Characterization of In Vitro Expanded Virus-Specific T cells for Adoptive Immunotherapy against Virus Infection

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INTRODUCTION
Viral infections are major causes of severe morbidity and mortality in immunocompromised hosts, such as patients undergoing hematopoietic cell transplantation (HCT) or patients with AIDS. Cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (AdV) infections, which are frequently asymptomatic in immunocompetent hosts, are potentially life-threatening. Adoptive transfer of virus-specific T cells as immunotherapy against some viral infections has become an effective preventive and therapeutic treatment following allogeneic HCT (1–4). Virus-specific T cells can be expanded in vitro from donor peripheral blood mononuclear cells (PBMCs) following stimulation with overlapping peptides for viral proteins of interest (5,6). The virus-specific T cells were stimulated with these peptides and virus-specific CD4+ and CD8+ T cells were expanded in vitro in the presence of interleukin (IL) 4 and IL7. A median of 13 (minimum–maximum, 2–46) peptides was recognized in the cohort. Both fresh and cryopreserved PBMCs were used for in vitro expansion. The expansion and breadth of T cell responses were not significantly different between the 2 PBMC sets. We assessed viral regions frequently recognized by T cells in a Japanese cohort that could become pivotal T cell targets for immunotherapy in Japan. We tested epitope prediction for CD8+ T cell responses against a common target region using a freely available online tool. Some epitopes were considered to be predictive.

SUMMARY: Adoptive transfer of virus-specific T cells has emerged as a promising therapeutic approach for treatment of virus infections in immunocompromised hosts. Characterization of virus-specific T cells provides essential information about the curative mechanism of the treatment. In this study, we developed a T cell epitope mapping system for 718 overlapping peptides spanning 6 proteins of 3 viruses (pp65 and IE1 from cytomegalovirus; LMP1, EBNA1, and BZLF1 from Epstein-Barr virus; Penton from adenovirus). Peripheral blood mononuclear cells (PBMCs) from 33 healthy Japanese donors were stimulated with these peptides and virus-specific CD4+ and CD8+ T cells were expanded in vitro in the presence of interleukin (IL) 4 and IL7. A median of 13 (minimum–maximum, 2–46) peptides was recognized in the cohort. Both fresh and cryopreserved PBMCs were used for in vitro expansion. The expansion and breadth of T cell responses were not significantly different between the 2 PBMC sets. We assessed viral regions frequently recognized by T cells in a Japanese cohort that could become pivotal T cell targets for immunotherapy in Japan. We tested epitope prediction for CD8+ T cell responses against a common target region using a freely available online tool. Some epitopes were considered to be predictive.
MATERIALS AND METHODS

Study population: PBMCs were obtained from 33 Japanese healthy volunteers of unknown CMV, EBV, and AdV sero-status. All participants gave written informed consent. The study was approved by the institutional review boards of the National Institute of Infectious Diseases (574), the Institution of Medical Science at the University of Tokyo (25-73-0310), and Tokyo Medical Dental University (1428).

HLA typing: HLA class I typing was performed by Luminex assays (WAKFlow HLA Typing Kit, Wakanaga Pharmaceutical, Osaka, Japan).

Peptides: We used a commercially available mixture of 15-mer overlapping-peptides (OLPs), overlapping by 11 amino acids spanning pp65 and IE-1 in CMV; EBNA1, BZLF1, and LMP1 in EBV; Penton in AdV (JPT Peptide Technologies, Berlin, Germany) for in vitro stimulation of PBMCs. The number of peptides that covers each protein was as follows: 138 (OLP1-138) for pp65, 120 (OLP139-258) for IE1, 122 (OLP259-380) for LMP2, 139 (OLP381-519) for EBNA1, 59 (OLP520-578) for BZLF1, and 140 (OLP579-718) for Penton. For epitope mapping, the same OLPs included in the peptide mix were synthesized (Eurofin Genomics, Tokyo, Japan), and working solutions were prepared for each peptide.

Design of peptide matrices: All 718 OLPs from 6 viral proteins were tested in a peptide pool matrix to facilitate comprehensive screening of T cell target as previously described (10,11). The OLPs were included in 3 different peptide matrix systems in this study. Within a given matrix, each peptide was represented in 2 different peptide pools, allowing for the identification of the respective peptide by responses in the 2 corresponding pools.

