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A simple and efficient method of generating HCMV pp65-specific T cells using overlapping peptides

S.-U. HWANG[1#], J.-H. LEE[1#], S.-Y. CHOI[2#], Y.-T. LEE[1], S.-Y. PARK[2], H. S. LEE[1], J. W. JAE[1], N.-C. JUNG[1], Y. WANG[3], D.-S. LIM[2*]

[1]Pharosvaccine Inc., Dunchon-daero, Jungwon-gu, Seongnam-si, Gyeonggi-do 462-807, Republic of Korea; [2]Department of Biotechnology, CHA University, 335 Pangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do 13488, Republic of Korea; [3]Immunotech Applied Science Ltd., Beijing 100176, P. R. China

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Summary. – The methods for expansion of human cytomegalovirus (HCMV)-specific T lymphocytes are limited due to the complex culture process, long culture duration, and human leukocyte antigen (HLA) restriction. Here, we report that *in vitro* stimulation with pp65 kDa phosphoprotein (pp65)-derived overlapping synthetic peptides rapidly generates large numbers of HCMV-specific cytotoxic T lymphocytes from peripheral blood mononuclear cells (PBMCs) regardless of HLA type. Treatment of PBMCs from healthy volunteers expressing HLA-A*02:01 or HLA-A*24:02 with 138 pp65 overlapping peptides (OLP) resulted in an expansion of HCMV pp65 NLVPMVATV (NLV) pentamer-specific $CD8^+$ T lymphocytes that expressed interferon (IFN)-γ, but the pp65 NLV peptide did not generate HCMV-specific $CD8^+$ T lymphocytes in PBMCs obtained from an HLA-A*24:02 donor due to HLA restriction. The OLP-induced T lymphocytes specific for HCMV derived from PBMCs of HLA-A*02:01- and HLA-A*24:02-expressing donors showed effective cytolytic responses against target cells loaded with OLP or the NLV epitope, but pp65 NLV peptide-induced T lymphocytes did not. Phenotypic analyses demonstrated that OLP increased the frequency of $CD3^+$ $CD8^+$ cells, but not $CD3^+$ $CD4^+$, $CD14^+$, or $CD56^+$ cells, in donor PBMCs. Thus, this study provides evidence that *in vitro* stimulation with OLP efficiently generates sufficient numbers of HCMV pp65-specific cytotoxic T lymphocytes for adoptive cell therapy.

Keywords: human cytomegalovirus; cytotoxic T lymphocyte; overlapping peptides; pp65; cytotoxicity

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an effective treatment for various diseases, including malignant lymphoma, multiple myeloma, refractory autoimmune disease, some solid tumors, and leukemia. However, due to immune suppression, transplant patients are at a high risk of death due to infectious complications; more than 30% die from opportunistic infections with viruses such as human cytomegalovirus (HCMV), adenovirus, Epstein-Barr virus (EBV), and the varicella zoster virus (Koc *et al*., 2000; Sahin *et al*., 2016; Chan and Logan, 2017). Of these, HCMV is one of the most common infectious complications of transplantation (Aandahl *et al*., 2004; Varani *et al*., 2009). This partly reflects the fact that it is a ubiquitous pathogen that chronically infects hosts for life. Although this chronic infection is normally controlled by the immune system, it is associated with dysfunctional immune responses in older individuals, including weakened responses to new pathogens and

*Corresponding author. E-mail: dslim@cha.ac.kr; phone: +82-31-881-7122. #These authors contributed equally to this study.

Abbreviations: APCs = antigen-presenting cells; BLCLs = B lymphoblastic cell lines; CTLs = cytotoxic T lymphocytes; EBV = Epstein-Barr virus; GvHD = graft versus host disease; HCMV = human cytomegalovirus; HSCT = hematopoietic stem cell transplantation; OLP = overlapping peptides; PBMCs = peripheral blood mononuclear cells

vaccines (Aiello and Simanek, 2012). Moreover, despite substantial medical advances, it remains a major cause of morbidity as well as mortality in patients, who receive T cell-depleted grafts, HSCT, or solid organ transplants (SOT), or who undergo prolonged immunosuppression. Indeed, it frequently precedes graft versus host disease (GvHD); these effects are due to both direct adverse effects caused by viral invasion of the organ system and indirect effects on the immune system, which increase the risk of other infections and promote acute graft rejection (Gallez-Hawkins *et al*., 2009; Hasanzamani *et al*., 2016).

To resolve this problem, transplant patients can be treated with antiviral agents that reduce virus load; these agents include ganciclovir, foscarnet, cidofovir, and acyclovir (Gerna *et al*., 1994; Cihlar *et al*., 1998; Biron, 2006). However, prolonged antiviral treatment may delay the recovery of virus-specific immune responses and can promote the emergence of resistance. Moreover, antiviral drug treatment often cannot be sustained because of complicating myelosuppression or nephrotoxicity (Chou, 2001).

Several additional strategies have been devised to treat HCMV-related diseases. One of these is the adoptive transfer of HCMV-specific T cells. This approach is based on animal studies with murine CMV that showed that $CD8^+$ CMV-specific cytotoxic T lymphocytes (CTLs) play a key role in controlling CMV replication (Reddehase *et al*., 1987). Moreover, Potena *et al*. showed that in the absence of preventive ganciclovir therapy, only 50% of patients who underwent allogeneic HSCT had detectable CTL responses 3 months after transplantation (Potena *et al*., 2016). In addition, a randomized clinical trial showed that the frequency of CMV-specific CTLs correlated positively with protection from HCMV infectious complications, namely GvHD (Li *et al*., 1994). These findings suggest that adoptive immunotherapy, where the patient is transfused with HCMV-specific CTLs, may prevent HCMV infection or reactivation.

