Identification of new \( T_h \) peptides from the cytomegalovirus protein pp65 to design a peptide library for generation of CD4 T cell lines for cellular immunoreconstitution


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Keywords: allodepletion, CD4 cell line, cellular immunotherapy, \( T_h \) epitope

Abstract

CD8 and CD4 lymphocytes control cytomegalovirus (CMV) infection in immunocompetent individuals, while patients with defective cellular immunity are prone to endogenous reactivation of latent CMV or, like seronegative subjects, prone to primary infection. Administration of CMV-specific CD8 lymphocytes was beneficial for immunocompromised hemopoietic stem cell (HSC) graft recipients. Since CD4 cells contribute to expansion of cytotoxic T lymphocytes (CTL), we defined new \( T_h \) peptides on the immunodominant protein pp65 recognized by CD4 cells from HLA-typed subjects, in the perspective of complementing CTL administration with CMV-specific \( T_h \) cells. Screening by ELISPOT on CD4 and CD8 subsets using overlapping peptides identified 10 novel CD4 peptides. To simplify procedures to generate T cell lines, we used a CD4 peptide library for T cell stimulation instead of ill-defined viral lysates, without the requirement of dendritic cells. This library stimulated CMV-specific CD4 cells. In fact, peptide-induced CD4 cells responded to pp65 and to the viral lysate. These cells were also devoid of alloreactivity after one stimulation cycle. Since Good Manufacturing Procedure-grade peptides can be synthesized, culture conditions are simplified and alloreactivity is rapidly lost, these procedures based on peptide stimulation can facilitate implementation of adoptive reconstitution of CD4 responses in immunocompromised patients also in the case when the HSC allodonor is available for generation of the T cell line.

Introduction

Cytomegalovirus (CMV) is an opportunistic pathogen that infects \textit{de novo} (1,2) or endogenously reactivates in immunocompromised patients (3). These include recipients of solid organ (4) or hemopoietic stem cell (HSC) grafts (5,6) and AIDS patients (7,8). This clinical experience shows that defects of cellular immunity make patients prone to CMV reactivation.

Experiments in animals have shown that latent CMV is controlled by specific cytotoxic T lymphocytes (CTL) and \( T_h \) cells (9). Studies in mice (10) and humans (11–14) introduced the concept of adoptive immunoreconstitution with specific CTL.

Simpler procedures to generate specific CTL have been described (15–23). The original reports used CMV-infected fibroblasts as antigen-presenting cells (APC) (13,24), but fibroblasts may not be available and infectious CMV had to be used. Several studies have identified CTL epitopes on the
immunodominant protein pp65 (25–34) and on other proteins (35–40). Generation of CTL lines using dendritic cells instead of fibroblasts has been reported (17,20–23,29,41,42).

T<sub>h</sub> cells are equally crucial for antiviral immunoreconstitution (43–47). The scant information on CMV Th epitopes (21,32,48±51) corresponds to only five peptides described so far on pp65 (21,32,50), insufficient for our purposes. Therefore, we mapped this protein to identify new Th peptides and their restriction elements. These new peptides, in association with the previously reported ones, were used as an antigenic library to produce CMV-specific CD4 T cell lines without the need for dendritic cells. The peptide-stimulated T cells were highly enriched in pp65-specific cells and devoid of allo-reactivity, even after one cycle of in vitro selection.

Here, we show that pp65 peptides containing Th epitopes can be used to generate CD4 T cell lines that respond to viral antigens. Since the T cell lines are depleted in alloreactive precursors, they can be obtained from the same allogeneic donor who provided the grafted HSC preparation.

**Methods**

**Antigens and media**

CMV antigen lysate (strain AD169; Microbix, Toronto, Canada) and protein pp65 (His-tagged, recombinant protein expressed in Escherichia coli) (52) were used at 5 and 1 μg/ml. Peptides from pp65 (SwissProt P06725) were 15mers overlapped by 11 residues (30). The15 selected CD4 peptides were pooled to prepare the peptide library used for proliferation experiments. A MultiSyn-Tech Syro synthesizer (Witten, Germany) was used for Fmoc chemistry. Purity was assessed by HPLC and MALDI-TOF spectrometry. Peptides were used at 1 μg/ml. Cultures were performed in RPMI 1640 (Biowhittaker, Verviers, Belgium) (10 mM L-glutamine, 5 × 10<sup>–6</sup>M 2-mercaptoethanol and 5% autologous plasma) without antibiotics. Human IL-2 (Proleukin; Chiron, Emeryville, CA) was used at 30 U/ml.