In vitro expansion of PBMCs with overlapping peptides: Fresh or cryopreserved PBMCs were stimulated with peptide mix spanning all 6 viral proteins (n = 718 OLPs) at a final concentration of 100 ng/mL for each peptide, and cultured for 2–3 weeks in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine (R10) with 800 U/mL interleukin (IL)4 and IL7 were used in the expansion medium. These cytokines induce efficient expansion of polyclonal, Th1-polarized virus-specific T cells in both CD4+ and CD8+ T cell subsets (5). The median cell expansion (fold-increase of cell number after stimulation) was 3.8 (minimum–maximum, 1.4–9). Both fresh (n = 18) and cryopreserved PBMCs (n = 15) were used for in vitro expansion. Cell expansion was not significantly different between fresh and cryopreserved PBMCs (median, 13.5–16 days) to expand virus-specific T cells. Because CMV-specific CD4+ T cells are important for the persistence of adoptive transfer of CD8+ T cells in the recipient (15), as in the case of EBV-specific T-cell therapy (3), IL4 and IL7 were used in the expansion medium. These cytokines induce efficient expansion of polyclonal, Th1-polarized virus-specific T cells in both CD4+ and CD8+ T cell subsets (5). The median cell expansion (fold-increase of cell number after stimulation) was 3.8 (minimum–maximum, 1.4–9). Both fresh (n = 18) and cryopreserved PBMCs (n = 15) were used for in vitro expansion. Cell expansion was not significantly different between fresh and cryopreserved PBMCs (median, 4.1 [minimum–maximum, 1.7–9] and 3.5 [minimum–maximum, 1.4–7.7], respectively; p = 0.15; Fig. 2A).

Epitope mapping of virus-specific T cells: To assess target regions of the virus-specific T cells, peptide matrix approaches have been employed (10,11). We performed the ELISpot assay on the in vitro expanded T cells using matrices consisting of the OLPs from these viral proteins. The peptides located at the intersection of rows and columns with a positive response were subjected to the single-OLP-based ELISpot assay to determine reactive peptide(s). Among 718 OLPs from 6 viral proteins, 283 OLPs (39.4%) were recognized by PBMCs from at least single subject of the 33 donors. T cell epitopes restricted by HLA class I are 8 to 11 amino acids long (16), most of which are included in the overlapping single peptides in the presence of costimulatory antibodies (anti-CD28 and anti-CD49d at 1 μg/mL) and momecin (GolgiStop; BD Biosciences, Franklin Lakes, NJ, USA). Following incubation for 6 h, ICS was performed as previously described (12). Dead cells were removed by LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoScientific, Tokyo, Japan). Fluorochrome-conjugated monoclonal antibodies to CD3, CD8, and IFN-γ (BioLegend, San Diego, CA, USA) were used. IFN-γ-producing cells greater than 0.05% of T cells were considered as positive responses. Data were collected on a FACSCanto II or Fortessa flow cytometer (BD Biosciences), and analyzed using FlowJo 9.9.3 software (TreeStar, Ashland, OR, USA).

Epitope prediction: The HLArestrictor-1.2 T cell epitope prediction tool that is available on the internet was utilized (13).

Statistical analysis: Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Mann-Whitney test were used for all analyses. A P-value < 0.05 was considered statistically significant.

RESULTS

HLA typing: We performed HLA class I typing for all samples used in this study. The allele frequency in the current study (n = 33) and in a Japanese population (n = 18,604) are shown in Fig. 1. The results indicate that the HLA distribution of our samples represents the Japanese population.

