Rapid expansion of cytomegalovirus-specific cytotoxic T-lymphocytes by artificial antigen presenting cells expressing a single HLA allele.

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Running title: Expansion of CMV-specific CTLs by AAPCs processing pp65

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Abstract
Cytomegalovirus (CMV) is a major threat in patients undergoing allogeneic bone marrow transplantation. The adoptive transfer of CMV-specific cytotoxic T lymphocytes (CTLs) expanded from the blood of CMV-seropositive donors has been shown to effectively control CMV infection. However, the requirement for safe and effective antigen-presenting cells (APCs) for each patient precludes broad applicability of this successful form of therapy. Here we analyze the ability of artificial APCs (AAPCs) to activate and expand CMV-specific CTLs from peripheral blood of seropositive HLA A2.1+ donors. We demonstrate that AAPCs expressing the CMV P495 peptide or the full-length pp65 protein stimulate P495-specific CTLs at least as effectively as autologous, peptide-pulsed, peripheral blood mononuclear cells or EBV transformed B-cells. Starting from 100ml of blood, the AAPCs reliably yield clinically relevant CTL numbers after a single stimulation. CTLs activated on AAPCs effectively kill CMV-infected fibroblasts, and have a Tc1 memory effector phenotype, identical to that of CTLs generated with autologous APCs. AAPCs thus offer a rapid, controlled, convenient and highly reproducible system for expanding CMV-specific CTLs. Furthermore, the CTL expansion obtained with AAPCs encoding full length pp65 indicate that AAPCs may be used to present known as well as unknown CTL epitopes in the context of the AAPC’s HLA.

Introduction
The reactivation of latent cytomegalovirus (CMV) is a major cause of morbidity and mortality in allogeneic bone marrow transplantation (BMT) recipients. In spite of the availability of effective antiviral agents, the survival of CMV infected patients remains less than that of seronegative patients. The risk of developing CMV disease is increased in recipients of matched unrelated or mismatched grafts, T-cell depleted grafts, and in patients with graft versus host disease (GVHD). Viral reactivation is directly related to the deficiency in CMV-specific cytotoxic T lymphocytes (CTLs). The adoptive transfer of CMV-specific CTLs can rapidly restore immunity and has proven to be safe and efficacious for the prophylaxis or treatment of CMV complications. However, the process whereby virus-specific CTLs are generated stands as a major obstacle to the broad applicability of this form of therapy, which requires the generation of antigen presenting cells (APCs) and CTLs on an individual patient basis.
The ex vivo activation and expansion of CMV-specific CTLs restricted to the recipient's MHC class I molecules require that CMV antigens be presented by an MHC-matched antigen-presenting cell (APC). Several APCs, using various antigen sources, have been successfully used for expanding CMV-specific CTLs. These include autologous skin fibroblasts infected with live CMV\(^{10,11}\), Epstein-Barr virus (EBV)-transformed B cells (BLCLs) retrovirally transduced to express CMV antigens\(^{12}\), and immature monocyte-derived dendritic cells (DCs) pulsed with CMV antigen derived from a CMV infected human lung fibroblast cell line\(^{13}\). While highly effective, these methodologies nonetheless have crucial limitations. The first requires the establishment of an autologous fibroblast culture and entails the manipulation of live CMV stocks; the second requires the immortalization of B cells with a transforming virus (EBV) for every patient; the third requires the repeated establishment of PBMC cultures and entails the use of poorly defined viral extracts. Thus, in addition to the time and labor involved in the preparation of autologous DCs or BLCLs, these approaches raise various concerns as to their safety, antigen-specificity, reproducibility and logistics out of the context of a highly specialized clinical environment. The availability of immortalized, MHC-matched APCs would therefore be extremely useful in providing a stable, virus-free system for expanding CMV-specific CTLs.

In the present study, we evaluated the efficacy of artificial APCs (AAPCs) that express a single HLA class I molecule to expand CMV-specific CTLs and compared it to that of autologous APCs. The AAPCs were derived from murine fibroblasts and stably expressed, in addition to a specific HLA-peptide complex, the human co-stimulatory molecules B7.1 (CD80), ICAM-1 (CD54), and LFA-3 (CD58)\(^{14}\). To efficiently present specific HLA-peptide complexes to CTLs, the single HLA molecule HLA A2.1 was co-expressed with human ß2 microglobulin and a single, genetically encoded antigen, either the full length CMV matrix protein pp65 (which is recognized by more than 70% of CMV specific CTLs\(^{15,16}\)) or the pp65-derived, HLA A2.1-restricted immunodominant peptide P495\(^{17,18}\). We focused on the HLA A2.1\(^+\) phenotype because of its high frequency in all ethnic groups\(^{19}\). We compared autologous EBV-transformed B cells and erythrocyte-rosette-negative (ER) PBMCs to AAPCs for their ability to activate and expand CMV-specific CTLs from peripheral blood T lymphocytes harvested from HLA A2.1\(^+\), CMV seropositive donors. We demonstrate that AAPCs efficiently expand P495-specific CTLs, at least as well as the autologous APCs. These CTLs have a Tc1 cytokine profile and an effector memory phenotype. In comparison to both autologous APCs, the AAPCs give the highest yield
of P495-specific CTLs after a single round of stimulation. Interestingly, AAPCs expressing the whole pp65 protein efficiently presented P495, suggesting that the AAPCs could be used for presentation of known or unknown peptides in different HLA backgrounds. Finally, we demonstrate that P495-specific CTLs, which account for 10 to 50% of the CD8+ T cells after a single round of stimulation, can be efficiently enriched using tetramers (to 95% purity or more), and retain their cytolytic activity. AAPCs thus offer a very convenient and rapid system for the specific activation and expansion of CMV-specific CTLs for adoptive immunotherapy.