**Peripheral blood mononuclear cell (PBMC) lymphoproliferation**

IgG-CMV-seropositive donors (age 18–55) were HLA-typed serologically for class I antigens (BAG, Lich, Germany) and molecularly for class II antigens (Dynal, Bromborough, UK). Their initials and HLA-DR typing are given in Table 1. Heparinized blood was spun on Biocoll (Biochrom, Berlin, Germany). PBMC in RPMI 1640 (2

<table>
<thead>
<tr>
<th>Initials</th>
<th>DR alleles</th>
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<tbody>
<tr>
<td>ALC</td>
<td>1, 7</td>
</tr>
<tr>
<td>CM</td>
<td>2(15), 7</td>
</tr>
<tr>
<td>EM</td>
<td>2(16), 5(11)</td>
</tr>
<tr>
<td>ES</td>
<td>1, 2(15)</td>
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<tr>
<td>FDM</td>
<td>2(15), 4</td>
</tr>
<tr>
<td>FI</td>
<td>3(17), 7</td>
</tr>
<tr>
<td>FM</td>
<td>2(15), 6</td>
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<tr>
<td>FSF</td>
<td>1, 5(11)</td>
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<tr>
<td>GC</td>
<td>5(11), 6(13)</td>
</tr>
<tr>
<td>GG</td>
<td>7, 5(11)</td>
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<tr>
<td>GLP</td>
<td>5(11), 7</td>
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<tr>
<td>LC</td>
<td>1, 1</td>
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<tr>
<td>LM</td>
<td>4, X</td>
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<tr>
<td>MB</td>
<td>1, 5(11)</td>
</tr>
<tr>
<td>MM</td>
<td>1, 3</td>
</tr>
<tr>
<td>MIM</td>
<td>5(11), 6(13)</td>
</tr>
<tr>
<td>MP</td>
<td>1, 7</td>
</tr>
<tr>
<td>RF</td>
<td>6(14), 5(11)</td>
</tr>
</tbody>
</table>

**ELISPOT assay**

PVDF plates (Millipore, Bedford, MA) were coated with anti-IFN-γ mAb (ELISPOT-hIFNγ, Mabtech, Nacka, Sweden), following the manufacturer’s instructions. PBMC (2 × 10<sup>6</sup>) or T cells from established lines (2 × 10<sup>4</sup>) were dispersed in 200 μl with or without antigen. The plates were incubated for 20 h, processed as indicated by the manufacturer and developed (alkaline phosphatase-conjugate kit; Bio-Rad, Hercules, CA). Spot-forming units (s.f.u.) were enumerated under a dissecting microscope. Responses with >20 spots/well and positive/negative ratio >3 were scored as positive. Results are shown as s.f.u./10<sup>6</sup> cells.

**CD4 and CD8 fractionation**

Magnetic beads coated with anti-CD4 or anti-CD8 antibodies (Dynal, Oslo, Norway) were used for negative fractionation of PBMC, following the manufacturer’s instructions. Standard separations were performed on 5 × 10<sup>6</sup> PBMC. Residual CD4 or CD8 cells were always lower than 0.5 and 1.2%.