In vitro expansion of virus-specific T cells: PBMCs from 33 Japanese healthy donors were stimulated in vitro with peptides spanning 6 viral proteins from CMV (pp65, IE1), EBV (LMP2, EBNA1, BZLF1), and AdV (Penton) for 2–3 weeks (median 15 days, IQR: 13.5–16 days) to expand virus-specific T cells. Because CMV-specific CD4+ T cells are important for the persistence of adoptive transfer of CD8+ T cells in the recipient (15), as in the case of EBV-specific T-cell therapy (3), IL4 and IL7 were used in the expansion medium. These cytokines induce efficient expansion of polyclonal, Th1-polarized virus-specific T cells in both CD4+ and CD8+ T cell subsets (5). The median cell expansion (fold-increase of cell number after stimulation) was 3.8 (minimum–maximum, 1.4–9). Both fresh (n = 18) and cryopreserved PBMCs (n = 15) were used for in vitro expansion. Cell expansion was not significantly different between fresh and cryopreserved PBMCs (median, 4.1 [minimum–maximum, 1.7–9] and 3.5 [minimum–maximum, 1.4–7.7], respectively; p = 0.15; Fig. 2A).
A specific T cell response was not detected against OLP87 in the ICS analysis, probably due to low sensitivity of this assay (low positive response was observed in the ELISpot as well). Of 33 Japanese healthy donors, both CD4+ and CD8+ T cell responses were detected against all 6 viral proteins tested (Fig. 3D). The most common target peptide was OLP123 AGILARNLVPM-ATV, located at amino acid position 489–503 in CMV pp65. The in vitro expanded T cells from 14 of 33 (42%) donors targeted OLP123 (Fig. 3D). Of 12 out of 14 responders for which ICS analysis was carried out, 3 of 12 and 6 of 12 donors showed CD4+ and CD8+ T cell responses, respectively. The other 3 donors had both CD4+ and CD8+ T cell responses, indicating that this peptide includes both HLA class I- and II-restricted epitopes.

The well-known pp65 epitope, NLVPMVATV, which is restricted by HLA-A*02:01 (17), is incorporated in OLP123. Eight of 9 donors with CD8+ T cell responses expressed HLA-A*02:01 or A*02:06, suggesting that a part of the CD8+ T cell responses are specific for the epitope. Although some HLA-DR-restricted epitopes have also been reported in this peptide (9), these alleles are rare in the Japanese population (14). In addition, no
Characterization of Virus-Specific T Cells

A) Single-OLP-based ELISpot was performed for all positive wells in the ELISpot using peptide matrices. Data from donor-14 are shown. B) Breadth of T cell responses in 33 donors. Graph legends correspond for both A and B. C) Representative FACS profile of IFN-γ intracellular staining. Data for donor-14 is shown. D) Number of responses for each OLP. CD4+ and CD8+ T cell responses are black and dark gray, respectively. The responses where T cell subset type could not be determined are shown in light gray.

Fig. 3. Virus-specific T cell responses in in vitro expanded T cells. A) Single-OLP-based ELISpot was performed for all positive wells in the ELISpot using peptide matrices. Data from donor-14 are shown. B) Breadth of T cell responses in 33 donors. Graph legends correspond for both A and B. C) Representative FACS profile of IFN-γ intracellular staining. Data for donor-14 is shown. D) Number of responses for each OLP. CD4+ and CD8+ T cell responses are black and dark gray, respectively. The responses where T cell subset type could not be determined are shown in light gray.
responder expressed any of these HLA-DRB1 molecules (data not shown), implying that epitopes in this peptide are restricted by an as-yet-unknowm HLA class II molecule(s). Regions covered by sequential OLPs recognized by more than 5 donors are listed in Table 1.

**Epitope prediction:** Accumulating information on epitopes and their HLA restriction has enabled the development of epitope prediction tools (13,18,19). The regions covered by OLP26-31, SICPSQEPMSVYALPLKNLNPVATVQGQN, located at 101–135 in pp65, were targeted by 19 T cell responses in 12 donors, in which 10 of 19 were CD8+ T cell responses (Table 1). All CD8+ T cell responses from 7 donors are shown in Fig. 4A. We performed epitope prediction of peptides within the OLP26-31 region by HLArestrictor-1.2 (13). Predicted epitopes restricted by the HLA class I alleles expressing in the responders are shown in Fig. 4B. Donor-3, -7, and -16, all expressing HLA-B*51:01, had CD8+ T cell responses against OLP28, in which the HLA-B*51-restricted epitope LPLKMLNI has been reported (20). Similarly, OLP30-31 targeted by PBMCs from donor-1 and donor-3 expressing HLA-B*35:01 included HLA-B*35:01-restricted epitope, IPSINVHHY, which has also been previously reported in the literature (21). Both epitopes were predicted as a strong binder by HLArestrictor (Fig. 4B). These data suggest that the responses to OLP28 and OLP30 are expected to be against the reported epitopes, though we cannot exclude possibility that the responses targeted other unknown peptides. CD8+ T cell responses against OLP26-27 were detected in 3 donors (donor-10, -16, and -19) sharing HLA-B*54:01 and HLA-C*01:02 (Fig. 4A). The epitopes restricted by HLA-B*54:01 and -C*01:02 were predicted in either OLP26 or OLP27 (EPMSIYVYA and EPMSIYYVAL for HLA-B*54:01 and -C*01:02) (Fig. 4B). Other CD8+ T cell responses detected in this study, responses against OLP29 in donor-2 and donor-10, also had predictive epitopes restricted by HLA expressing the responders in the target OLP (HLA-A*24:02-restricted VY ALPLKMLNI and HLA-B*54:01-restricted LPLKMLNI, LPLKMLNIPS, and LPLKMLNIPS).