At present, adoptive immunotherapy against HCMV is hampered by a number of immunological and practical issues. First, the optimal HCMV target protein(s)/peptide(s) remain unclear. Sylwester *et al*. showed that healthy HCMV-infected adults (but not seronegative adults) generally exhibited strong *in vitro* $CD8^+$ T cell responses to a subset of 13,687 peptides that overlapped HCMV open reading frames; on average, 10.2% of the total memory $CD8^+$ T cell repertoire of these subjects recognized a median of eight (range, 1–30) HCMV peptides. Indeed, up to 30% of the memory $CD8^+$ T cells of some subjects responded to HCMV peptides (Sylwester *et al*., 2005). However, there was extensive variability among individuals in terms of the magnitude of their anti-HCMV T cell response. The reasons for this variability are not fully understood but may include the dose and timing of the HCMV infection, major histocompatibility class restriction, and competition between high- and low-affinity T cell clones during *in vivo* expansion (the high-avidity cells are preferentially selected early after infection and retain their dominance throughout latency). The second issue hampering adoptive immunotherapy against HCMV is that it takes weeks to months to prepare sufficient numbers of clinical-grade CTLs *in vitro* for transfer. First, the antigen-presenting cells (APCs) must be prepared; these are either dendritic cells (Peggs *et al*., 2001) or EBV-transformed B lymphoblastic cell lines (BLCLs) (Ramadan, 2008). Thereafter, the CTLs must be stimulated with the peptide-loaded APCs (Vannucchi *et al*., 2001; Leen *et al*., 2006). Lilleri *et al*. showed recently that within 3 months of transplantation, the majority of seropositive HSCT recipients (62%) exhibited an increase in HCMV DNA in the blood, prompting pre-emptive treatment (Lilleri *et al*., 2012). The costs associated with CTL production and the complexity of production also hamper adoptive immunotherapy in HSCT recipients (Leen *et al*., 2010).

pp65 is the most abundant tegument protein and the major tegument protein responsible for modulating/evading the host cell immune response during HCMV infections (McLaughlin-Taylor *et al*., 1994; Chevillotte *et al*., 2009). This viral structural protein has been identified as a target antigen for HCMV-specific class I MHC restricted CTL derived from the peripheral blood of most asymptomatic HCMV-seropositive individuals. Also, pp65 mainly targets both humoral and cellular immunity and serves as the dominant targeting antigen of cytotoxic T lymphocytes. (McLaughlin-Taylor *et al*., 1994; Tomtishen, 2012). The overlapping peptide, which was mainly used to search for immunogenic epitopes on immune cells, was used as an antigen. Unlike the epitope peptide, the overlapping peptide mixture is not limited to HLA type, and it is known that it can stimulate CD8+ T cells more effectively than when using viral lysates or proteins recognized as exogeneous antigens (Holden T *et al*., 2001). The aim of this study was to determine whether HCMV-specific CTLs can be effectively expanded from peripheral blood mononuclear cells (PBMCs) using a mixture of peptides that overlap the HCMV pp65 protein. Here, we show that this approach is feasible and may be useful for adoptive immunotherapy of HCMV-infected HSCT recipients.

Materials and Methods

Donors. Nine healthy volunteers (eight expressing HLA-A*02:01 and one expressing HLA-A*24:02) were enrolled in this study. All procedures involving the human participants

Table 1. Overlapping peptide pool of 138 peptides derived from a peptide scan (15 mers with 11 amino acid overlap) through HCMV pp65 (Swiss Prot ID: P06725)

