Summary: Characterization of human cytomegalovirus-specific T cells (CMV-T) is of critical importance for their potential use in adoptive immunotherapy after allogeneic hematopoietic stem cell transplantation. Background frequencies of CMV-T in peripheral blood mononuclear cells (PBMCs) of CMV-seropositive healthy subjects are usually very low, hence the requirement for prolonged culture time and multiple stimulations to expand them. The evaluation of the end-culture specificity and composition has sometimes been neglected or difficult to assess in these settings. We explored the identity and the functionality of pp65-specific and IE1-specific T cells, enriched in short-term cultures from PBMCs. Antigen-specific T cells were further isolated by IFN-γ capture system and/or CD154 microbeads. Frequency of IE1-specific cytotoxic T cells in PBMCs secreting IFN-γ was higher compared with the pp65-specific one, whereas the latter cell types showed a higher median CD107a expression among healthy donors. T cells were mainly of late effector stages but could reflect the observed individual CMV-specific cellular immunology. This heterogeneity raises points to be considered when approaching adoptive immunotherapy.

Key Words: allogeneic hematopoietic stem cell transplantation, CMV-seropositive donors, pp65-specific and IE1-specific T cells, adoptive immunotherapy

Preemptive and prophylactic strategies are effective in the control of reactivation of latent cytomegalovirus (CMV) occurring in allogeneic hematopoietic stem cell transplantation (HSCT), reducing the incidence of CMV disease to no more than 5%–8%. However, these strategies do not show a definitive improved overall survival in this setting. These poor results may be in part related to the associated toxicities of antiviral drugs as, for example, the myelosuppressive effects of ganciclovir. Furthermore, the advent of prophylaxis caused an alteration in the epidemiology of CMV disease, with an increase in late-onset CMV reactivation so that delayed or impaired antiviral sensitivity may lead to an increased incidence of CMV disease. In this setting, restoration of CMV immunity by adoptive transfer of CMV-specific T cells (CMV-T) has been shown to be effective and safe.

The goal of current trials based on cellular immunotherapy against CMV is to make treatment with CMV-T more broadly applicable using more rapidly available products from the donor, such as directly selected T lymphocytes. Indeed, increasingly sophisticated strategies show that the beneficial effect of adoptive T-cell transfer (ACT) relies on an in vivo expansion in the presence of the antigen after rapid in vitro isolation.

Major drawbacks of these procedures remain the limitation in the characterization of the T-cell product before infusion and the best administration schedule because of the low quantity of T-specific lymphocytes, so that any method to expand these cells to an extent sufficient to carry out experimental work would be really important. In this paper, we aimed to evaluate the biology of antiviral CD4+ and CD8+ T cells in healthy subjects, shortly enriched in vitro upon a single antigenic stimulation with the viral epitopes pp65 and IE1 [mixed lymphocyte-peptide cultures (MLPCs)]. We have assessed the specificity of the cells subjected to expansion, final yield, antigen-specific proliferation and degranulation potential, cytokine secretion patterns, and T-cell subpopulations phenotype. We have compared the results obtained from 8 peripheral blood mononuclear cells (PBMCs) samples from CMV-seropositive normal donors after activation with either one or the other CMV antigen. To avoid any bias, we have split each lot from the same donor to perform a legitimate data comparison between the disparate antigen-specific T cells. We focused on the diverse functionality of the respective reactive pp65-specific and IE1-specific T cells. The diversified behavior of these CMV-T cells when applied in adoptive cellular therapies protocols is even more considered as key for the patients’ adaptive immunity recovery after HSCT.

MATERIALS AND METHODS

Blood Donors and Cell Samples

Blood samples from 8 CMV-seropositive healthy volunteers were obtained with written informed consent and...
stored or transported at room temperature (RT). PBMCs were isolated within 4 hours from blood drawing by density gradient separation using Ficoll-Paque Premium (GE Healthcare BioSciences AB, Uppsala, Sweden). Platelets were removed by centrifugation of cells at 200g for 15 minutes at RT. Cells were cryopreserved in RPMI 1640 (Sigma-Aldrich, St Louis, MO) with 50% human serum albumin (Albital 200 g/L; Kedrion, Lucca, Italy) and 10% dimethylsulphoxide (DMSO; Sigma-Aldrich, St Louis, MO) according to standard protocols, for use in scheduled experiments as well as a source of autologous feeder cells. For use cryopreserved PBMCs were rapidly thawed in a water bath at 37°C and washed once in the so-called CTL medium, which consists of RPMI 1640 medium supplemented with 1% Pen/Strep (both Life Technologies, Paisley, UK) and 10% human AB serum (Sigma-Aldrich). After thawing, PBMCs were rested for at least 4 hours in a V-shaped 50 mL tube (Falcon BD, Heidelberg, Germany) in CTL medium, at a concentration of 5–10×10⁶/mL, and incubated at 37°C in a humidified atmosphere at 5% CO₂. To allow gas exchange the tube cap was loosened. Afterward, living PBMCs were counted using trypan blue staining.

**Generation of pp65-specific and IE1-specific T Cells by MLPCs**

In this study, we analyzed 8 CMV-seropositive normal donors after stimulation with either pp65 or IE1 CMV antigens and observed the biological profiles within the same donors. The induction of CMV-T-cell lines was performed as previously described⁹ with some modifications. PBMCs were resuspended in CTL medium at 10×10⁶/mL. Either CMV-pp65 or CMV-IE1 antigenic peptide pool was directly added to PBMCs (20 μL of stock solution at 50 μg/mL for 10⁷ cells) in a V-shaped 50-mL Falcon tube and the cultures were maintained at 37°C and 5% CO₂ for 2 hours. To allow gas exchange the tube cap was loosened. Upon stimulation, 2 consecutive washing steps in CTL medium were performed. Cells have been plated in 24-well plates (BD Falcon) at a density of 3×10⁶/mL in a final volume of 1 mL (day 0). On day 3, recombinant human interleukin (IL)-2 (50IU/mL) was added. Starting on day 5, half-medium change and supplementation of IL-2 were performed every other day until day 12.