Materials and Methods

T cell preparation

Approval for the study was obtained from the Memorial Sloan Kettering Institutional Review Board. Peripheral blood (100ml) was collected from healthy donors after obtaining informed consent. CMV serostatus was determined by serologic techniques in the Clinical Microbiology Laboratory at MSKCC. HLA genotyping was established by PCR in the HLA Typing Laboratory at MSKCC. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on lymphocyte separation medium (Accurate Chemical & Scientific Corporation, Westbury, NY). Highly purified populations of T-cells were prepared by positive selection using sheep red blood cells (Colorado serum company, Denver, Co) as described.

After lysis of sheep red cells, the erythrocyte-rosette positive (ER+) fraction was washed with phosphate buffered saline (PBS) with 2% fetal calf serum (FCS). If CD56 cells comprised >2% of the ER+ fraction, depletion of B cells, activated T cells, natural killer cells and monocytes macrophages was done by panning as described.

Preparation of antigen presenting cells

The construction of AAPCs restricted to HLA A2.1 (AAPC A2) has been described. The pp65 sequence was kindly provided by Dr. N. Cereb and cloned into the SFG vector as shown in Figure 1. The P495 vector was generated by fusing the sequence encoding P495 (NLVPMVATV) to the human CD8α sequence as previously described. After selection with puromycin (10μg/ml), expression of pp65 was verified by indirect immunofluorescence using the Light Diagnostics CMV pp65 Antigenemia Immunofluorescence Assay (Chemicon International, Inc, Temecula, Ca) according to the manufacturer’s instructions. Expression was
maintained under selective pressure with puromycin (10µg/ml). AAPC\textsuperscript{A2}, which do not express any relevant immunogenic antigen, were used as negative control in all experiments.

ER\textsuperscript{PBMCs} were prepared as described for T cell preparation by negative selection using sheep red blood cells. ER\textsuperscript{PBMCs} are highly enriched in B cells, monocytes and macrophages.

EBV-transformed B-cell lines (BLCLs) were generated from PBMCs (2\times10^6) infected with viral supernatant for 2 hours at 37\textdegree C. The B95-8 EBV producing line was used for culture of EBV. PBMCs were cultured in RPMI (Mediatech) with 10\% FCS and cyclosporin A at a concentration of 2µg/ml in 24-well plates. The BLCLs were expanded in 25cm\textsuperscript{2} and 75 cm\textsuperscript{2} flasks over a period of 4 to 6 weeks. BLCLs expressing pp65 (BLCL\textsuperscript{pp65}) or the HLA A2.1-restricted influenza matrix protein derived immunodominant peptide (BLCL\textsuperscript{flu}) were generated by transduction with recombinant retroviruses pseudotyped with the gibbon ape leukemia virus envelope as described\textsuperscript{22}. Selection of the transduced cells was performed using puromycin at a concentration of 1µg/ml.

**Peptide synthesis**

Peptides were synthesized at the peptide synthesis facility at MSKCC or Research Genetics (Invitrogen, Carlsbad, Ca). Peptides were resuspended in 50\% (vol/vol) RPMI-dimethylsulfoxide (Sigma) and stored at −20\textdegree C. The HLA A2.1-restricted peptides used in this study were the pp65-derived P495 (NLVPMVATV) and the influenza matrix protein-derived peptide flu (GILGFVFTL).

**Tetrameric HLA A2.1 peptide complexes**

Soluble MHC peptide tetramers for staining of epitope-specific cells were generated as previously described\textsuperscript{23}, at the MSKCC tetramer core facility.

**Activation of CMV-specific CTLs**

Cocultures of T cells with antigen presenting cells, were established in 24-well plates using 0.75 to 1\times10^6 donor T cells/well as responders. Interleukin-2 (IL-2, Chiron, St Louis, Mo) was added to the coculture after 7days (20 IU/ml) and every third day thereafter. The same supplementation with IL-2 was used for AAPC and autologous presenting cells. Specific conditions were as follows: Coculture of AAPC with donor T cells has been described\textsuperscript{14}. AAPCs were maintained in culture in Dulbecco’s modified Eagle medium (DMEM; Mediatech, Herndon, VA), with 10\% donor calf serum (DCS, Hyclone, Logan, UT). One day prior to the beginning of the coculture AAPCs were plated at 10^5cells/well in 24-well plates in 0.5ml AIM-V medium supplemented
with 5% DCS. The following day the monolayer of AAPP was irradiated (1,500 Gy) prior to addition of 1.0x10^6 cells/well donor T cells in 0.5ml AIMV medium. ERPBMCs were prepared fresh for each coculture. ERPBMCs were pulsed with P495 peptide at a concentration of 100µg/ml for 2 hours at room temperature. Autologous BLCLs were pulsed with P495 peptide at a concentration of 100µg/ml for 2 hours at room temperature and irradiated (12,000 Gy).