**Generation of CD4 cell lines**

CD4 cell lines were generated by stimulation of PBMC with autologous APC, followed by IL-2 expansion (53,54). PBMC were pulsed with proteins or peptides at 10 × 10<sup>5</sup>/ml for 4 h at 37°C and plated at 10<sup>5</sup>/ml in 24-well plates, 2 ml/well. Four days later, IL-2 was added. Cells were split to 0.2 × 10<sup>5</sup>/ml when the concentration exceeded 10<sup>6</sup>/ml. After 3 weeks, 5 × 10<sup>5</sup> T cells were re-stimulated with 10<sup>5</sup> autologous irradiated (30 Gy) PBMC pulsed with antigens in 2 ml/well. IL-2 was added after 2 days and the lines were split as above. Re-stimulations were performed every 3 weeks. These conditions select CD4 lymphocytes (>96% after two cycles). Proliferation was checked by seeding 2 × 10<sup>4</sup> T cells plus 10<sup>5</sup> irradiated PBMC with or without antigen. Cultures pulsed on day 2 were harvested as for PBMC lymphoproliferation. Alloreactivity of T cell lines was tested by replacing autologous irradiated
PBMC with an equal amount of 30 Gy irradiated PBMC from a fully HLA-mismatched subject. The wells containing autologous irradiated PBMC were the negative control. Cultures were processed as for antigen-specific proliferation.

Results

ELISPOT on PBMC with pp65 peptides

Proliferation to CMV lysate and to purified pp65 was determined on 30 CMV-seropositive donors. Twenty-eight (93%) CMV responders (>2 klc.p.m.) proliferated to pp65 (correlation coefficient between proliferation to CMV and to pp65 of 0.65). The panel of 138 peptides of pp65 was used to screen PBMC from 20 pp65 responders. Figure 1 shows representative ELISPOT profiles, with examples of broad responses (left panels) and focused responses (right panels). Screening on these donors, as shown in Fig. 2, identified regions of pp65 that were frequently recognized (solid bars) or regions that were never recognized (open bars). This assay did not discriminate between CD4 and CD8 responses, but indicated peptides deserving further analysis.

ELISPOT on selected CD4 and CD8 cells

To define Th and CTL peptides, PBMC were fractionated with magnetic beads to obtain CD4 and CD8 cell subsets. Preliminary experiments were run to ascertain that ability of separated CD4 and CD8 cells to functionally recognize antigen was retained after the selection procedures. Figure 3 shows examples of CD8 and CD4 subsets tested with peptides encompassing Th or CTL epitopes. Responses to pp65 tested by ELISPOT were CD4-mediated in donors FI, CM and ES, while donor LM responded to pp65 also with the CD8 subset (Fig. 3A). This was somewhat unexpected, since the uptake of protein antigens is targeted to the endosomal MHC class II-restricted compartment (55). This suggests a spill-over of antigen from the class II to the class I compartment (56,57). This was reported in the CMV system by others, who were able to induce CD8 cell expansion with protein viral antigens administered to APC (14,22) or by cross-presentation (58,59). Peptides were recognized by CD4 (peptides 57, 62 and 128) or CD8 cells (peptides 104 and 123) with mutually exclusive patterns (Fig. 3B and C). Peptides TM10 (HLA-B7 restricted) and NV9 (HLA-A2 restricted), within peptides 104 and 123 respectively, already reported as CTL peptides, confirmed recognition by CD8 cells.

Novel Th peptides of pp65 for definition of a CD4 peptide library

Peptides defined in Fig. 2 were tested on CD4 subsets from the 18 donors listed in Table 1, which shows their HLA-DR alleles. Ten CD4 peptides were identified here for the first time, out of 15 that were recognized by CD4 lymphocytes (Table 2). Five peptides (peptides 11, 71, 92, 123 and 128) have already been described (21,32,50), but this number was too limited to prepare a peptide library for the goals we had in mind. Table 2 shows the peptide number with amino acid residues and sequence, the potential DR restriction alleles, and the number of individuals responding to the peptide versus the number of individuals in the panel carrying the allele.

Fig. 1. ELISPOT assays on PBMC with pp65 peptides. A panel of 138 overlapping peptides was used to screen pp65 responders. Response to two adjacent peptides was considered as a single response to the 11-amino-acid overlapping region. Representative profiles show examples of broad responses (five or more stimulatory peptides, left panels) or focused responses (three or fewer stimulatory peptides, right panels). Background s.f.u. values (<50 s.f.u./10^6 in all cases) were subtracted from the graphs.