**Evaluation of the Enrichment of CMV-T Cells by Flow Cytometry**

After a total of 12–13 days’ culture, an aliquot of MLPC was harvested and the frequency of peptide-specific CD3⁺ T cells was evaluated by flow cytometry by either CD154⁺ upregulation or by intracellular cytokine staining (ICS) in combination with CD107α degranulation assay.

**CD154 Activation Marker Expression**

About 5×10⁶ PBMCs seeded in 200 μL were stimulated for 6 hours in flat-bottomed 96-well culture plates (BD Falcon, Devon, UK) with 1 μg/mL of CMV-pp65 or CMV-IE1 Peptivator and incubated at 37°C/5% CO₂. Cultures were stimulated in the presence of 1 pg/mL anti-CD40 antibody (clone HB14; CD40 pure—functional grade; Miltenyi Biotec), to prevent the downregulation of CD154 expression on T cells induced by interaction with CD40 on antigen-presenting cells (APCs). Samples for negative control were left untouched, whereas human Cytostim (polyclonal stimuli that cause rapid activation of T cells; Miltenyi Biotec) was used at 1 μg/mL as positive control.⁹ Samples were taken at 6 hours and stained with anti-CD154-PE, anti-CD3-EC, anti-CD4-FITC, and anti-CD8-PECy7 as described further.

**ICS Combined With CD107α Degranulation Assay**

Degranulation of activated T cells is a necessary precursor of cytolysis and involves the marker CD107α, present in the membrane of cytotoxic granules and exposed onto the cell surface as a result of T-cell activation.¹⁰ Simultaneous characterization of cytolytic and cytokine-secreting effector functions can elucidate the functional patterns of CMV-pp65 and CMV-IE1-specific CTLs.¹¹ The cytotoxic activity of CMV-T was evaluated by CD107α degranulation assay targeted on IFN-γ-producing T cells as recently described¹² with some modifications. The assay has been performed on PBMCs at day 0, on MLPCs at day 11, and at the end of the culture. Peptide was directly added to the aliquot of T-cell lines without any APC and cytokines.

Cells were harvested and seeded at 5×10⁶ cells per well in a 96-well U-bottom plate (BD Falcon) and cocultured for 1 hour with either PeptiVator CMV-pp65 or PeptiVator CMV-IE1, or left unloaded as control. After 1 hour, Brefeldin A (Sigma-Aldrich) and GolgiStop (BD Biosciences), a Golgi-blocking agent to preserve the generated cytokines within the cytoplasm, were added to each well. The cells were stained with CD107α APC immediately (all but isotype control). Phorbol 12-myristate 13-acetate (PMA)/Ionomycin (both from Sigma-Aldrich) was used to obtain a positive control.

After 18 hours of incubation cells were harvested, permeabilized, and stained for intracellular IFN-γ apart from surface markers before subjection to flow cytometry analysis. Antibodies for CD3, CD8, and CD4 were added and incubated for 15–20 minutes at 4°C in the dark. Two consecutive washing steps were performed. Cells were permeabilized by adding 250 μL of 1× Permeabilizing Solution 2 (Perm2; BD Biosciences) for 12 minutes at RT in the dark. IFN-γ PE was added directly in the left flow after 2 washing steps and incubated for 20 minutes at 4°C in the dark. At the end, cells were suspended in 200 μL of washing buffer and subjected to flow cytometry analysis.

**Analysis of T-Cell Subsets**

We analyzed the T-cell phenotypes of CMV-T upon paired pp65-specific and IE1-specific MLPCs within the same subject and compare it with the cognate PBMCs before stimulation (n = 8). Surface staining was performed with saturating conditions of the following antibodies: anti-CD62L (clone DREG56) and anti-CD45RA (clone 2HG-RD1). Analysis was performed in the CD3⁺ CD8⁺ and CD3⁺ CD4⁺ T-cell gates. Naive T-cell/T-memory stem cell (T₅₈/T₃₇) were defined as CD45RA⁺CD27⁻, central memory T cells (T₇₄) as CD45RA⁻CD27⁺, effector memory T cells (T₅₈) as CD45RA⁻CD27⁻, and late effector T cells (T₄₅) as CD45RA⁺CD27⁻.

**Antibodies and Flow Cytometric Analysis**

Antibodies used for phenotyping were as follows: anti-CD3-EC (clone UCHT1), anti-CD4-FITC (clone 13B8.2), anti-CD8-PECy7 (clone T8), anti-CD56-APC (clone N901), anti-CD14-APC (clone RM052), anti-CD45RA-PE (clone 2HG-RD1; all from Beckman Coulter,
Isolation of CMV-T Cells After MLPCs

In selected experiments, on the basis of the entity of the proliferation of CMV-T during MLPC, restimulation of the whole culture was performed on day 13 by using the same peptide pool as of day 0, followed by positive selection either by IFN-γ Secretion Assay—Cell Enrichment and Detection Kit or by CD154 MicroBead Kit (both Miltenyi Biotec), performed according to the manufacturer's instructions. In general, a good enrichment of CMV-T was feasible when starting with a frequency of CD3+ antigen-specific T cells at the end of the MLPC ≥ 10%.