**Cytotoxicity assays**

Cytotoxicity was tested in triplicate in standard ^{51}Cr release assays. Transfer associated with antigen processing (TAP) protein-deficient HLA A2.1^+ T2 cells were loaded with the peptide (10µM, 2 hours at room temperature in RPMI without serum) before pulsing with ^{51}Cr for 1 hour at 37°C. Autologous or allogeneic mismatched HLA A2.1^+ BLCL^{pp65} or BLCL^{flu} were pulsed with ^{51}Cr for 2 hours. The human fetal lung HLA A2.1^+ cell line MRC5 (ATCC) was cultured in the presence of 100 units/ml IFN-γ for 24 hours prior to infection with supernatant of CMV AD169 overnight and subsequent labeling with ^{51}Cr for 2 hours. Uninfected MRC5 were used as controls. A total of 2500 targets per well were used in 96 well plates at different effector to target cell (E:T) ratios for 4 hours. Specific ^{51}Cr release was calculated using the formula \[\left(\frac{^{51}Cr \text{ release - spontaneous release}}{\text{maximum release - spontaneous release}}\right) \times 100.\]

**Phenotyping of CTLs**

For intracellular cytokine staining, after 10 to 14 days of coculture, 10^6 CTLs/ml of RPMI were incubated for 5 hours at 37°C on 24-well flat-bottom plates containing a fresh monolayer of AAPP^{A2P495} or coated with anti-CD3 mAb (10µg/ml) with 1mg/ml brefeldin A (BD Pharmingen, San Diego, Ca). Cells were fixed by incubation with Cytofix/Cytoperm (BD Pharmingen) at 4°C for 20 minutes. Thereafter cells were washed twice with Perm/Wash buffer (BD Pharmingen) and stained at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti IFN-γ, FITC-conjugated anti TNF-α or FITC conjugated IgG isotype control. Finally cells were washed with 1X Perm/Wash buffer and resuspended in PBS with 2% FCS for flow cytometric analysis. Isotype matched control of irrelevant specificity at concentration equal to anti cytokine antibody (<0.5 µg/10^6 cells) was used as negative control.

For immunophenotyping, CTLs were stained using standard methods with phyco-erythrin (PE)- and or FITC-labeled antibodies for the surface molecules and analyzed by flow cytometry (BD). At least 10000 events in the gate were acquired for the analysis. Isotype-specific negative controls were included in all experiments. Antibodies against the following molecules were
used: CD3, CD4, CD45RA, CD8, CD62L (Pharmingen), CCR7, CXCR4 (R&D Systems Minneapolis, MN), all FITC conjugated. HLA A2.1 peptide tetrameric complexes were PE-conjugated. The limit of detection for tetramer positivity was 0.1%. Controls for tetramer specificity included staining with irrelevant (flu) tetramer as well as staining of cells activated on “empty” AAPC<sup>A2</sup> with the P495 tetramer.

**Purification and expansion of P495-specific CTLs using P495-tetramer and anti-PE microbeads.**

After one round of stimulation on AAPC<sup>A2</sup>P<sub>495</sub> or AAPC<sup>A2</sup>p<sub>65</sub>, T cells were washed twice and incubated with tetramer for 20 minutes, in PBS with 2% FCS at 4°C. One μl of P495-tetramer was used for 1 million T cells in a volume of 100μl. After washing twice the cells in PBS with 2% FCS at 4°C, the anti-PE microbeads were used according to the manufacturer’s instructions (Miltenyi, Auburn, CA), maintaining the cells at 4°C. Right after purification, negative and positive fractions of T cells were cocultured with AAPC<sup>A2</sup>P<sub>495</sub> or AAPC<sup>A2</sup>p<sub>65</sub> for 7 to 10 days at a concentration of 500,000 cells per well in 24-well plates, prior to performing tetramer staining and cytotoxic assays. IL-2 (20 IU/ml) was added at the start of the coculture and every third day afterwards.

**Results**

**AAPCs efficiently stimulate CMV-specific cytotoxic T cells.**

Our first objective was to establish whether AAPCs expressing HLA A2.1 and CMV antigens could stimulate CMV-specific CTLs. First we generated AAPC<sup>A2</sup>P<sub>495</sub> using a replication-defective bicistronic retroviral vector encoding P495 and puromycin-N-acetyltransferase (Figure 1). To investigate whether AAPCs can process the full-length pp65 protein and present the P495 peptide in the context of HLA A2.1, we also generated AAPC<sup>A2</sup>p<sub>65</sub> by retroviral transduction with another vector encoding pp65 (Figure 1). By immunofluorescence, more than 95% of the AAPCs stained strongly positive for pp65 after selection with puromycin (data not shown).
We next compared the efficiency of AAPC\textsuperscript{A2P495} and AAPC\textsuperscript{A2pp65} to stimulate CMV-specific CTLs. A representative example is shown in Figure 2. Starting from peripheral blood T cells harvested from HLA A2.1\textsuperscript{+} CMV seropositive donors, 15\% and 36 \% of the T cells stained positive with P495 tetramers after 10 to 14 days of coculture on AAPC\textsuperscript{A2P495} and AAPC\textsuperscript{A2pp65} respectively (Figure 2, upper panel). These CTLs efficiently lysed T2 cells pulsed with the P495 peptide (Figure 2, lower panel). The non-specific killing of T2 cells pulsed with a control peptide (flu) was consistently low, even at the highest E:T ratios.
Figure 2.