**DISCUSSION**

Adoptive transfer of donor-derived, virus-specific T cells has been demonstrated to be an effective strategy to control CMV, EBV, and AdV infections after allogeneic HCT (4, 22–25). Peptide mix spanning multiple viral proteins has the advantages of stimulating virus-specific T cells and eliciting broad virus-specific T cell responses regardless of HLA phenotype. However, there are some difficulties in characterizing these responses with respect to quality, breadth of the responses, and HLA restriction. Cellular immune response by cytotoxic T lymphocytes (CTL) is a critical defense mechanism against virus infection. Both the magnitude and breadth of T cell responses are important for virus control (26,27). In HIV infection, broader T cell responses against Gag, a highly conserved region, but not other viral proteins that tolerate amino acid change, are associated with viral control (26), suggesting that target region of virus-specific T cells is critical for T cell mediated anti-viral activity. Although the genetic stability of the viral genome is much higher in DNA viruses compared to RNA viruses, such as HIV and HCV, escape mutants from CTL recognition by amino acid substitutions in CTL epitopes or dele-

### Table 1. Common target regions of in vitro expanded virus-specific T cells in Japanese population

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<tr>
<th>virus</th>
<th>protein</th>
<th>OLP aa position</th>
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<th>CD4</th>
<th>CD8</th>
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<td>pp65</td>
<td>5-6</td>
<td>17-35 PISGHVLKA VFSRGDTPVL</td>
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1): undetermined.
Characterization of Virus-Specific T Cells

A) CD8+ T cell responses, measured by ELISpot, against OLP26-31. All T cell responses that were confirmed as CD8+ T cell responses by IFN-γ–IFC are shown. Alleles in HLA-A, B, and C loci for each donor are shown. B) Epitopes predicted by HLArestrictor-1.2. The peptides predicted as “strong binder (%) Rank is less than 0.5) to the cognate HLA” are shown. “Affinity” shows predicted binding affinity (IC50 [nM]) to the cognate HLA molecule. NA, not assessed. The references are shown if the epitope has been reported.

Fig. 4. Epitope prediction. A) CD8+ T cell responses, measured by ELISpot, against OLP26-31. All T cell responses that were confirmed as CD8+ T cell responses by IFN-γ–IFC are shown. Alleles in HLA-A, B, and C loci for each donor are shown. B) Epitopes predicted by HLArestrictor-1.2. The peptides predicted as “strong binder (% Rank is less than 0.5) to the cognate HLA” are shown. “Affinity” shows predicted binding affinity (IC50 [nM]) to the cognate HLA molecule. NA, not assessed. The references are shown if the epitope has been reported.

Although epitope databases and prediction tools provide useful information, genes of the HLA are the most variable coding loci in the human genome, and the allele distribution is ethnically variable. These tools are less informative for rare HLA alleles in well-studied populations, such as Caucasoids. The prevalence of HLA-B*54:01 is much higher in Asia including Japan where the allele frequency is 7.63%, the 6th most prevalent HLA-B allele. However, HLA-B*54:01 is very rare in Caucasian and African populations (less than 0.1% in both ethnicities) (14), and there is no HLA-B*54:01-restricted epitope information in CMV, EBV, and AdV in an epitope database (9). In the case of Perton in AdV, only 3 epitopes have currently been reported. Accumulation of epitope information would improve the accuracy of epitope prediction tools. The accumulation from broad geographical regions might be useful for clinical application of the T cell immunotherapy for virus infection.

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Conflict of interest  None to declare.

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