Fig. 2. Profile of T cell stimulatory peptides of pp65 by ELISPOT assay. Results of the peptide screening are shown as the number of responding individuals out of 20 CMV-seropositive subjects examined. Stimulatory peptides cluster in certain regions (solid bars), whereas other regions of the pp65 sequence contain no T cell epitopes (open bars) recognized by the donor panel.
Assignment of potential DR restriction alleles to given peptides was more consistent for peptides recognized in association with the DR alleles most frequently expressed by our donor panel (e.g. peptide 30, DR1; peptide 92, DR11; peptide 112, DR1; peptide 123, DR11; peptide 128, DR1). Furthermore, testing peptide-specific T cell lines devoid of alloreactivity with HLA-typed APC from different donors can provide additional information and indicate unequivocally the restriction element used by the given peptide (60). Several peptides, such as peptides 30, 66, 71, 104, 123 and 128, were presented by more class II alleles, suggesting non-unique associations between peptides and DR alleles.

Expansion of specific CD4 T cells with the CD4 peptide library

The Th peptides in Table 2 were mixed to prepare the CD4 peptide library. PBMC were pulsed with the antigenic pool so that each peptide was present at 1 μg/ml, corresponding to 0.5 μM for an average mol. wt of 1.8 kDa for the 15mer peptides. Experiments were performed to validate the use of the peptide pool for the generation of CD4 T cell lines responsive to CMV and to test whether alloreactive precursors were diluted out after peptide stimulation. One representative pp65 responder was used for this analysis. The cultured cells lost alloreactivity after one stimulation cycle as shown in Fig. 4. In particular, PBMC (Fig. 4, left panel) responded to the CD4 Th pool, and to peptides 30, 45, 62 and 128, consistently with the donor’s class II typing (DR3, DR7). Responses to purified protein derivative (PPD) and to allogeneic responses after one stimulation cycle (Fig. 4, middle panel). Notably, CMV and pp65 were recognized, indicating that peptides had expanded the relevant CD4 T cells. This cannot be taken for granted, since we have previously observed in the HIV system that not all peptide-generated CD4 lines respond to the corresponding viral proteins gp120 and reverse transcriptase (61).

Table 2. Th peptides of CMV pp65 identified by this study

<table>
<thead>
<tr>
<th>Pep. no.</th>
<th>Residues</th>
<th>Sequence</th>
<th>Potential DR restriction</th>
<th>Responders</th>
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<tr>
<td>...</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>41–55</td>
<td>LLQTGIHVRVSQPSL</td>
<td>15</td>
<td>2 vs 4</td>
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<tr>
<td>30</td>
<td>117–131</td>
<td>PLKMLNPSINVHYY</td>
<td>1</td>
<td>3 vs 7</td>
</tr>
<tr>
<td>43</td>
<td>169–183</td>
<td>TROQONWKEPDVYYT</td>
<td>1</td>
<td>2 vs 3</td>
</tr>
<tr>
<td>45</td>
<td>177–191</td>
<td>EPDVYTTAFVFPTK</td>
<td>7</td>
<td>1 vs 7</td>
</tr>
<tr>
<td>57</td>
<td>225–239</td>
<td>KYLESFCEVPSHGK</td>
<td>15</td>
<td>2 vs 6</td>
</tr>
<tr>
<td>62</td>
<td>245–259</td>
<td>TLGSDVEEDLMTRN</td>
<td>3</td>
<td>2 vs 4</td>
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<tr>
<td>66</td>
<td>261–275</td>
<td>QPFMPHERNGFTVL</td>
<td>13</td>
<td>1 vs 3</td>
</tr>
<tr>
<td>71</td>
<td>281–295</td>
<td>IIKPKSHMDVLA</td>
<td>4</td>
<td>2 vs 2</td>
</tr>
<tr>
<td>94</td>
<td>365–379</td>
<td>EHPFTSOLYIQGKL</td>
<td>11</td>
<td>1 vs 6</td>
</tr>
<tr>
<td>94</td>
<td>373–387</td>
<td>YRIQGKLEHTPRW</td>
<td>3</td>
<td>4 vs 9</td>
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<tr>
<td>104</td>
<td>413–427</td>
<td>TERKTPRTGGGAMA</td>
<td>14</td>
<td>1 vs 3</td>
</tr>
<tr>
<td>108</td>
<td>429–443</td>
<td>ASTSAGRKRSASA</td>
<td>11</td>
<td>1 vs 2</td>
</tr>
<tr>
<td>112</td>
<td>445–459</td>
<td>ACTSGVTRGLKAE</td>
<td>1</td>
<td>2 vs 9</td>
</tr>
<tr>
<td>123</td>
<td>489–503</td>
<td>AGILARNLVPMTAV</td>
<td>11</td>
<td>5 vs 7</td>
</tr>
<tr>
<td>128</td>
<td>509–523</td>
<td>KYQEFFWDANDIYRI</td>
<td>1</td>
<td>1 vs 3</td>
</tr>
</tbody>
</table>