No.	Amino acid sequence	No.	Amino acid sequence	No.	Amino acid sequence
1	MESRGRRCPEMISVL	47	AFVFPTKDVALRHVV	93	FTSQYRIQGKLEYRH
2	GRRCPEMISVLGPIS	48	PTKDVALRHVVCAHE	94	YRIQGKLEYRHTWDR
3	PEMISVLGPISGHVL	49	VALRHVVCAHELVCS	95	GKLEYRHTWDRHDEG
4	SVLGPISGHVLKAVF	50	HVVCAHELVCSMENT	96	YRHTWDRHDEGAAQG
5	PISGHVLKAVFSRGD	51	AHELVCSMENTRATK	97	WDRHDEGAAQGDDDV
6	HVLKAVFSRGDTPVL	52	VCSMENTRATKMQVI	98	DEGAAQGDDDVWTSG
7	AVFSRGDTPVLPHET	53	ENTRATKMQVIGDQY	99	AQGDDDVWTSGSDSD
8	RGDTPVLPHETRLLQ	54	ATKMQVIGDQYVKVY	100	DDVWTSGSDSDEELV
9	PVLPHETRLLQTGIH	55	QVIGDQYVKVYLESF	101	TSGSDSDEELVTTER
10	HETRLLQTGIHVRVS	56	DQYVKVYLESFCEDV	102	DSDEELVTTERKTPR
11	LLQTGIHVRVSQPSL	57	KVYLESFCEDVPSGK	103	ELVTTERKTPRVTGG
12	GIHVRVSQPSLILVS	58	ESFCEDVPSGKLFMH	104	TERKTPRVTGGGAMA
13	RVSQPSLILVSQYTP	59	EDVPSGKLFMHVTLG	105	TPRVTGGGAMAGAST
14	PSLILVSQYTPDSTP	60	SGKLFMHVTLGSDVE	106	TGGGAMAGASTSAGR
15	LVSQYTPDSTPCHRG	61	FMHVTLGSDVEEDLT	107	AMAGASTSAGRKRKS
16	YTPDSTPCHRGDNQL	62	TLGSDVEEDLTMTRN	108	ASTSAGRKRKSASSA
17	STPCHRGDNQLQVQH	63	DVEEDLTMTRNPQPF	109	AGRKRKSASSATACT
18	HRGDNQLQVQHTYFT	64	DLTMTRNPQPFMRPH	110	RKSASSATACTSGVM
19	NQLQVQHTYFTGSEV	65	TRNPQPFMRPHERNG	111	SSATACTSGVMTRGR
20	VQHTYFTGSEVENVS	66	QPFMRPHERNGFTVL	112	ACTSGVMTRGRLKAE
21	YFTGSEVENVSVNVH	67	RPHERNGFTVLCPKN	113	GVMTRGRLKAESTVA
22	SEVENVSVNVHNPTG	68	RNGFTVLCPKNMIIK	114	RGRLKAESTVAPEED
23	NVSVNVHNPTGRSIC	69	TVLCPKNMIIKPGKI	115	KAESTVAPEEDTDED
24	NVHNPTGRSICPSQE	70	PKNMIIKPGKISHIM	116	TVAPEEDTDEDSDNE
25	PTGRSICPSQEPMSI	71	IIKPGKISHIMLDVA	117	EEDTDEDSDNEIHNP
26	SICPSQEPMSIYVYA	72	GKISHIMLDVAFTSH	118	DEDSDNEIHNPAVFT
27	SQEPMSIYVYALPLK	73	HIMLDVAFTSHEHFG	119	DNEIHNPAVFTWPPW
28	MSIYVYALPLKMLNI	74	DVAFTSHEHFGLLCP	120	HNPAVFTWPPWQAGI
29	VYALPLKMLNIPSIN	75	TSHEHFGLLCPKSIP	121	VFTWPPWQAGILARN
30	PLKMLNIPSINVHHY	76	HFGLLCPKSIPGLSI	122	PPWQAGILARNLVPM
31	LNIPSINVHHYPSAA	77	LCPKSIPGLSISGNL	123	AGILARNLVPMVATV
32	SINVHHYPSAAERKH	78	SIPGLSISGNLLMNG	124	ARNLVPMVATVQGQN
33	HHYPSAAERKHRHLP	79	LSISGNLLMNGQQIF	125	VPMVATVQGQNLKYQ
34	SAAERKHRHLPVADA	80	GNLLMNGQQIFLEVQ	126	ATVQGQNLKYQEFFW
35	RKHRHLPVADAVIHA	81	MNGQQIFLEVQAIRE	127	GQNLKYQEFFWDAND
36	HLPVADAVIHASGKQ	82	QIFLEVQAIRETVEL	128	KYQEFFWDANDIYRI
37	ADAVIHASGKQMWQA	83	EVQAIRETVELRQYD	129	FFWDANDIYRIFAEL
38	IHASGKQMWQARLTV	84	IRETVELRQYDPVAA	130	ANDIYRIFAELEGVW
39	GKQMWQARLTVSGLA	85	VELRQYDPVAALFFF	131	YRIFAELEGVWQPAA
40	WQARLTVSGLAWTRQ	86	QYDPVAALFFFDIDL	132	AELEGVWQPAAQPKR
41	LTVSGLAWTRQQNQW	87	VAALFFFDIDLLLQR	133	GVWQPAAQPKRRRHR
42	GLAWTRQQNQWKEPD	88	FFFDIDLLLQRGPQY	134	PAAQPKRRRHRQDAL
43	TRQQNQWKEPDVYYT	89	IDLLLQRGPQYSEHP	135	PKRRRHRQDALPGPC
44	NQWKEPDVYYTSAFV	90	LQRGPQYSEHPTFTS	136	RHRQDALPGPCIAST
45	EPDVYYTSAFVFPTK	91	PQYSEHPTFTSQYRI	137	DALPGPCIASTPKKH
46	YYTSAFVFPTKDVAL	92	EHPTFTSQYRIQGKL	138	GPCIASTPKKHRG

in this study were conducted in accordance with the ethical standards of institutional and national research committees and with the Helsinki Declaration and its later amendments. The experiments were approved by JW CreaGene (certificate No. JWC-IRB-2014-01). Written, informed consent was obtained from all volunteers in advance.

Generation of the NLV peptide and overlapping peptides. The 9-mer peptide that spans the HLA-A*02:01 binding epitope of the HCMV pp65 protein (495–504; NLVPMVATV) was synthesized by JPT Peptide Technologies. The same company also generated 138 15-mer peptides that spanned the entire HCMV pp65 protein (Swiss Prot ID: P06725) and overlapped their neighbors by 11 amino acids. The NLV epitope is contained in full within peptides 123 and 124 (Table 1). Each peptide was diluted with DMSO to a concentration of 100 mg/ml. The reconstituted overlapping peptides (OLP) were then combined into a mixture, in which each peptide was present at a final concentration of 1 mg/ml. The NLV peptide was also diluted to a concentration of 1 mg/ml. The diluent in all cases was RPMI 1640 (Lonza, Verviers, Belgium).