Magnetic Selection by IFN-γ Capture (CCS)

All steps, but the secretion period, have been performed by keeping cells cold and by using precooled solutions to prevent capping of antibodies on the cells surface and a nonspecific cell labeling. Upon stimulation with peptides for 4 hours, the cells have been labeled with IFN-γ Catch Reagent and incubated on ice for 5 minutes. Warm medium was then added to allow cytokine secretion, cells were incubated in a closed tube for 45 minutes at 37°C, and turned every 5 minutes to resuspend them. Labeling was performed for 10 minutes on ice using 20 μL of IFN-γ Detection Antibody-PE per 10^7 total cells in 80 μL of CliniMACS buffer, the cell suspension was enriched using MS or LS columns on a MiniMACS or MidiMACS.

Antigen-specific Proliferation

To assess whether the generated CMV-T can appropriately divide after stimulation with endogenously processed antigen, CMV-T obtained immediately upon isolation by CD154 magnetic enrichment from MLPCs (day 13) were labeled with carboxyfluorescein diacetate succimidyl ester (CFSE; CellTrace CFSE Cell Proliferation Kit; Invitrogen, Molecular Probes, Eugene, OR) and were cocultured for 6 days with autologous feeder cells [mitomycin C (Sigma-Aldrich)-treated PMBCs from the unlabeled negative fraction]. Fluorescence labeling of PBMCs and generated T-cell lines was achieved as described14 with some modifications. Briefly, cells were washed in RPMI 1640 medium containing 10% AB serum and labeled at a density of 1 × 10^6 cells/mL with 1 μM of CFSE. Cells were incubated with the dye at 37°C for 10 minutes. Staining was quenched by the addition of 5 volumes of ice-cold CTL medium to the cells. After 5 minutes of incubation on ice, cells were centrifugated at 400g for 10 minutes at 4°C. Labeled cells were washed a further 2 times and plated in a round-bottomed 96-well plate (BD Falcon) with autologous myotymcin-treated PBMCs preincubated with 1 μg/mL of CMV PepTivator at a ratio of 1:10. CTL medium included 50 IU/mL IL-2 and 1 μg/mL phytohemagglutinin-t. Data were evaluated in a paired manner using the Student t test. Reported P-values are 2-sided and were considered statistically different if *P < 0.05, **P < 0.005, and ***P < 0.001.
RESULTS

Antiviral CD8+ and CD4+ T Cells in Healthy Subjects

We tested PBMCs and MLPCs in peptides titration assays to identify possible effects of peptide pools concentration on antigen sensitivity. Frequencies of IFN-γ-secreting CMV-T in PBMCs were not significantly different upon stimulation with either 0.5 or 1 μg/mL of both peptide pools (Fig. 1A). The number of responding Th cells was higher for each CMV epitope at both peptide concentrations, compared with CMV-reactive CD8+ T cells (IFN-γ+/CD8+ cells: 1.59% ± 1.44%; IFN-γ+/CD4+ cells: 0.11% ± 0.08%). Progressive decreases in CMV-IE1 peptide pools concentration stimulated lower numbers of antigen-specific cells in MLPC; overall antiviral CD8+ T-cell responses decreased upon exposure to peptide pool concentrations ranging from 1 to 0.01 μg/mL (Fig. 1B). Accordingly, we decided to use 1 μg/mL of peptide mix for both CMV epitopes during in vitro stimulation of either PBMCs or MLPCs.

We were able to detect the frequencies of CD3+ CMV-T in PBMCs from 8 healthy donors, upon stimulation with CMV-pp65 and CMV-IE1 peptide pools (Table 1). The percentage of peripheral blood helper and cytotoxic T cells were estimated based on the expression of double-positive CD3+CD4+ and CD3+CD8+, respectively. PBMCs contained a median of Th cells of 63.4% (range, 51.5%–72.2%) and of CTLs of 26.1% (range, 19.5%–34.3%) of the overall lymphomonocytes gate. These levels coincide with normal reference values in healthy subjects for T lymphocytes.15,16 pp65-specific and IE1-specific CD8+ T cells ranged from <0.01% to 1.56% (median: 0.17%) for the former and from 0.05% to 3.61% (median: 0.66%) for the latter. Median frequency of pp65-specific and IE1-specific CD3+CD8+ T cells was 0.34% (range, <0.01%–2.89%) and 0.17% (range, <0.01%–4.79%), respectively.

Heterogeneity of Degranulating CMV-specific CD8+ T Lymphocytes in PBMCs

We applied a combined assay to provide simultaneous assessment of 2 critical CD8+ T-cell effector functions: cytokine production and CD107a degranulation in response to stimulation with the antigen. CD107a is a marker present in the membrane of cytotoxic granules and it is exposed onto the cell surface as a result of degranulation. Following in vitro stimulation, CMV-specific CD8+ T lymphocytes were heterogeneous with regards to the composition of cells positive for CD107a. There was no linear relationship of IFN-γ secretion and CD107a degranulation in the CD3+CD6+ population from the same sample (Fig. 2). Indeed, even if expressing CD107a at high levels, CD8+ T cells in the same gated population were poorly positive for IFN-γ (Fig. 2, bottom right quadrant). The most frequent situation involves the bottom left panel in the Figure 2, where are plotted either pp65-specific or IE1-specific polyfunctional CTLs expressing very low levels of both IFN-γ and CD107a activation markers. The 80% of analyzed antigen-specific CTLs that expressed a fair amount of IFN-γ (in the range of 1%–4%), whereas almost negative for CD107a (range, 0.2%–0.4%) were IE1 CTLs (Fig. 2, upper left quadrant). Median degranulation of CTLs in PBMCs upon stimulation with CMV-pp65 and CMV-IE1 peptides was 0.46% (range, <0.01%–6.51%) and 0.13% (range, <0.01%–7.99%), respectively. CD3+CD4+ antigen-specific T cells were negative for CD107a (data not shown).