These results indicated that AAPCs could efficiently stimulate viral antigen specific CTLs, thus extending our earlier findings obtained with HLAA2.1 influenza matrix protein-specific CTLs\textsuperscript{14}. Importantly, these results established that AAPC\textsuperscript{A2pp65} presented the P495 peptide in the context of HLA A2.1. Furthermore AAPC\textsuperscript{A2pp65} generated a significantly higher number of P495-specific CTLs compared to AAPC\textsuperscript{A2P495} (p=0.02). Starting from 1 x 10\textsuperscript{6} of T cells, we generated a median of 516 x 10\textsuperscript{3} P495-specific cells on AAPC\textsuperscript{A2pp65} (range 400-800 x10\textsuperscript{3}) and 172 x 10\textsuperscript{3} on AAPC\textsuperscript{A2P495} cells (range 163-546 x 10\textsuperscript{3}) after a single round of stimulation. P495 tetramer positive cells, ranging from undetectable (<0.1%) to 2% at the onset of the coculture, reached a median 32% (range 28-36) and 12% (11-18) at the end of the coculture on AAPC\textsuperscript{A2pp65} and AAPC\textsuperscript{A2P495} respectively, in 3 different donors (n=12 experiments).

**CMV-specific CTLs generated on AAPCs kill CMV infected targets.**

We tested the cytotoxicity of CTLs against CMV infected fibroblasts in a standard \textsuperscript{51}Cr release assay. The HLA A2.1\textsuperscript{+} human cell line MRC5 was infected with the laboratory strain CMV AD169. Expression of pp65 by the CMV-infected MRC5 was verified by immunofluorescence staining for pp65 prior to cytotoxicity assays (Figure 3A).
At an E:T ratio of 40 to 1, we observed 45% to 65% specific killing of CMV-infected MRC5 above background killing of uninfected MRC5 (Figure 3A). To further establish that cytotoxicity was directed against pp65, we tested the cytotoxicity against HLA A2.1⁺ BLCL<sub>pp65</sub>. Expression of pp65 in BLCL<sub>pp65</sub> was verified by immunofluorescence staining prior to cytotoxicity assays (Figure 3B). The CTLs specifically killed BLCL<sub>pp65</sub>, but not BLCL<sub>flu</sub> controls (Figure 3B). This data therefore established that the CTLs induced by either AAPC<sub>A2P495</sub> or AAPC<sub>A2pp65</sub> efficiently lysed HLA A2.1⁺ cells expressing pp65, including CMV-infected targets.

**Comparison of activation and expansion of P495 specific CTLs by AAPCs and autologous APCs.**

Having established that AAPCs efficiently expand P495-specific CTLs, we could compare the engineered cells to autologous APCs. The ER⁻ fraction of PBMCs was obtained during the isolation of T cells from peripheral blood. This fraction is highly enriched in B cells, monocytes and macrophages. BLCLs were established for three HLA A2.1⁺ donors and used in parallel in all experiments. We first optimized the ratio of APCs to T cell responders in the different cocultures. We determined an optimal ratio of 1:1 for ER⁻ PBMCs and of 1:3 for BLCLs (data not shown). An important factor for efficient ex vivo generation of CTLs is the availability of a sufficient supply of APCs. The generation of CTLs using ER⁻ PBMCs was restricted by the number of available PBMCs. The range of ER⁻ PBMCs obtained per 100ml of blood was 16 to
33 x 10\(^6\), while the number of T cells we obtained ranged from 35 to 70 x 10\(^6\). On the other hand, there was no restriction imparted by the number of available BLCLs or AAPCs.

Starting from 100 ml of peripheral blood, we compared ER\(^-\) PBMCs and BLCLs pulsed with the P495 peptide to AAPCs encoding P495 or pp65 for their ability to activate and expand CMV-specific CTLs (Figure 4A).

**Figure 4**

The percentage of P495 specific CTLs after 10-14 days of coculture on AAPC\(^{A2pp65}\) was significantly higher than that of CTLs activated on ER\(^-\) PBMCs or BLCLs (p<0.03). CTLs activated on AAPC\(^{A2P495}\) and ER\(^-\) PBMCs contained comparable percentages of P495 specific CTLs (p>0.05) (table 1). CTLs activated on AAPCs or autologous APCs specifically lysed T2 targets pulsed with P495 in proportion to the absolute number of P495 tetramer positive T cells (Figure 4B, C).

Next, we examined the expansion of P495-specific CTLs after 10 to 14 days of coculture (Table 1). AAPC\(^{A2pp65}\) gave a significantly higher number of P495-tetramer positive T cells than BLCLs (p=0.004). ER\(^-\) PBMCs and BLCLs generated a comparable number of P495-specific
CTLs when normalized to the number of input responder cells (Table 1). Starting from 100 ml of peripheral blood, we generated a total population of P495 tetramer positive CTLs of 6 to 7 x 10^6 with ER^- PBMCs, 6 to 20 x 10^6 with BLCLs, 10 to 17 x 10^6 with AAPC^{A2P495} and 28 to 32 x 10^6 with AAPC^{A2pp65} (Table 1).