Expansion of HCMV-specific T cells. In total 15–20 ml peripheral blood was collected from healthy volunteers. The PBMCs

were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare Life Sciences, NJ, USA), and 2 × 10^6 PBMCs were suspended in 1 ml RPMI 1640 supplemented with 3% autologous plasma, 2 mM Glutamax, and 50 µM 2-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA, USA). The cells were then placed in 14 ml polypropylene round-bottom tubes and pulsed with 1 µg/ml of the NLV epitope or 100 ng/ml of the OLP mixture. The cells were incubated at 37°C in a 5% CO_2 humidified incubator for 2 days, after which 1 ml fresh culture medium containing 100 IU/ml recombinant human (rh)IL-2 was added. On day 6, the culture medium was replaced with fresh medium containing the same concentration of rhIL-2. This was repeated every 2–3 days for a total of 14 days of incubation.

Flow cytometry analysis. To identify HCMV-specific CTLs in PBMCs or cell lines, the PBMCs/cell lines were stained with APC-anti-CD8 (clone RPA-T8; BD Pharmingen, San Jose, CA, USA; diluted 1:200) and PE-HCMV-pp65 495–504 pentamer (ProImmune, UK; diluted 1:200). For surface phenotype analyses, the cells were stained with anti-CD3 (clone: UCHT1), anti-CD14 (clone: M5E2), anti-CD19 (clone: HIB19), anti-CD56 (clone: B159), and anti-CD69 (clone: FN50) (all from BD Pharmingen; all diluted 1:200) on ice for 20 min, and then washed with PBS containing 1% bovine serum albumin (BSA). To determine the percentage of cells expressing interferon gamma (IFN-γ), the cells were washed and stained with APC-anti-CD8, and then fixed and permeabilized using an intracellular staining kit (BD Biosciences), and stained with FITC-anti-IFN-γ (clone: 4S.B3; BD Pharmingen; diluted 1:200). Flow cytometry was performed on a FACSCalibur instrument (BD Biosciences). All data were analyzed with FlowJo software (TreeStar Inc., San Carlos, CA, USA).

Cytotoxicity assay. To assess the cytotoxicity, the HCMV-specific CTLs were cultured with target cells (autologous BLCLs or HLA-matched T2 cells). To generate autologous BLCLs, donor PBMCs were cultured for 3 weeks in a 37°C CO_2 incubator with 1 µg/ml cyclosporine A and EBV-containing supernatant harvested from a marmoset cell line (B98-8). The autologous BLCLs and the HLA-A*02:01-expressing T2 cells were then pulsed overnight at 37°C in a CO_2 incubator with or without 10 µg/ml of the NLV peptide or OLP. The cells were plated in 96-well round-bottom plates at effector:target (E:T) ratios of 5:1, 10:1, and 20:1. After another 4 hours of incubation, the cells were pelleted by centrifugation, and the lactate dehydrogenase (LDH) activity in cell-free supernatants was measured by using the CytoTox96 non-radioactive assay kit (Promega, WI, USA) according to the manufacturer's protocol.

Statistical analysis. The data are expressed as the mean ± SEM. Statistical comparisons were performed using Student's *t*-tests or one-way analysis of variants (ANOVA) followed by Newman-Keul's tests. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were performed on GraphPad Prism V7.0 software (GraphPad Software, San Diego, CA, USA).

Results

Generation of HCMV pp65-specific CTLs

The PBMCs of eight volunteers expressing HLA*02:01 were first assessed for the presence of $CD8^+$ T cells that were specific for the HLA*02:01-presented NLV epitope of HCMV pp65 by staining with anti-CD8 and the HCMV-pp65 495–504 pentamer. Three donors (#2, #7, and #8) showed NLV epitope-specific CTLs in the PBMCs, ranging in frequency from 0.2% to 0.37% (Fig. 1a), but NLV-specific T cells were detected at a lower frequency (<0.2%) in the other donors. Based on the pentamer staining data, we selected those three donors (#2, #7 and #8) to use for the generation of NLV-specific T cells by stimulation with OLP. The HCMV-specific CTLs that were generated from these three donors with OLP that spanned the entirety of pp65 protein are referred to as OLP-T. The method that was employed is depicted in Fig. 1b. In five independent experiments using PBMCs from the same three donors, after 14 days of stimulation with OLP in the presence of IL-2, the number of NLV pentamer$^+$ $CD8^+$ T cells increased from 0.2–0.37% to 30% on average (Fig. 1c,d). Thus, by the end of the 14 day culture period, the population of NLV pentamer$^+$ $CD8^+$ T cells had expanded by 78.4-fold (Fig. 1d). These results show that the method depicted in Fig. 1b reliably and effectively expands HCMV-specific CTLs.

Cytotoxicity of the HCMV pp65-specific CTLs

In an independent set of experiments, HCMV pp65-specific CTLs from the same three donors (#2, #7, and #8) were generated using the NLV peptide and are referred to as NLV-T. The NLV epitope expanded NLV-specific CTLs as effectively as the OLP (Fig. 2a). Moreover, the NLV epitope and the OLP generated similar frequencies to IFN-γ^+ $CD8^+$ T cells on day 14 of culture (Fig. 2b). Thus, stimulation with OLP generated HCMV pp65 epitope-specific and IFN-γ-expressing $CD8^+$ T cells as efficiently as the epitope peptide itself.