Composition of the MLPCs and Variable Enrichment of Viral Antigen-specific T Cells

In PBMCs from healthy donors, background expression of specific activation markers or secretion of cytokine (ie, CD154, CD137, IFN-γ, IL-2, TNF-α) in the unstimulated sample are generally very low.17,18 We induced MLPCs to expand cognate antigen-specific T cells from healthy donors serologically positive for CMV before selection by both CCS and CD154 microbeads. PBMCs were prepared after density gradient separation with 12.5% (n = 5). Absolute PBMCs cell number stimulated with either pp65 or IE1 peptide pools at day 0 was 31.8 ± 12.3 × 106 and 23.4 ± 6.4 × 106, respectively.

The ratio of CD4+ and CD8+ T cells within the T-cell populations was determined by flow cytometry before stimulation with peptides (PBMC at day 0) and after the short-term MLPCs (at day 11–13). A predominance of CD4+ T cells was shown in all cases, either at day 0 or after 11–13 days, regardless of the CMV epitope used as stimulant to begin the antigen-specific culture (Fig. 3). MLPC obtained by priming with pp65 peptide pools were composed of 26.05% ± 8.82% CTLs compared with 67.15% ± 12.55% Th. MPLCs obtained by priming with IE1 peptide pool were composed of 28.60% ± 10.86% CTLs compared with 57.53% ± 5.09% Th. The magnitude of responses varied depending on the epitope, peptide, and donor (Table 2). Median cell viability from all samples at day 11 upon CMV-pp65 stimulation was 31.6% (range, 6.0%–84.3%), whereas only 13.8% (range, 2.3%–81.7%) upon CMV-IE1 stimulation. Cells stimulated with pp65 were 10.5 ± 8.5 × 106 and only 4.7 ± 6.1 × 106 were alive upon IE1 induction of MLPC. In the latter case, all cultured cells were used for the IE1-specific frequency at flow cytometry. As shown in Figure 4, by considering the absolute CMV-T numbers we could better appreciate the wide spectrum of fold increase, upon MLPC, among the donor-specific and antigen-specific cell lines (pp65 specific vs. IE1 specific) and the way to check for their specificity (IFN-γ+ vs. CD154+). The rate of increase of pp65-specific T cells was 1- to 33-fold, whereas IE1-specific T cells ranged from 1- to 961-fold under the same experimental conditions.

Good enrichment of CMV-pp65-specific cells was achieved in 3 out of 8 samples (Table 2), when starting with a frequency of CD3+ antigen-specific cells ≥10% before restimulation and selection (results are shown for 1 representative T-cell line in Fig. 5). We failed to further expand CMV-T in 62% of cases despite improving the total recovery by performing a repeated purification step with a new column, for each sample (unlabeled cells from the first column were loaded onto a new one; the obtained positive fractions from each column were combined before coculturing them with feeder cells).

Pp65-specific and IE1-specific T Cells Show a High Proliferation Rate After Antigenic Rechallenge

To assess whether the generated CMV-T can appropriately divide after restimulation with endogenously processed antigen, T cells were labeled with CFSE and were cocultured for 6 days (from day 1 to day 7 after selection)
FIGURE 1. Titration of the CMV-pp65 and CMV-IE1 peptide pools for the stimulation of PBMCs (A) and MLPC (B). A, Histograms show the IFN-γ secretion by CMV-pp65-specific and CMV-IE1-specific CD4+ and CD8+ T cells for given peptides concentrations in PBMCs (day 0). Background values as determined in unstimulated controls are subtracted before calculating the % of CMV-specific T cells. Data shown are mean ± SD from 3 independent assays (paired samples between virus epitopes). B, Specific T-cell lines generated with CMV-IE1 peptide pool were restimulated with the indicated concentrations of the same antigen after 13 days of MLPC, and the percentages of IFN-γ+ cells among the CD3+ population after 6 hours were plotted. From the top to the bottom, a progressive decrease of the peptide pool amount determines a lower IFN-γ secretion by both CD3+ CD8+ (left panels) and CD3+ CD4+ (right panels) lymphocytes. One representative experiment out of 2 with similar results is shown.
Antiviral T-cell responses in terms of IFN-γ secretion as assessed by flow cytometric analysis (frequencies assessed by ICS) in a cohort of 8 healthy CMV-seropositive individuals. Controls include the unstimulated and the unlabeled samples; positive control was obtained with PMA/Ionomycin. Background values as determined in unstimulated controls are subtracted before calculating the % of CMV-specific T cells. Levels of cytokine-secreting CMV-T are epitope dependent and show disparate numbers among donors.