Interestingly, the mean immunofluorescence intensity of P495-specific CTLs by tetramer staining was similar in all groups, suggesting similar avidities for cognate MHC-peptide complexes. (Figure 4B). Furthermore, the level of specific killing of T2 cells in the different groups was proportional to the number of tetramer positive cells (Figure 4C). For all donors, the highest absolute number of P495 specific CTLs and the highest specific killing at ratio 10:1 were observed with AAPC^{A2pp65} (Table 1).

**Comparison of cytotoxicity against mismatched and autologous BLCLs.**

To further assess cytolytic specificity, including potential alloreactivity, the CTL cultures were tested against allogeneic HLA A2.1^+ BLCLs targets mismatched at 3 HLA loci or more. The cytolysis of BLCL^{pp65} and BLCL^{flu} at the E:T ratio of 10 to 1 is shown for 3 different donors in Figure 5.
CTLs induced on AAPCs consistently exhibited the lowest background killing. CTLs activated on BLCLs displayed the highest nonspecific killing compared to CTLs activated on ER- PBMCs or AAPCs (p<0.05). CTLs activated on ER- PBMCs had consistently higher nonspecific killing compared to AAPCs at all E:T ratios tested. This difference reached statistical significance at E:T ratios of 20 and 40 (p=0.05).

The three different APCs also differed in the degree of background activity on autologous HLA A2.1+ BLCL flu. CTLs activated on AAPCs showed the lowest level of nonspecific killing (less than 5%) at an E:T ratio of 10 to 1. This was significantly lower (p<0.05) than the levels observed for CTLs stimulated by ER- PBMCs (15-20%) or BLCLs (20-35%). Cytotoxicity directed against EBV antigens presented by the BLCLs in an autologous fashion likely accounted, at least in part, for the higher killing of autologous BLCLs
Expanded P495-specific CTLs have an effector memory phenotype with a Tc1 cytokine profile.

We compared the phenotype of P495-specific CTLs from peripheral blood to that of CTLs generated ex vivo on ER− PBMCs, BLCLs or AAPCs. All specific CTLs were CD62L−, CCR7−, CD45RA− and CXCR4−, consistent with an effector memory phenotype (Figure 6A). CTLs activated on AAPCs showed strong intracellular staining for interferon-γ and TNF-α, consistent with a Tc1 phenotype (Figure 6B). CTLs activated on autologous APCs had a similar intracellular cytokine staining profile (data not shown).

Figure 6
P495-specific CTLs can be purified using P495-tetramer and anti-PE microbeads and rapidly expanded on AAPCs.

We purified P495-specific T cells from the CTL cultures using Miltenyi microbeads (n=3 donors). The cell recovery right after purification was always in the range of 50 to 60%. The expansion of purified CTLs after coculture on a new monolayer of AAPCs for 7 to 10 days was between 10 and 15 fold. The P495 tetramer-positive cells were always greater than 95% in the positive fraction and below detection level in the negative fraction (Figure 7). Importantly, the P495-specific T cells highly and specifically lysed T2 cells pulsed with P495 and HLA A2.1 BLCL<sup>pp65</sup>. The P495-tetramer negative fraction was not cytotoxic against the same targets (Figure 7).

Figure 7
Discussion

The implementation of a rapid, efficient, safe and reproducible approach to adoptive immunotherapy of CMV disease hinges on the availability of adequate APCs to present relevant antigens. Whereas several approaches to generate CMV-specific CTLs have been successfully introduced in clinical experimental protocols, the establishment of a broadly applicable process is still lacking. One of the major hurdles to overcome lies in the preparation of the APCs. As CMV reactivation occurs early after BMT, the prompt availability of CTLs for CMV prophylaxis is critical\textsuperscript{2,5}. The generation of APCs from autologous fibroblasts, EBV-immortalized B cells, and monocyte-derived dendritic cells is labor intensive, requiring from 12 days to several weeks.

The need to repeatedly generate such APCs is also liable to the variability that is inherent to customized cell processes. Furthermore, the generation of APCs requires both a specialized laboratory and appropriate quality assurance and control. In principle, autologous APCs are ideal because they possess all the MHC molecules to which the donor’s CTLs are restricted and because they do not present host antigens, which could stimulate T cells capable of causing GVHD. However, reliance on autologous APCs requires that they be generated for every donor as a prerequisite to expanding virus-specific CTLs. Alternatively, one could envisage the use of an immortalized human APC bearing a matched MHC haplotype, but this approach would require an enormous bank of APCs and carry the risk of stimulating potentially deleterious T cells directed against minor antigens\textsuperscript{24,25}. Immortalized APCs presenting specific antigens in the context of a single HLA molecule thus present a highly attractive alternative.