The HCMV-specific cytotoxicity of all generated T cells was assessed using T2 cells or autologous BLCLs that had been pulsed with OLP or the NLV peptide as the targets. The three donors differed in terms of the cytotoxicity of their generated CTLs, irrespective of the peptide(s) used to generate them; for example, Donor 8-derived OLP-T killed NLV-loaded T2 cells (NLV-T2) more efficiently than Donor 2- and 7-derived OLP-T. Notably, OLP-T killed NLV-T2 almost as well as NLV-T (Fig. 2c). Moreover, OLP-T killed OLP-loaded autologous BLCLs (OLP-BLCL) as well as NLV-loaded autologous BLCLs (NLV-BLCL) (Fig. 2d). In addition, we generated OLP-T from the HLA-A*24:02-expressing donor (Donor 9). Donor 9-derived OLP-T exerted

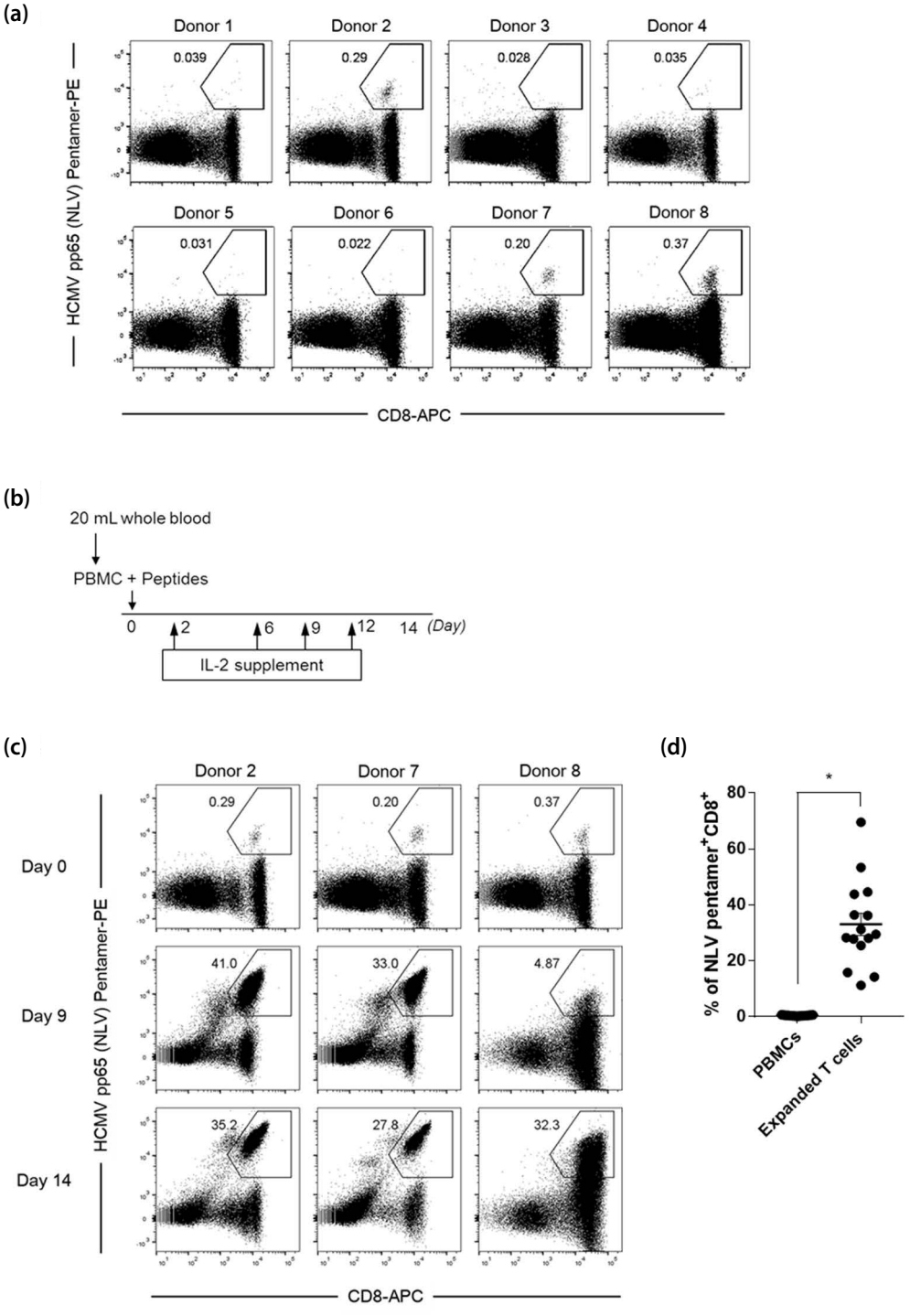

Fig. 1

Generation of HCMV pp65-specific CD8⁺ T cells using overlapping peptides

(a) The frequency of HCMV pp65 NLV-specific CD8⁺ T cells in peripheral blood mononuclear cells (PBMCs) of healthy volunteers. Blood samples were obtained from eight healthy HLA-A*02:01-expressing donors. The cells were stained with anti-CD8 and an HCMV pp65 NLV epitope pentamer, and analyzed by flow cytometry. **(b)** Schematic depiction of the method used to generate HCMV pp65-specific CD8⁺ T cells. **(c)** Change over time in the frequency of NLV-specific CD8⁺ T cells in PBMCs cultured with OLP as described in Fig. 1b. PBMCs from the subjects