CMV-T indicates CMV-specific T cells; ICS, intracellular cytokine staining; PBMCs, peripheral blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate.

with autologous feeder cells (mitomycin-C-treated PMBCs from the unlabeled negative fraction) in 1:20–1:60 ratios. Proliferation potential was assessed on CMV-T obtained upon enrichment by MLPC and selection by CD154 microbeads. The isolated CMV-T showed high antigen-specific proliferation compared with the negative control (Fig. 6). As shown, autologous feeder cells did not proliferate during 6 days of expansion. Mitomycin-C treatment was accountable for this lack of proliferation; indeed, peptide-preloaded autologous PMBCs grew out up to 50% during culture (Fig. 6, upper right vs. upper middle). In contrast, >95% of the isolated and expanded CD3+

### TABLE 1. Percentage of Lymphocytes Specific for IE1 and Pp65 Epitopes in PBMCs From CMV-Seropositive Donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>IE1 CMV-T (%)</th>
<th>pp65 CMV-T (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD3+ CD8+ CD3+ CD8+ CD4+ CD4+</td>
<td>CD3+ CD8+ CD3+ CD8+ CD4+ CD4+</td>
</tr>
<tr>
<td>1</td>
<td>2.29</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.16</td>
<td>1.67</td>
</tr>
<tr>
<td>3</td>
<td>0.16</td>
<td>0.18</td>
</tr>
<tr>
<td>4</td>
<td>0.09</td>
<td>4.79</td>
</tr>
<tr>
<td>5</td>
<td>&lt; 0.01</td>
<td>2.89</td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>0.16</td>
</tr>
<tr>
<td>7</td>
<td>0.19</td>
<td>0.07</td>
</tr>
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</table>

Healthy volunteers were the same as used to assess CMV-T frequencies in PBMCs at day 0 (Table 1). Samples no. 1 and 6; frequencies assessed by ICS; samples no. 4, 5, 7, and 8; frequencies assessed by CD154 staining. Background values as determined in unstimulated controls are subtracted before calculating the % of CMV-T. Gray shadows point out samples that further allowed CMV-T expansion after MLPC restimulation. MLPC failed in 2 out of 8 cases because the cells had died after 12 days of culture (donor number 2 and 3). We can presume that the standard predetermined peptide load, used for the first stimulation of PBMCs, was not optimal for those specific lots. Indeed, it has been shown in previous reports focused on T cells immunosassays and epitopes discovery that the HLA repertoires show different affinity toward immunodominant epitopes upon stimulation with peptides.19,21,22

### TABLE 2. Percentage of Lymphocytes Specific for IE1 and Pp65 Epitopes After MLPCs

<table>
<thead>
<tr>
<th>Donor</th>
<th>IE1 CMV-T (%)</th>
<th>pp65 CMV-T (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3+ CD8+ CD3+ CD8+ CD4+ CD4+</td>
<td>CD3+ CD8+ CD3+ CD8+ CD4+ CD4+</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>NA</td>
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<tr>
<td>3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>2.10</td>
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</tr>
<tr>
<td>5</td>
<td>0.40</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>6</td>
<td>1.10</td>
<td>8.70</td>
</tr>
<tr>
<td>7</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>0.02</td>
<td>2.05</td>
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</tbody>
</table>

FIGURE 2. CD107a and cytokine-producing CD3+CD8+ double-positive T cells in PBMCs. The cytotoxic potential of CMV-T was estimated by means of CD107a degranulation assay targeted on IFN-γ-producing CTLs. Background values as determined in unstimulated controls are subtracted before calculating the % of degranulating and IFN-γ-secreting T cells. The diagram is shown in a logarithmic scale. The x-axis refers to values of CD107a+ CTLs (%); y-axis refers to values of IFN-γ+ CTLs (%). Percentages of double-functional (IFN-γ+CD107a+) CTLs were always >0%, mostly weakly positive (range, <0.01%–0.66%) in the case of pp65 CTLs (bottom left quadrant), whereas CD107a weakly positive with a good secretion of IFN-γ (range, 1%–4%) in the case of IE1 CTLs (upper left quadrant). IE1 CTLs indicates IE1-specific cytotoxic T cells; pp65 CTLs, pp65-specific cytotoxic T cells.

FIGURE 3. CTL/Th cell ratio within PBMCs and the different MLPCs. The ratio of CD4+ and CD8+ T cells within the T-cell populations was determined by flow cytometry before stimulation with peptides (PBMCs at day 0) and after a short-term expansion period (MLPC at day 11–13). Data shown are mean ± SD from 6 independent donors. MLPC_IE1 indicates the mixed lymphocytes peptide culture obtained upon stimulation of PBMCs with CMV-IE1 peptide library; MLPC_pp65, the mixed lymphocytes peptide culture obtained upon stimulation of PBMCs with CMV-pp65 peptide library; PBMCs, peripheral blood mononuclear cells.
CMV-T underwent at least 1 cell division, as measured by loss of intensity in the CFSE signal after a short period of expansion. The extent of this latter proliferation was not dependent on the CMV-T:feeder cells ratio, in our hands, as the proliferation rate was almost identical at 1:20, 1:40, and 1:60 (data not shown).

Multicytokine Repertoire After MLPCs was IFN-γ+ TNF-α+ Polarized

The cytokines secretion by MLPCs was analyzed to compare the CMV epitope-specific expression profile in samples at 2 different time points. MLPCs from PBMCs of CMV-seropositive healthy subjects were stimulated with pp65 or IE1 overlapping peptides spanning the entire immunodominant CMV proteins. After 6 and 18 hours, aliquots of supernatants were taken and evaluated for the release of IL-2, TNF-α, IFN-γ, IL-4, IL-5, and IL-10. The secretion repertoire was mainly IFN-γ+ TNF-α+ polarized with high levels of IL-4 and IL-10 upon pp65 and IE1 reload, respectively (Fig. 7). Median IFN-γ level was higher at 18 hours’ collection time compared with earlier collection time for both CMV antigens. In contrast, TNF-α secretion was less appreciable at 18 hours for both pp65 and IE1. Anyhow, TNF-α absolute concentration was higher upon pp65 stimulation. Median IL-2 concentration was unexpectedly very low regardless of CMV epitope and collection time, when considering both mean and median values (Fig. 7 and Table 3), whereas IL-5 secretion seems appreciable only after 18 hours stimulation period, albeit representing <10% of the overall Th1/Th2 secretion pattern. In general, we observed a wide and heterogeneous secretion pattern among different MLPCs and, from an absolute quantitative point of view (cytokine concentration) the amount of each measured cytokine was even higher in the pp65-specific cultures. As expected, in the case of supernatants upon pp65 and IE1 restimulation of MLPC with no detectable CMV-T (< 0.01%), there was no expression of Th1/Th2 cytokines, alike those from unstimulated MLPCs (data not shown).