We compared AAPCs to autologous APCs, either ER\textsuperscript{−} PBMCs or pre-established BLCLs. Starting with PBMCs harvested from HLA A2.1\textsuperscript{+} CMV-seropositive donors, we first show that AAPC\textsuperscript{A2P495} efficiently activate and expand P495-specific CTLs. We further demonstrate that AAPC\textsuperscript{A2} expressing the full-length pp65 protein stimulate P495-specific CTLs, even more efficiently than AAPC\textsuperscript{A2P495}. The percentage of P495-specific CTLs was statistically significantly higher (p<0.05) after coculture with AAPC\textsuperscript{A2pp65} (mean=32\%) in comparison to autologous ER\textsuperscript{−} PBMCs (mean=17\%) or BLCLs (mean=12\%). AAPC\textsuperscript{A2P495} gave comparable results to ER\textsuperscript{−} PBMCs or BLCLs. P495 tetramer positive cells were most of the time less than 0.1\% at the onset of the coculture. The expansion of P495-specific CTLs after one round of stimulation on AAPCs was therefore at least 300 to 600-fold.
The absolute number of CMV-specific CTLs we obtained after one round of stimulation, starting with 100 ml of blood, is well within the range required for effective immunotherapy\textsuperscript{26-28}. Importantly, the phenotype and the cytolytic abilities of the CTLs obtained with AAPCs were identical to those obtained with autologous APCs, indicating that the AAPCs did not preferentially expand a distinct type of CTLs.

The infusion of allogeneic cytotoxic T cells early after transplantation carries the risk of inducing GVHD, depending on the number of alloreactive T cells\textsuperscript{24,25}. We therefore investigated the alloreactivity of the T cell populations expanded on either autologous ER\textsuperscript{-} PBMCs, autologous BLCLs or HLA A2.1-matched AAPCs. The AAPCs generated CTLs with the highest specificity as shown by the extremely low killing of allogeneic HLA A2.1\textsuperscript{+} BLCLs. Furthermore, CTLs activated on AAPCs also had the lowest cytolysis against autologous BLCL\textsuperscript{flu} (less than 5% at an E:T ratio of 20 to 1), establishing that the AAPCs did not significantly stimulate auto-reactive T cells, if at all.

Additionally, we established a rapid and reliable method for purifying P495-specific CTLs. A 10-15-fold expansion of P495-specific T cells could be obtained by restimulation on AAPCs for 7 to 10 days, with a purity that remained greater than 95%. Importantly, the purified, re-expanded T cells remained functional in cytotoxic assays against T2 targets pulsed with P495 or BLCL\textsuperscript{pp65}. This high purity compares favorably with others’ results obtained by tetramer purification (75-86%)\textsuperscript{29} or by the interferon-\gamma capture assay (66-99%)\textsuperscript{30}. In another approach based on purification of CD25\textsuperscript{+} CTLs after exposure to P495 peptide and prior depletion of CD25\textsuperscript{+} cells, Vie and colleagues efficiently generated CMV specific CTLs using allogeneic feeder cells for T cell expansion. However the content of P495 tetramer positive cells was not measured in this study\textsuperscript{31}. In all of the above mentioned approaches, the purified cells required multiple stimulations with allogeneic PBMC as feeders. The single HLA allele expressed by our AAPCs may account for the higher specificity of CTLs we obtained after cell purification and a single restimulation (>95%).

Several strategies based on autologous APCs have been successfully used for the generation of CMV-specific CTLs for adoptive immunotherapy\textsuperscript{10,11,32-34}. In their initial approach, Riddell and colleagues activated CTLs using CMV-infected fibroblasts and subcloned CMV-specific CTLs to remove alloreactive T cells\textsuperscript{11}. In the approach suggested by Lucas and colleagues, CTLs are activated on autologous BLCLs retrovirally transduced with pp65\textsuperscript{35}, a
strategy that may yield polyclonal T-cell lines with both CD8 and CD4 T cells directed against pp65 epitopes. In addition to the time required for the generation of autologous APCs, both approaches require several weeks of culture to reach a number of CTLs that is relevant for adoptive immunotherapy. Furthermore, the use of live CMV or transforming EBV requires additional time and effort for safety testing. The difficulties associated with the use of live viruses could be alleviated by using CMV antigens. However, CMV antigens derived from CMV infected cell lines carry the potential for viral transmission and also require safety testing. In two recent clinical trials, CTLs were activated on immature monocyte-derived DCs or PBMCs pulsed with CMV antigens. The yield of P495-specific CTLs obtained after 3 weekly stimulations by these approaches was highly variable. The poor standardization of CMV antigens and possible immunosuppressive effect of some CMV gene products may have accounted for the low yield of P495 specific CTLs. Extensive analyses of CTLs activated on AAPCs showed the former to be functionally equivalent to CTLs generated using autologous APCs. The definitive demonstration of the therapeutic equivalence of CTLs activated on AAPCs will require a clinical trial.

To further take advantage of the AAPC system, we investigated whether AAPCs could expand CTLs specific for an epitope derived from a full length precursor protein. We show here that AAPC-A2pp65 were very effective in stimulating P495-specific CTLs. The processing of an antigen and presentation of immunogenic peptides are an extremely attractive attribute of the AAPC system which could therefore be used to activate CTLs against multiple known or unknown epitopes. These data suggest that AAPCs could be generated for an array of different antigens and HLAs, including the structural CMV proteins pp65 and pp150, and other common HLA A and B molecules so as to cover the majority of patients with a small panel of AAPCs.