with high frequencies (>0.2%) of NLV-specific CD8⁺ T cells (Donors 2, 7, and 8) were stimulated with OLP, and CD8/pentamer double-positive cells were counted at days 0, 9, and 14 by flow cytometry. The data shown are representative of five independent experiments with each of the three donors. **(d)** Frequencies of NLV-specific CD8⁺ T cells on days 0 and 14, as determined by flow cytometry. The data from five independent experiments with each of the three donors are shown. *P <0.05.

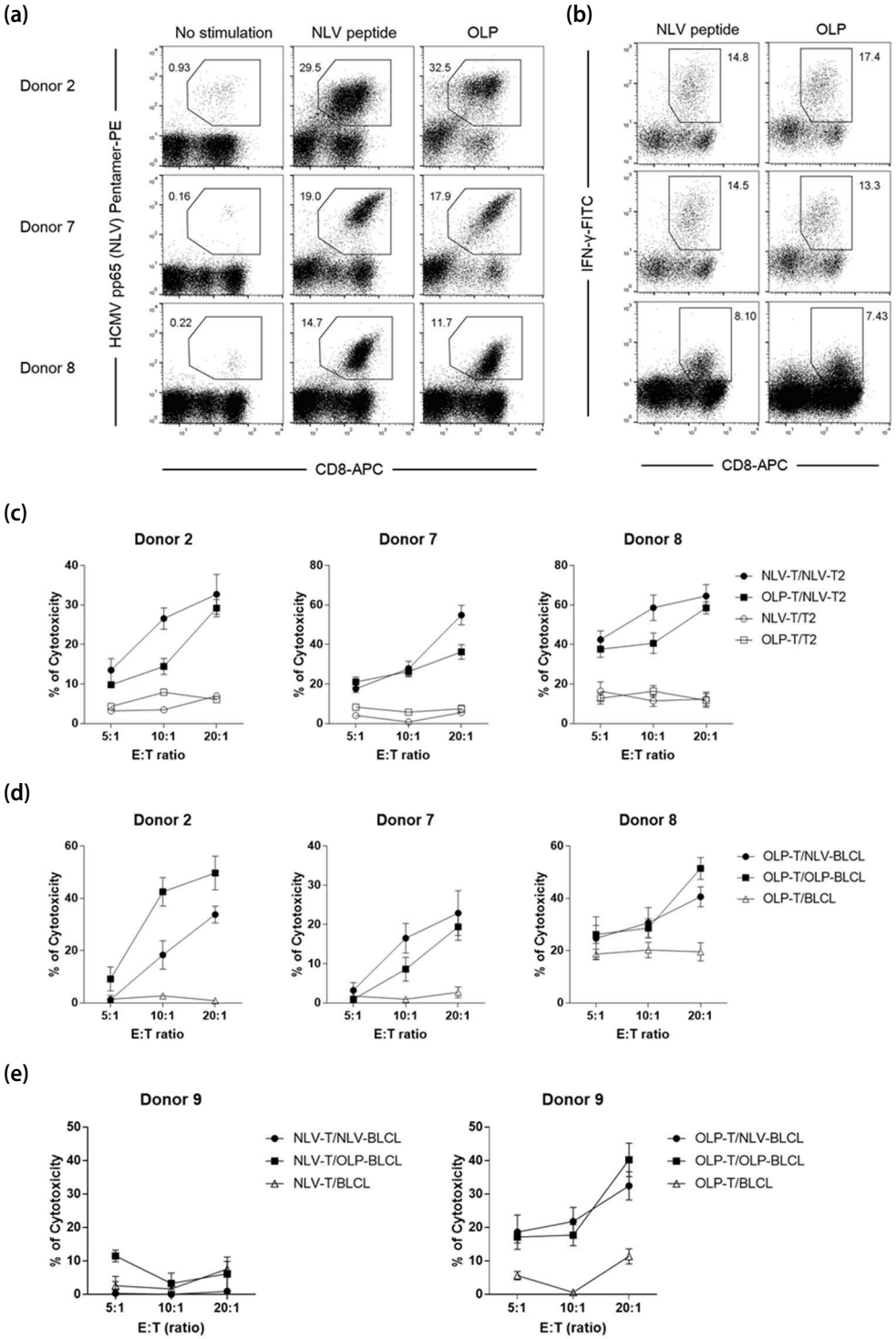

Fig. 2

Cytotoxicity of the HCMV pp65-specific CD8⁺ T-cells generated with OLP or the NLV peptide

(a) Frequency of NLV-specific CD8⁺ T cells generated from PBMCs from Donors 2, 7, and 8 by stimulation with the immunodominant NLV peptide or OLP. The cultured cells were analyzed at day 14 by flow cytometry using an anti-CD8 antibody and the HCMV pp65 pentamer. **(b)** The frequency of IFN-γ⁺ CD8⁺ cells was determined by flow cytometry. Data from a representative experiment are shown. **(c)** Cytotoxic activity against NLV-T2 cells. **(d)** The cytotoxic activity of OLP-T against either NLV-BLCL or OLP-BLCL. **(e)** Cytotoxic activity of CTLs generated from the PBMCs of an HLA-A*24:02-expressing donor using the NLV peptide or OLP. The cytotoxic activity of the CTLs against NLV-BLCL or OLP-BLCL was measured. Results are expressed as the mean ± SEM. **P* <0.05.