Single Antigenic Stimulation Significantly Reduces the Level of pp65-specific and IE1-specific Late Effector CD8+ T Cells

To evaluate and compare the distribution of different T-cell subsets among antigen-specific cultures, we analyzed the phenotypic profile of MLPCs and their cognate PBMCs. The largest subpopulation in the T-cell phenotypes of both PBMCs and MLPCs was formed by T cells of late effector stages (Fig. 8A). Expanded CMV-T had significantly lower level of Temra CTLs compared with PBMCs before CMV stimulation (MLPC_pp65: 78.48% ± 11.05%, P < 0.005; MLPC_IE1: 77.82% ± 9.03%, P < 0.001; PBMCs: 97.5% ± 1.4%). Temra Th were less represented upon MLPC_IE1 (51.07% ± 11.52%, P < 0.05%; PBMCs: 71.0% ± 13.6%). CMV-T expansion was accompanied by a significant increase of Tn/scm CTLs (MLPC_pp65: 8.23% ± 4.28%, P < 0.001; MLPC_IE1: 14.2% ± 4.91%, P < 0.001; PBMCs: 10.0% ± 0.9%). Tn/scm Th counterpart significantly increased only upon stimulation with IE1 (20.25% ± 9.67% vs. 5.1% ± 3.6%, P < 0.005). Tcm made up the smallest subpopulation upon expansion (MLPC_pp65: 3.32% ± 3.63%; MLPC_IE1: 4.88% ± 2.34%).

The phenotypes of magnetically enriched CD154+ fractions from IE1 and pp65 cultures were investigated after 6 days of expansion by culture with different amount
FIGURE 5. Expression level of CD154 activation marker after antigen-specific activation and reloading with CMV peptide pools. Example of kinetics of CD154 after stimulation with CMV-pp65 and CMV-IE1 at different culture times. Upper panels: cells were gated based on the forward scatter (FSC) versus side scatter (SSC) profile, underlying progressive morphologic changes during culture (upper left and upper middle) and after MACS selection (upper right). Variable settings of the FSC/SSC gate are due to progressive increase in size and granularity of activated T cells that may shift out of the measurement if the lymphocyte gate is set too narrow. CD154 T-cell surface molecule is strongly upregulated after T-cell activation so it can be used for the selection of antigen-specific T cells. CD154, which is transiently expressed on activated CD4+ T cells (lower panels) and to a lesser extent on activated CD8+ T cells (middle panels) after antigen contact, needs the presence of anti-CD40 antibody in the culture medium during the stimulation period. One representative experiment out of 3 with similar results is shown.
of antigen reloaded autologous feeder cells (Fig. 8B). The overall CMV-T population encompasses 2 designated regions in the FSC/SSC gate, the smallest named “T1” and the contiguous named “T2” of wider area, holding activated and bigger CMV-T at higher granularity. CMV-T after magnetic enrichment displays an effector phenotype with cells that belong to T1 region equally composed of Tem and Temra cells and CMV-T that belongs to T2 gate, mainly Tem, with a lower proportion of Temra (Figs. 8B, C). As shown in Figure 8B (left panels), the CMV-T:feeder cells ratio did not affect neither the expansion outcome nor the composition of the final T-cell subpopulations.

**DISCUSSION**

This study focuses on the biological effects of antigen-specific T cells stimulation in PBMCs from CMV-seropositive healthy donors. We evaluated the in vitro behaviors of the enriched pp65 and IE1 CMV-T after a short expansion period and compared the functional patterns among the obtained cells.

CMV-T activation relies on the recognition of strongly immunogenic viral epitopes differently involved in the reactivation of the virus and with heterogenous level of expression. 21-24 To date, both pp65 and IE1 are considered dominant T-cell targets, whereas the past research mainly concentrated on pp65 only. Some studies confirmed a positive correlation between immune reconstitution after HSCT and rising number of pp65-specific T cells. 25,26 Unfortunately, IE1-specific CD3+ T cells were not included in these investigations or, when included, they were mixed with the cognate pp65-specific ones in the data analysis, or even earlier, so that at the end pp65-specific and IE1-specific T cells were generically called “CMV-specific T cells.”