In summary, we show that AAPCs are a potent tool to generate CTLs against an immunodominant CMV antigen. Efficient CTL expansion (200 to 600-fold) requires only a 10-14 coculture on a monolayer of immortalized AAPCs presenting an antigen that is constitutively expressed and presented in the context of a single HLA molecule. Clinically relevant CTL numbers are thus reached without need for restimulation. In by-passing the need for generating autologous APCs, AAPCs represent a very rapid and reliable potential approach to generating specific CTLs for adoptive immunotherapy.
References


Figure Legends

Figure 1. Vectors used to engineer AAPC$^{A2}$ AAPC$^{A2P495}$ and AAPC$^{A2pp65}$.
Monocistronic retroviral vectors were used to express human $\beta_2$ microglobulin (h$\beta_2$m) and the costimulatory molecules CD80 (B7.1), CD54 (LFA-3) and CD58(ICAM 1) (A). Bicistronic vectors were used to express HLA A2.1 (B), the CMV protein pp65 (C) and the CMV peptide P495 (D) using an internal ribosomal entry site (IRES) linked to either neomycin phosphotransferase (neo$^R$), or puromycin-N-acetyltransferase (puro$^R$). SD, splicing donor; SA, splicing acceptor; $\Psi^+$, extended packaging signal.

Figure 2. Tetramer reactivity and cytotoxicity profile of CTLs activated on HLAA2.1/AAPCs.
Upper panels: staining with anti-CD8 (FITC-labeled, x-axis) and HLAA2.1/P495 tetramer (PE-labeled, y-axis). Lower panels: cytotoxicity assays performed against peptide pulsed TAP-deficient A2.1$^+$ T2 targets with CTLs shown above. Squares: T2 cells pulsed with P495. Diamonds: T2 cells pulsed with irrelevant peptide.
(A) CTLs cultured on “empty” AAPCs. Staining with P495 tetramer was lower than 0.1%. (B) CTLs activated on AAPC$^{A2P495}$. (C) CTLs activated on AAPC$^{A2pp65}$. The latter two efficiently kill T2 targets pulsed with P495. Cytolysis at a given E:T ratio is higher for CTLs activated on AAPC$^{A2pp65}$ compared to AAPC$^{A2P495}$. Results are representative for three different donors.

Figure 3. CMV-specific CTLs activated on AAPCs kill CMV-infected fibroblasts and recognize endogenously processed pp65.
(A) Left: Immunofluorescence staining for pp65 of uninfected control A2.1$^+$ MRC5 fibroblasts (MRC5) or MRC5 infected with the CMV strain AD169 (MRC5/CMV). More than 90% of MRC5/CMV stain positive for pp65 indicated by the green fluorescence. Staining was done immediately prior to cytotoxicity assays. Right: cytotoxicity assays performed against MRC5 fibroblasts. CTLs activated on AAPC$^{A2pp65}$ specifically lyse MRC5/CMV but not uninfected MRC5. Diamonds: uninfected MRC5. Squares: MRC5 infected with CMV.
(B) Left: pp65 immunofluorescence staining in A2.1$^+$ BLCLs transduced with the irrelevant flu peptide (BLCL$^{flu}$) or with pp65 (BLCL$^{pp65}$). More than 95% of the BLCL$^{pp65}$ stain positive.
Right: Cytotoxicity assays performed against BLCLs shown on the left. CTLs specifically lyse BLC^{pp65} but not BLCL^{flu}. Diamonds: BLCL^{flu}. Squares: BLCL^{pp65}. Similar results were obtained for three different donors. A representative experiment is shown. Values represent average of triplicates.

Figure 4. Comparison of CTLs activated on ER\(^{-}\) PBMCs, BLCLs, AAPC\(^{A2P495}\) or AAPC\(^{A2pp65}\).

(A) T cells were purified from PBMCs by positive selection using sheep red cell rosetting (SRCR). T cells were used as responders in cocultures with ER\(^{-}\) PBMCs, BLCLs or AAPCs. All cocultures were started with the same number of responder cells in 24-well tissue culture plates (1x10\(^{6}\) T cells/well). IL-2 was added on day 7 to all cocultures and every third day afterwards. Cytotoxicity assays and immunophenotyping were performed on day 10 to 14.

B. CD8 and HLAA2.1/P495 tetramer staining on the same day for all cocultures. The percentage of P495 specific CD8 cells in the culture is 20% for ER\(^{-}\) PBMCs, 9% for BLCLs, 14% for AAPC\(^{A2P495}\) and 34% for AAPC\(^{A2pp65}\).

C. Cytotoxicity assays against T2 targets done on the same day as immunophenotyping (B) are shown for CTLs activated on autologous ER\(^{-}\) PBMCs pulsed with P495, autologous BLCLs pulsed with P495, AAPC\(^{A2P495}\) or AAPC\(^{A2pp65}\). Diamonds: T2 cells pulsed with flu. Squares: T2 cells pulsed with P495. CTLs activated on ER\(^{-}\) PBMC lyse T2 cells pulsed with P495 but also T2 cells pulsed with flu at the higher E:T ratio. CTLs activated on AAPC\(^{A2pp65}\) show the highest level of cytolysis of T2 cells pulsed with P495 and very low cytolysis of T2 cells pulsed with flu. Similar results were obtained with three different donors.