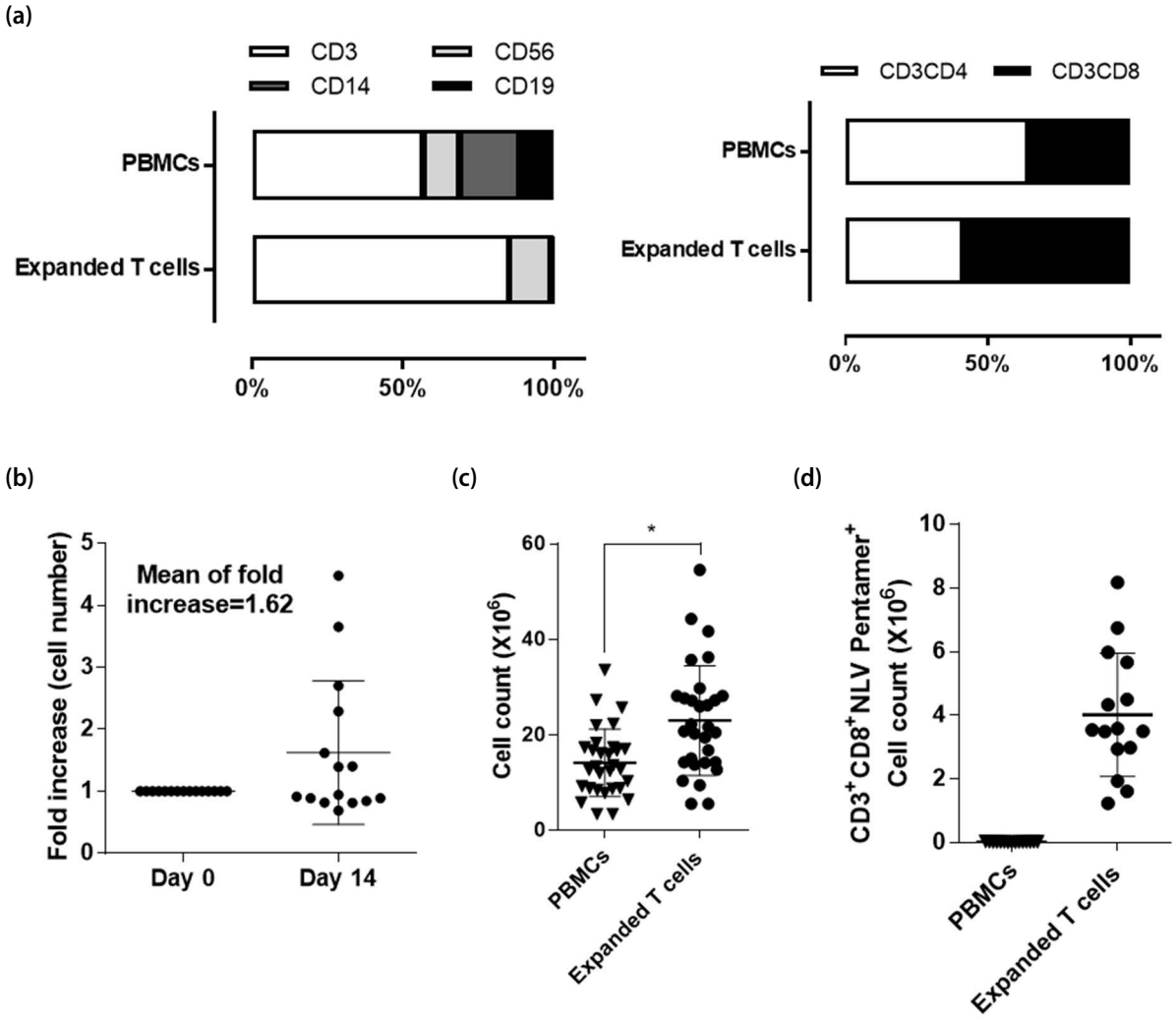

Fig. 3

Phenotypic analysis of the T cells

(a) Phenotypic analysis of freshly isolated PBMCs and the expanded T cells after 14 days of culture with OLP. The percentages of cells expressing CD3, CD4, CD8, CD56 (NK cells), CD14 (monocytes), and CD19 (B cells) was assessed by flow cytometry. *P <0.05. **(b)** The relative increase in $CD3^+$ T cells generated using the OLP (day 14) compared with the numbers in freshly isolated PBMCs (day 0). Data from five independent experiments with each of the three donors are shown. **(c)** Absolute numbers of PBMCs and expanded T cells 14 days after expansion with OLP. **(d)** Analysis of $CD3^+$ $CD8^+$ NLV pentamer$^+$ cells before and after culture with OLP for 14 days. Data from five independent experiments with each of the three donors are shown. *P <0.05, ***P <0.001.

cytotoxic activity against autologous NLV-BLCL and OLP-BLCL, but NLV-T did not (Fig. 2e). These results suggest that the OLP induced functional HCMV-specific CTLs from healthy donors bearing HLA-A*02:01 or HLA-A*24:02.