FIGURE 6. Proliferation potential by CFSE staining of antigen-specific T cells isolated after MLPC according to their expression of CD154. IE1-specific T cells were labeled with CFSE immediately upon selection by anti-CD154 microbeads, performed after the 13-day MLPC period, and incubated for 6 days with mitomycin-treated autologous feeder cells (PBMCs from the unlabelled negative fraction after selection). For exclusion of unspecific proliferation, feeders alone (upper middle panel) and autologous negative fraction (upper right panel) were used as negative controls. Levels of proliferation were determined by CFSE fluorescence loss in flow cytometry (lower panels). Loss of the CFSE intensity is shown for the gated region in the morphologic FSC/SSC plot at both day 3 and day 6 after MACS selection. The overlay of the plots at day 3 and 6 shows that almost all CMV-T proliferated at the end of the assay. This experiment has been performed 2 times with either pp65-specific or IE1-specific T cells and similar results have been obtained. CFSE indicates carboxyfluorecein diacetate succimidyl ester; FSC/SSC, forward scatter/ side scatter.
lymphocytes.”27–29 Finally, a recent finding showed that dominance and magnitude (relative frequencies) of the IE1-specific but not the pp65-specific CD8+ T-cell response correlate with protection from CMV disease in 27 heart and lung transplant recipients, even though factors apart from the entity of the CD8/IE1 response were conferring protection.30 A major strength of our study was the comparison between IE1-specific and pp65-specific T cells biological features obtained from paired samples. Our data showed that IE1-specific CTLs were more than pp65-specific CD3+ CD8+ T cells (Table 1), in agreement with the literature.31 Despite this, the median CD107a degranulation by IE1-specific CTLs was lower than the pp65-specific cytotoxic counterpart and there was no correlation between the frequency of IFN-γ-producing T cells and their cytotoxic potential (Fig. 2). Although the CD107a degranulation assay does not directly provide cytotoxicity of CMV-T, it measures a functional response to antigen-specific stimulation so it reflects the activation status of CMV-T. As expected and in keeping with other studies, our results showed heterogeneity, supporting that HLA may have a major effect on immune response.

**FIGURE 7.** Cytokine secretion upon antigenic rechallenge. Quantitative assessment of Th1/Th2 cytokines in the supernatants of MLPCs after 6 and 18 hours of CMV-pp65 and CMV-IE1 stimulation was performed by cytometric bead array. Median values (%) from 6 lots expressed as the contribution of each cytokine is shown. Paired samples between virus epitopes and time of supernatants collection. IL indicates interleukin.

**TABLE 3.** Absolute Quantities of Th1 and Th2 Cytokine Repertoires Secreted in MLPCs Supernatants Upon Stimulation With pp65 and IE1 Epitopes

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Mean ± SD (pg/mL)</th>
<th>Median (Range) (pg/mL)</th>
<th>Mean ± SD (pg/mL)</th>
<th>Median (Range) (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>3710.8 ± 2846.3</td>
<td>5535.6 (340.0–6049.1)</td>
<td>2563.7 ± 2998.5</td>
<td>1218.3 (107.8–6870.8)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5162.6 ± 4449.4</td>
<td>6277.1 (182.8–10605.1)</td>
<td>1557.7 ± 2855.2</td>
<td>293.6 (21.6–6649.0)</td>
</tr>
<tr>
<td>IL-2</td>
<td>203.7 ± 302.7</td>
<td>98.3 (0.0–194.5)</td>
<td>84.1 ± 188.1</td>
<td>0.0 (0.0–420.5)</td>
</tr>
<tr>
<td>IL-4</td>
<td>4148.3 ± 3813.5</td>
<td>5785.9 (0.0–8256.2)</td>
<td>635.1 ± 950.5</td>
<td>367.1 (0.0–2289.3)</td>
</tr>
<tr>
<td>IL-5</td>
<td>155.9 ± 163.3</td>
<td>129.1 (0.0–359.6)</td>
<td>44.2 ± 55.0</td>
<td>15.8 (0.0–120.0)</td>
</tr>
<tr>
<td>IL-10</td>
<td>9586.1 ± 13156.1</td>
<td>690.9 (0.3–27851.2)</td>
<td>1506.3 ± 1779.9</td>
<td>1537.9 (0.0–4359.7)</td>
</tr>
</tbody>
</table>

Details of quantitative assessment of Th1/Th2 cytokines in the supernatants of cultures after 6 and 18 hours of specific CMV-pp65 or CMV-IE1 stimulation. The assay was performed by cytometric bead array. Results from 6 donors for each condition are shown.

IFN indicates interferon; IL, interleukin; Th, T helper; TNF, tumor necrosis factor.
Stimulation with peptide pools (spanning the entire CMV protein sequence), including broadly immunogenic and conserved pp65 and IE1 epitopes, seems to be especially important for viral T-cell expansion to recruit effector cells with different precursor frequencies and affinities for the target antigen, which may reduce the risk of viral escape mutants. Another advantage of overlapping peptide pools is that both CD4+ and CD8+ virus-specific T cells can be obtained, which may promote the survival and persistence of the generated T cells after ACT. Using overlapping peptides, CD3+ T-cell responses can be detected to multiple epitopes, regardless of HLA type. Here we used pp65 and IE1 libraries as stimulating antigens to generate a coordinated and simultaneous activation of both CD8+ and CD4+ CMV-T response from CMV-seropositive donors’ peripheral blood and to be independent from donors’ HLA type, as the latter was not known before stimulation. However, healthy donors differ in HLA background. Epitope dominance and variability in the relative numbers of CD4 and CD8 effector cells and IFN-γ production among normal donors could reflect individual CMV-specific cellular immunity. These variables would likely result in differences in the degree of T-cell responsiveness to epitopes presented in the peptide pool, some of which might have not been optimally presented in our experimental setting. Indeed, cell viability after MLPC_IE1 was only 13.8%, the range of the observed viability among cellular lots was really wide (2.3%–81.7%), and CMV-T fold-increase upon short-term culture showed a distinct behavior based on the stimulus originally used to activate them and on the way their functionality (ICS vs. CD154 upregulation) was measured.