Peptides recognized by CD4 lymphocytes are listed according to the consecutive number in the panel, with residues and sequences. The potential DR restriction alleles are indicated. The number of peptide responders versus the number of subjects in the panel carrying the same DR allele is shown in the Frequency column. More than one potential allele was attributed when a peptide responder was negative for the more frequently utilized allele. Peptides 11, 71, 92, 123 and 128 were originally reported by others (21,32,50). The initials of the subjects responding to the different peptides are also shown in the last column.
Testing the same T cell lines after four stimulation cycles with the peptide library showed that a T cell line can be extensively expanded in vitro with preservation of the same reactivity profile (Fig. 4, right panel).

Discussion

Following primary infection (2), latent CMV is controlled by the immune system in spite of its persistence. Antibodies (62), that limit primary infection, and CD8 cells, that in association with CD4 cells maintain viral latency, demonstrate the efficacy of the immune responses (9,45). Several conditions result in altered cellular immunity (4–8) followed by opportunistic infections. CMV, in particular, is responsible for severe complications (4–8). Animal experiments have shown that cellular immunity maintains CMV latency (1,9,10), while studies on immunocompromised patients have linked control of CMV disease to specific cellular responses examined at the single-cell level (8,23,63,64). This is the rationale for monitoring specific CTL (63,64) and for using CMV-specific T lymphocytes as supportive treatment (45). The appearance of chemotherapy-resistant strains (65) and the increased risk for CMV infection in T-depleted HSC transplants (14) have triggered a growing interest in adoptive immunoreconstitution.

Initial studies focused on CTL for protection (11–13). Thereafter, it appeared that CD4 lymphocytes were equally relevant, since they favor the persistence of re-infused CTL (14,43,45) or may contribute to endogenous reconstitution of the CMV-specific CD8 repertoire. Furthermore, CD4 cells exhibit antiviral activity by producing cytokines (49,66).

To overcome the technically demanding procedures of the methods originally devised for the generation of CMV-specific CTL (11,13,24), alternative approaches have been investigated in numerous studies.

Dendritic cells have been used as APC to replace fibroblasts. Instead of infectious CMV, methods have been designed to present CMV antigens to CTL, such as antigen pulsing (18–22,26,29,34,67) or transfection (39), infection (17,21,42) and transduction (15,16,29,68,69). Epitopes have been mapped on proteins to replace complex protein antigens with synthetic peptides (26–34,36–40). To accelerate the production of T cell lines, specific T cells have been enriched using tetramers (18) or activation markers (21,51).

CMV lysates can expand CD4 and CD8 cells (14,22), but these antigenic preparations are produced for diagnostic purposes and should not be used for ex vivo T cell selection. Stimulation with recombinant proteins (67) or with APC transduced or infected with antigen-encoding vectors also carries regulatory limitations. CTL lines were generated with APC producing endogenous pp65, with antigen or peptide-pulsed APC, or by selection methods. These are admittedly improvements over protocols using CMV-infected fibroblasts, but they introduce infectious agents or reagents that are not suitable for ex vivo use. Therefore, we developed simpler procedures using peptide pools to generate CMV-specific T cell lines, without the requirement of dendritic cells.