Phenotype of HCMV pp65-specific CTLs

The PBMCs and the corresponding expanded T cells generated using OLP were assessed for cell surface phenotype by flow cytometry. Compared with freshly isolated PBMCs, there was a marked increase in the frequency of $CD3^+$ $CD8^+$ T cells after 14 days of culture with OLP. In particular, there was a sharp increase in the frequency of $CD3^+$ $CD8^+$ T cells (from 21.5% to 53.5%). By contrast, the frequencies of $CD3^+$ $CD4^+$ T cells and $CD56^+$ NK cells did not change significantly, and the $CD14^+$ monocytes and $CD19^+$ B cells almost disappeared (0.5% and 0.8% at day 14, respectively) (Fig. 3a). Therefore, only $CD3^+$ $CD8^+$ T cells expanded in response to OLP. In addition, there was an increase in the frequency of activated $CD3^+$ T cells, as indicated by staining for CD69 (data not shown). When the PBMCs were cultured with OLP for 14 days, the number

of total cells increased on average by only 1.62-fold (Fig. 3B). Nevertheless, the numbers of $CD8^+$ T cells increased by 3.8-fold on average (data not shown). Even more strikingly, the numbers of HCMV pp65 pentamer$^+$ cells rose by an average of 127.6-fold (data not shown). On average, therefore, 20 ml blood yielded 1.4×10^7 PBMCs, and the protocol shown in Fig. 1b generated 2.3×10^7 $CD3^+$ T cells (Fig. 3c). In addition, the OLP stimulation induced a 308-fold increase in the numbers of NLV pentamer$^+$ $CD8^+$ T cells after 14 days compared with the numbers in freshly isolated PBMCs (Fig. 3d). These results suggest that OLP induces NLV-specific CTLs but not NLV-specific $CD4^+$ T cells.

Discussion

CMV disease continues to result in high mortality in recipients of T cell-depleted grafts subjected to prolonged immunosuppression. In particular, GvHD and delayed immune reconstitution after HSCT or solid organ transplants are associated with a significant risk of post-transplant infection or reactivation (Chan and Logan, 2017).

Several clinical trials have assessed the efficacy of adoptive immunotherapy with antigen-specific T cells in preventing HCMV disease after transplantation (Bao *et al.*, 2012). However, this treatment relies on standard *ex vivo* culture methods, whereby not only HCMV-specific CTLs, but also BLCLs or dendritic cells must be produced. This restriction has significantly hampered research into the effectiveness of this therapy in various patient groups (Ramadan, 2008). Therefore, a more rapid and effective method for generating HCMV-specific CTL is required.

We first performed experiments with PBMCs isolated from nine healthy donors, but only three donors were used for further experiments. Because the frequency of HCMV-specific T cells in healthy volunteers is very low and highly variable, the PBMCs were screened for NLV epitope-specific CTLs to verify the induction and proliferation of CTLs, and a frequency of at least 0.2% NLV epitope-specific $CD8^+$ T cells was used as a cut-off.

In this study, we established a culture method for producing HCMV-specific CTLs. In this process, PBMCs are stimulated for 14 days in the presence of IL-2 with OLP that span the HCMV pp65 protein. This method is independent of the separate production of APCs and eliminates the need for a viral expression system. As a result, it is simple, fast, and economical. Furthermore, the use of OLP as the antigen exposes the PBMCs to a variety of epitopes, which reduces the problem posed by HLA restriction. This method increased the number of HCMV pp65 pentamer$^+$ cells by more than 100-fold. Moreover, the peptide-expanded T lymphocytes exerted cytolytic activity. HCMV-specific T cells generated by OLP stimulation (OLP-T) killed an HLA-A*02:01-expressing cell line (T2 cells) loaded with NLV peptide (NLV-T2), as well as autologous BLCLs loaded with either the NLV peptide (NLV-BLCL) or OLP (OLP-BLCL). The cytolytic activity was specific for peptide- or OLP-loaded target cells, even at the highest the E:T ratio. In addition, we demonstrated the cytolytic activity of CTLs generated from an HLA-A*24:02-expressing donor. OLP-T generated from an HLA-A*24:02 donor killed NLV-BLCL and OLP-BLCL, but HCMV-specific T cells generated by NLV peptide stimulation (NLV-T) did not kill either NLV-BLCL or OLP-BLCL. These data suggest that stimulation with OLP induces antigen-specific T lymphocytes without HLA restriction.

In addition, on average, this protocol generated 2.3×10^7 T cells from 20 ml blood. In general, $1–10 \times 10^7$ T cells are transferred in adoptive therapy (Cobbold *et al.*, 2005; Rauser *et al.*, 2004; Robbins *et al.*, 2015). Thus, using this protocol, it is possible to derive the number of cells needed for adoptive therapy. Therefore, this protocol is suitable for further research on the merits of adoptive immunotherapy after HSCT.

More research is needed to determine whether CTLs can be derived from HCMV antibody-negative donors. It is also necessary to determine whether specific CTLs can be generated against the other viruses that cause complications after transplantation, including herpes simplex virus, EBV, varicella zoster virus, and adenovirus. The protocol we describe here is likely to be useful for selectively inducing CTL that are specific for these viruses as well. It may also be useful for preparing tumor-specific T cells.

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