Besides the evaluation of the enrichment of CMV-T during MLPCs by flow cytometer (Tables 1 and 2 and Fig. 4), we isolated, when feasible, CMV-T after MLPCs by either CCS or CD154 MACS selection. IFN-γ capture enables the rapid enrichment of both CD4+ and CD8+ virus-specific cells but only a subset of virus-specific
T cells secrete IFN-γ on activation and a significant number of human immunodeficiency virus epitope-specific CTLs detected by tetramer binding do not produce IFN-γ. Different T-cell surface molecules that are selectively expressed or strongly upregulated after T-cell activation could similarly be useful for the selection of antigen-specific T cells. Of these, CD137 and CD154 were recently the object of studies exploiting their antigen-induced expression. This aspect makes CD137 and CD154 promising candidates for the selection of CMV-T at a clinical level. We showed specificity and sensitivity of the CD154 upregulation-based CMV-T isolation method, which allowed simultaneous enrichment of CD4+ and CD8+ pathogen-specific T cells (Figs. 4-6 and our previous preliminary results). The purity of antigen-specific T cells after MACS selection with paramagnetic beads and after an additional short expansion phase seems to be correlated with the frequency of antigen-specific T cells before enrichment. Purity of the obtained CMV-T upon magnetic enrichment was not evaluated in this study. In samples with lower CMV-T at day 11 of MLPC (Table 2), a higher percentage of contaminating CD154 or IFN-γ-negative cells was supposed to affect the expansion outcome. To assess whether there was a correlation between the frequency of CMV-pp65 or CMV-IE1 reactive T cells in the starting population and the yield or purity of the enriched fractions after MLPC, the samples should have been evaluated by flow cytometer. Compared with the CCS, direct selection by CD154 microbeads appeared in our hands less laborious and with fewer critical steps. Indeed, despite the CMV-T isolation methodology based on cytokine secretion showed similar sensitivity and specificity along with a greater balance between CD4+ and CD8+ CMV-T isolation; it requires several additional washing steps and the use of ice-cold reagents to avoid unspecific background. In contrast, a protocol for the automated isolation of antigen-specific T cells based of IFN-γ secretion upon stimulation with clinical-grade CMV-pp65 or CMV-IE1 peptide pools is already available at clinical level since a decade, and CCS now moves toward clinical routine. In addition, the easy generation of pathogen-specific T-cell lines on the basis of IFN-γ+ and/or expression of CD137 and CD154 has been recently described, but both approaches result in different T-cell population, which may lead to divergent T-cell responses in vivo. Finally, the experimental need of the anti-CD40 antibody addiction to allow CMV-T selection by CD154 upregulation potentially limits its translatability.

IE1-specific or pp65-specific T cells were isolated in vitro according to their CD154 expression from MLPC at day 13–14. After 6 days of in vitro expansion, the specificity of the isolated T cells was confirmed by the expression of the activation marker CD154 (Fig. 5) and by the CFSE-based proliferation assay (Fig. 6).

We then explored the repertoire of cytokines secreted by pp65-specific and IE1-specific T lymphocytes upon antigenic reload. Stimulation with CMV-IE1 caused the release of a considerable amount of IL-10, beside the expected Th1-type cytokines IFN-γ and TNF-α, at both 6 and 18 hours collection times (Fig. 7). Each measured cytokine was specifically secreted upon stimulation, as no cytokine secretion was present without stimulus or without CMV-T in the MLPC. The finding that IL-10 secretion was so high in the MLPC_IE1 is consistent with the hypothesis that the Th lymphocytes that were part of the MLPC T-cell repertoire (Fig. 3) possess a regulatory-like phenotype. Indeed, some recent reports describe the existence in human blood of a type 1-T cells lineage producing both IFN-γ and IL-10, but not IL-2. The CBA assay does not allow to distinguish between CD4+ and CD8+ secreting T cells, as it is not an approach at single-cell level but population based. Even if we cannot get information on polyfunctionality of CMV-T, these results showed that IL-10 and/or IL-4 were secreted only when IFN-γ and/or TNF-α were also present (Fig. 7 and Table 3).

Finally, we investigated the phenotype of T-cell subsets by flow cytometer analysis of CD62L and CD45RA as markers of T-cell maturation, showing that CMV-T in the bulk of MLPC were mainly Tem (Fig. 8A) but >80% of activated CMV-T after magnetic enrichment was Tem (Figs. 8B, C). Historically, the most pressing technical issues in ACT therapies has been the generation of a sufficient quantity of antigen-specific T cells for transfer rather than to identify T cells subsets that possess superior traits for adoptive immunotherapy. However, extensive culturing of antigen-specific T cells has been shown to efficiently diminish alloreactivity and concurrently limit the proliferative capacity of the transferred T cells in vivo. It is now clear from relevant preclinical animal models and detailed retrospective analyses of human clinical trials that infusion of the less-differentiated T-naive, T-memory stem cells, and T-central memory subsets is associated with superior T-cell engraftment, persistence, and antigen recognition compared to T-effector memory and terminally differentiated short-lived effector T cells. Upon reexposure to antigen, memory T cells respond more rapidly and robustly than naive T cells, providing better clearance of pathogens. Recent reviews have reinforced the text-book view that memory T cells arise from effector cells. Although this is an appealing notion, emerging data are more consistent with a model where naive cells directly develop into memory cells without transitioning through an effector stage. Recent studies at single-cell level cast a spotlight on the issue of the lineage relationships between memory and effector cells, paving the way for profound implications in the development of effective T-cell-based therapies.

In conclusion, in the current study, we aimed at improving basic knowledge on pathogen-specific T cells biology and functional behavior. We established analytical methods focused on in vitro preclinical CMV-T generation and on T-cell monitoring techniques. As for many assays involving T-cell stimulation, outcome was variable, depending on multiple factors such as donor and viral epitope, experimental stimulation, and culture conditions. Notably, differences in HLA and epitope dominance and variability in the relative number of CD3+ effector cells and IFN-γ/CD154 expression among healthy donors could reflect the observed individual CMV-specific cellular immunity.

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CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES

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