Figure 5. Comparison of P495-specific and non specific killing of HLA A2.1\(^{+}\) allogeneic BLCLs by CTLs obtained with different antigen presenting cells.

Black bars: Cytolysis of allogeneic mismatched A2.1\(^{+}\) BLCL\(^{pp65}\). (Percentage of specific killing at an E:T ratio of 10 to 1 was calculated as the percentage killing of BLCL\(^{pp65}\) minus the percentage killing of BLCL\(^{flu}\)). Open bars: Cytolysis of BLCL\(^{flu}\) at an E:T ratio of 10 to 1 for the same assay. Top to bottom: CTLs activated on autologous ER\(^{-}\) PBMCs, autologous BLCLs, AAPC\(^{A2P495}\) and AAPC\(^{A2pp65}\). For the three donors, CTLs activated on AAPCs showed the lowest non specific killing.
Figure 6. CMV-specific CTLs have an effector memory phenotype and a Tc1 cytokine secretion profile.

A. CMV-specific CTLs have an effector memory phenotype. CTLs were characterized by staining for (left to right) CD8, CD62L, CD45RA, CCR7 and CXCR4 (all FITC-labeled antibodies, x-axis), against the P495 tetramer (PE-labeled, y-axis). Top to bottom: CTLs from peripheral blood prior to any stimulation (PBMC), CTLs activated on ER− PBMCs, BLCLs or AAPC<sup>A2pp65</sup> (AAPC). The immunophenotyping of CTLs from peripheral blood before any stimulation or after <em>in vitro</em> expansion using autologous presenting cells or AAPCs (either AAPC<sup>A2P495</sup> or AAPC<sup>A2pp65</sup>) was identical, and consistent with an effector memory phenotype for the three donors.

B. The P495-specific CTLs have a Tc1 cytokine secretion profile. Intracellular cytokine staining was performed after 10-14 day coculture with AAPC<sup>A2pp65</sup>. At the end of the coculture, 48% of the cells stain positive for P495 tetramer (left panel, y-axis). After a brief restimulation on a monolayer of AAPCs in the presence of brefeldin A, a comparable percentage of CD8 positive cells stained strongly positive for IFN-γ and TNF-α. Similar results were obtained with three APCs and for the three donors.

Figure 7. P495-specific CTLs remain highly cytotoxic after purification using tetramer and anti-PE microbeads.

(A) Tetramer reactivity and cytotoxicity profile of P495-depleted CTL fraction (P495−) after restimulation on AAPC<sup>A2pp65</sup>. CTLs are less than 0.5% P495 tetramer positive (upper). Cytotoxicity assays were performed starting from an E:T ratio of 80 to 1 against T2 cells (middle) or against A2.1<sup>+</sup> BLCLs (lower). Middle: Filled squares: T2 cells pulsed with P495. Open diamonds: T2 cells pulsed with flu. Lower: Filled squares: BLCL<sup>pp65</sup>. Open diamonds: BLCL<sup>flu</sup>.

(B) Tetramer reactivity and cytotoxicity profile of P495-specific CTL fraction (P495+) after restimulation on AAPC<sup>A2pp65</sup>. P495<sup>+</sup> CTLs remain more than 95% P495 tetramer positive (upper). Cytotoxicity assays were performed starting from an E:T ratio of 5 to 1 against the same targets as in (A). CTLs specifically killed T2 cells pulsed with P495 (middle) and BLCL<sup>pp65</sup> (lower). Similar results were obtained with T cells of three different donors.
Table 1. Yield of P495 Tetramer-positive CTLs and cytotoxicity after a single stimulation with ER<sup>-</sup> PBMCs, BLCLs and AAPCs.

The total number of T cells per 1x10<sup>6</sup> responder cells, the percentage of P495 tetramer positive cells and the absolute number of P495 tetramer positive cells from 100ml of peripheral blood are shown for 3 donors (A, J, Y). Values represent mean ± standard deviation after 10-14 day coculture on ER<sup>-</sup> PBMCs, BLCL, AAPC<sup>A2p495</sup> and AAPC<sup>A2pp65</sup>. Mean values represent at least 3 experiments.

* Number of P495 tetramer positive cells = [Total number of T cells] x [% of tetramer positive cells].

# Specific killing of T2 cells = [% killing of T2 cells pulsed with P495] minus [% killing of T2 cells pulsed with flu].
Table 1. Yield of P495 Tetramer + CTLs and cytotoxicity after a single stimulation with ER PBMCs, BLCLs and AAPCs

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<th>APC</th>
<th>Total number of T cells after coculture (x10^6)</th>
<th>Percentage of HLAA2.1/P495 tetramer positive cells</th>
<th>Absolute number of P495 tetramer + cells* (x10^6)</th>
<th>% killing of T2 cells# (E:T ratio 10:1)</th>
<th>Absolute number of P495 tetramer + cells obtained from 100 ml of blood (x10^-6)</th>
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Rapid expansion of cytomegalovirus-specific cytotoxic T-lymphocytes by artificial antigen presenting cells expressing a single HLA allele

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