The new Tₜₕ peptides identified in this study with their HLA-DR restriction alleles and the already known CD4 peptides (21,32,50) were used to prepare a library of 15 peptides that was tested in functional experiments to induce CD4 cell proliferation, in addition to IFN-γ secretion. It was important to demonstrate that peptide-activated CD4 T cells recognized pp65 either as recombinant protein or as a component of the viral lysate. Peptide stimulation resulted in depletion of CD4 lymphocytes responding to recall antigens and, most significantly, also resulted in depletion of alloreactive CD4 lymphocytes. If this finding is confirmed with other donors (work in progress), these culture procedures can promptly provide CMV-specific T cell lines from allogeneic donors.
that are safe for re-infusion in HSC recipients, after checking for lack of residual alloreactivity.

The protocols described here for the generation of short- and long-term CD4 T cell lines are simpler than other published procedures, in that dendritic cells are not required and positive selection procedures can be avoided. In addition, the use of well-defined peptides is preferable to the use of ill-defined viral lysates employed in previous reports (14,22). Viral lysates, in fact, are convenient and informative antigens for in vitro assays to evaluate cellular immunity, but cannot be applied for the generation of therapeutic T cell lines, since they are produced as diagnostic reagents and cannot be certified as Good Manufacturing Practice (GMP) reagents.

While reliable predictions for MHC class I allele restriction of CTL epitopes can be made (70), this is not the case for MHC class II associations with 

\[ \text{T_h} \] epitopes. For this reason we have examined the whole peptide panel of pp65 by using in vitro experiments rather than relying on a predictive approach. According to our study, peptides restricted by one DR allele were not recognized by all individuals bearing that allele. Thus, the potential to bind a given HLA allele does not necessarily imply the availability of peptide-specific T cells in the repertoire. On the other hand, peptides like 30, 71 and 123 were recognized in the context of more than one DR allele in different individuals, suggesting some degree of promiscuity in peptide restriction.

Generation of CMV-specific T cell lines from CMV-seronegative donors (20,29) is most appealing for allogeneic HSC recipients. In other viral systems, seronegative donors were able to mount CD8 responses in vitro against Epstein–Barr (71) or CD4 responses against HIV (72) when dendritic cells were used as APC. Also, according to our previous experience with HIV or HTLV-1 naive donors (60,73), peptides may be more effective than proteins for the expansion of naive CD4 cells from non-immune donors.

A selected peptide library derived from the pp65 sequence can stimulate CMV-specific T cells that maintain specificity for the protein antigen, as proposed here. The possibility of using the whole peptide panel can also be considered, but this approach is discouraged by the high cost of producing a large number of GMP grade peptides. Also the use of longer peptides can be envisaged for CD4 stimulation, in order to limit the number of peptides, yet also to have a broad coverage of epitopes less frequently recognized. In this case it should be kept in mind that degeneracy of extended peptides may be a drawback that requires accurate purification. With peptide-induced T cell lines, alloreactivity drops to undetectable levels according to lack of proliferation in a mixed lymphocyte reaction.

In conclusion, the simplified culture conditions reported here, based on peptides that can be synthesized with GMP grade, can make immunoreconstitution of the CMV-specific CD4 T cell repertoire more easily achievable as a therapeutic procedure.

Acknowledgements

This work was supported by: National Health Institute, Rome (AIDS grants 400.54, 500.18 and 450.1.17), National Research Council, Rome (Biotechnology Project), Ministry of Higher Education, Rome (FIRB-RBNE01-RB98.003), Ministry of Health (2002, N.149) and European Union, Brussels (FAIR-CT-97-3046, BHH4-CT97-2055, QLK2-CT-2000-01040 and QLK2-CT-2000-01321). We thank S. Barocci, S. Fiordoro, A. Tagliamacco and A. Nocera (San Martino Hospital, Genoa) for the help of Giorgio Langheim for graphics work and of Tom Wiley for revision of the English text. We are grateful to the donors who volunteered for repeated donations of blood samples for this investigation.

Abbreviations

- APC: antigen-presenting cell
- CTL: cytotoxic T lymphocyte
- CMV: cytomegalovirus
- GMP: Good Manufacturing procedure
- HSC: hemopoietic stem cell
- PBMC: peripheral blood mononuclear cell
- PPD: purified protein derivative
- s.f.u.: spot-forming unit

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