have already shown that these cells can be used to induce primary and secondary T-cell responses in vitro.[7,23] The expression of functional CCR7 and CXCR4 receptors and the production of T-cell–attracting chemokines suggests that these cells are also capable of migrating into T-cell–rich areas within secondary lymphoid organs, which would be a desired effect when applied in in vivo immunotherapy protocols.[24] Our experiments show that also B-lymphocytes of HIV-1–infected patients can be activated through the CD40-CD40L pathway and can be used as fully functional APCs, despite reports on the possible disruption of the B-lymphocyte development in HIV-1 infection.[25] Taken together, CD40-B cells might be considered as a valuable APC for use in the cellular immunotherapy protocols, alone or in combination/alternation with autologous DCs when multiple restimulations are mandatory.

Su et al[26,27] have demonstrated, both in vitro and in vivo, that DC transfected with mRNA encoding chimeric proteins carrying the endosomal/lysosomal sorting signal of the LAMP1 are capable of stimulating antigen-specific concomitant $CD8^+$ and $CD4^+$ T-cell responses without negatively affecting the intracellular generation and subsequent presentation of MHC class I epitopes. Our results now clearly show that Sig-CMV-LAMP1 mRNA-electroporated CD40-B cells are not only able to generate CMV pp65-specific $CD8^+$, but also $CD4^+$ T-cell responses that may be required to induce and maintain an optimal $CD8^+$ cytotoxic T-lymphocyte response against the immunodominant CMV pp65 epitope.[28] Moreover, the use of HLA haplotype-unrestricted mRNA has the advantage over peptide pulsing, because it has recently been shown that pp65 epitopes can be presented by a wide variety of HLA-A, HLA-B, and HLA-C alleles.[29] Our expanded CMV pp65-specific T-cells all had an effector (memory) phenotype, which is comparable to the IFN-γ–producing CMV-specific $CD4^+$ and $CD8^+$ T-cells that can be found during primary CMV infection.[28,30] The demonstration of a CMV pp65-specific dual IFN-γ/IL-2–secreting $CD8^+$ T-cell population with a $CD45RA^-CCR7^-$ phenotype after stimulation with CMV pp65 mRNA-electroporated CD40-B cells is in accordance with the findings of Zimmerli et al,[31] who have identified similar $CD8^+$ T-cell populations in Epstein-Barr virus and influenza infection. The induced single IFN-γ–secreting CMV pp65-specific $CD8^+$ T-cells were contained within the $CD45RA^-CCR7^-$ and $CD45RA^+CCR7^-$ effector populations, which also confirms their results.[31] In addition, CMV-specific immunotherapy using autologous mRNA-electroporated CB40-B cells can be considered a flexible approach, because the CMV-specific T-cell expansion can be performed in vivo or ex vivo using these APCs in an adoptive T-cell therapy strategy. Further investigation is required to determine which of these approaches is optimal to treat CMV reactivation in immunocompromised patients after hematopoietic stem cell transplantation or in HIV-infected patients with progressive disease.

Several studies have already shown the efficacy of using antigen-loaded autologous APCs for the induction of HIV-specific T-cell immune responses. In an animal model, adoptive transfer of autologous DCs loaded with inactivated simian immunodeficiency virus in infected Chinese macaques resulted in an increase of simian immunodeficiency virus-specific cellular immunity and a decrease of viremia.[32] Adoptive transfer of autologous DCs loaded in vitro with aldrithiol-2 inactivated HIV-1 induced protective antiviral immunity in hu-peripheral blood lymphocyte/severe combined immunodeficiency mice.[33,34] Moreover, a recent study by Lu et al[4] demonstrated the value of whole-virus pulsed DC vaccines for the induction of sustained viral suppression without the need for introduction of HAART in a group of untreated, chronically HIV-1–infected individuals. Using DCs loaded with inactivated autologous HIV virus, they were able to boost both $CD4^+$ and $CD8^+$ T-cell responses, as was observed by us in vitro. Our

consensus *gag* mRNA approach, however, holds less biosafety risks and does not require patient-specific preparations like isolating the autologous virus, which makes it a more generally applicable therapeutic vaccination strategy.

After electroporation of the CD40-B cells with (h)*gag* mRNA, the intracellularly produced Gag protein is processed by the proteasome into peptides that are loaded onto MHC class I molecules in the endoplasmatic reticulum. These stable peptide-class I MHC complexes are subsequently expressed on the cell surface. Some of the Gag protein is also secreted and endocytosed by the CD40-B cells, which is the pathway used to load MHC class II molecules, leading to antigen-specific $CD4^+$ T-cell activation. Our results correspond with the findings of Weissman et al,[35] who used nonhumanized *gag* mRNA to transfect DCs. This approach allows to activate and expand not only HIV-specific $CD8^+$, but also $CD4^+$ T-cells that are needed to induce effector-memory $CD8^+$ T-cells and to help the HIV-specific $CD8^+$ T-cells to control the viral replication.[36–38] Targeting the Gag protein in the setting of anti-HIV immunotherapy might also be beneficial because of the high frequency of Gag as an immunodominant epitope.[39] Using *gag* mRNA instead of a single Gag peptide or a peptide pool also offers the advantage of inducing cellular T-cell responses against multiple epitopes of the protein.[17] This increases the possibility of targeting the immunodominant epitope in all patients and decreases the risk of losing viremia control because of a single point mutation.[40] Moreover, the technique of mRNA electroporation is a safe transfection method that is applicable in clinical study protocols. No significant correlation between (h)*gag*-specific T-cell responses and HIV-1 plasma viral load was observed in this study. The absence of a negative correlation between viral load and IFN-γ production by HIV-1–specific T-cells is in line with the studies by Betts et al, but is in contrast to studies assessing responses to a limited number of epitopes or HIV-1 proteins that did show inverse correlations.[41,42] These discrepancies among the different study results may be due to the restriction of the analysis to responses directed against particular regions of the HIV-1 genome in different studies or due to differences in the study methods and studied population.[43] Because all our tested HIV-1–infected individuals were asymptomatic untreated patients, the effect of low $CD4^+$ cell counts on the observed immune response could not readily be evaluated. According to Castelli et al,[44] the $CD8^+$ cell antiviral responses decrease only when the $CD4^+$ cell count falls below 350 cells/μL, which was the case for only 1 patient in this study.

In conclusion, we have demonstrated that mRNA-electroporated autologous CD40-B cells are a valuable immunologic tool for the simultaneous induction of viral antigen-specific $CD4^+$ and $CD8^+$ T-cells. Additionally, our results provide evidence that this approach might be applied for the therapeutic immunization of CMV and HIV infection in seropositive individuals as an adjunct to classical drug therapy.

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