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Induction of the Epstein-Barr Virus Latent Membrane Protein 2 Antigen-specific Cytotoxic T Lymphocytes Using Human Leukocyte Antigen Tetramer-based Artificial Antigen-presenting Cells

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Abstract Cytotoxic T lymphocytes (CTLs) specific for the Epstein-Barr virus (EBV) latent membrane protein 2 (LMP2) antigen are important reagents for the treatment of some EBV-associated malignancies, such as EBV-positive Hodgkin's disease and nasopharyngeal carcinoma. However, the therapeutic amount of CTLs is often hampered by the limited supply of antigen-presenting cells. To address this issue, an artificial antigen-presenting cell (aAPC) was made by coating a human leukocyte antigen (HLA)-pLMP2 tetrameric complex, anti-CD28 antibody and CD54 molecule to a cell-sized latex bead, which provided the dual signals required for T cell activation. By co-culture of the HLA-A2-LMP2 bearing aAPC and peripheral blood mononuclear cells from HLA-A2 positive healthy donors, LMP2 antigen-specific CTLs were induced and expanded *in vitro*. The specificity of the aAPC-induced CTLs was demonstrated by both HLA-A2-LMP2 tetramer staining and cytotoxicity against HLA-A2-LMP2 bearing T2 cell, the cytotoxicity was inhibited by the anti-HLA class I antibody (W6/32). These results showed that LMP2 antigen-specific CTLs could be induced and expanded *in vitro* by the HLA-A2-LMP2-bearing aAPC. Thus, aAPCs coated with an HLA-pLMP2 complex, anti-CD28 and CD54 might be promising tools for the enrichment of LMP2-specific CTLs for adoptive immunotherapy.

Key words immunotherapy; cytotoxic T lymphocyte; artificial antigen-presenting cell; Epstein-Barr virus

The infusion of cytotoxic T lymphocytes (CTLs) for Epstein-Barr virus (EBV) antigens has been proved as safe and effective prophylaxis and treatment for EBV-associated diseases [1–4]. In the immunocompetent host, EBV is associated with malignancies that express a more limited array of viral genes. For example, in EBV-positive Hodgkin's lymphoma and nasopharyngeal carcinoma, only subdominant EBV antigens, such as latent membrane protein (LMP) 1 or 2 and Epstein-Barr nuclear antigen 1, are expressed. Of the potential CTL target antigens expressed in Hodgkin's lymphoma and nasopharyngeal carcinoma, LMP2 is the ideal target antigen for immunotherapy [5,6]. LMP2-specific CTLs can be generated *in vitro* using peptide-pulsed autologous dendritic cells (DCs) as antigen-presenting cells (APCs) [7,8]. However, this method is time-consuming and expensive due to the limited availability of donor-derived DCs. To date, several reports indicated effective stimulation of T cells by human leukocyte antigen (HLA)-peptide ligands coating on artificial antigen-presenting cells (aAPCs), such as liposomes [9,10] and microbeads [11–14]. The aAPCs simulate the natural APCs (such as DCs, macrophages and B cells), which are able to provide the dual signals (the antigen-specific and costimulatory signals) for T cell activation. These aAPCs can be prepared by coating the T-cell receptor (TCR) ligand, e.g. peptide-major histocompatibility complex (MHC), and costimulatory molecules, such as B7, on a cell-sized bead, which provides a practical and convenient approach to generating antigen-specific T cells for adoptive immunotherapy.

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In this study, we explored a simple and efficient method to induce LMP2-specific CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A2 positive healthy donors, using aAPCs prepared by coating an HLA-A2-pLMP2 complex, anti-CD28 antibody and CD54 molecule to a cell-sized latex microbead *in vitro*. The interaction of the T-cell receptor and the HLA-A2-pLMP2 complex provides the basis for antigen specificity. Signaling through the CD28 receptor provides a powerful costimulatory signal following engagement of the anti-CD28 antibody. The adhesion molecule CD54 provides a synergistic signal through the LFA-1 molecule expressed on T cells. Co-cultured with the aAPCs, the HLA-A2 positive PBMCs can be induced to generate LMP2-specific CTLs, which bear the antigen-specific cytolytic properties.

Materials and Methods

Cell line

T2 cell line was kindly provided by Prof. Nicholas ZAVAZAVA (Kiel University, Kiel, Germany). This cell line bears the HLA-A*0201 gene, but expresses a very low level of cell surface HLA-A2.1 molecules, and is unable to present endogenous antigens due to a deletion of most of the MHC class II region including the transporter associated with antigen processing, and genes encoding for immuno-proteasomal subunits. Cells was maintained in RPMI 1640 medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, USA) and antibiotics.

Synthetic peptides

The following HLA-A2-restricted peptides were used in this study: the LMP2-derived peptide CLGGLLTMV (referred to as pLMP2) [15], the tyrosinase peptide YMDGTMSQV (pTyr) [16], and the HIV-Gag peptide SLYNTVATL (pHIV) [17]. Peptides were synthesized by standard solid-phase chemistry and characterized by mass spectrometry. The purity of the synthetic peptides was more than 90% as indicated by analytical HPLC. Lyophilized peptides were dissolved in dimethylsulfoxide and stored at –80 ºC after dilution in phosphate-buffered saline (PBS).

Preparation of biotinylated HLA-A2-pLMP2 monomeric and tetrameric complexes

Synthesis of monomeric and tetrameric HLA-A2-pLMP2 complexes was carried out according to the protocol of Altman *et al*. [18]. Briefly, plasmids encoding HLA-A*0201 heavy chain molecule with a C-terminal biotinylation site and human β2m molecule were constructed by insertion of the target genes into pET28a, respectively. The heavy chain and β2m molecules were expressed in *Escherichia coli*, and purified from inclusion bodies, then refolded in the presence of excess pLMP2 to form HLA-A2-pLMP2 monomeric product. The folded product was then subjected to enzymatic biotinylation by BirA enzyme (Avidity, Denver, USA) at 25 ºC for 12 h. This biotinylated HLA-A2-pLMP2 monomer was used for the preparation of both the aAPCs (see below) and the HLA-A2-pLMP2 tetramer. The tetrameric complex of biotinylated HLA-A2-pLMP2 was produced by mixing the purified biotinylated monomer with fluorescein-isothiocyanate-labeled streptavidin (Sigma, St. Louis, USA) at a molar ratio of 4:1. HLA-A2-pHIV and HLA-A2-pTyr tetramers were also prepared according to the protocol.

Generation of aAPCs bearing HLA-A2-pLMP2

Five microliters of sulfate polystyrene latex beads (Interfacial Dynamics, Portland, USA) were incubated sequentially with streptavidin (1 μg/10^7 beads; Sigma), CD28-specific antibody and CD54 molecule (1 μg/10^7 beads and 1.5 μg/10^7 beads, respectively; BD PharMingen, San Diego, USA) for 30 min in 1 ml PBS at 4 ºC on a rotator. The beads were then incubated with biotinylated HLA-A2-pLMP2 monomer (2 μg/10^7 beads) for 1 h at 4 ºC on a rotator, which allowed the biotinylated HLA-A2-pLMP2 monomer to bind to the streptavidin coated on the surface of the beads. After each incubation step, these aAPC beads were washed with PBS, and stored in the PBS at 4 ºC.

In vitro CTL induction by co-culture of HLA-A2+PBMCs and aAPCs

PBMCs from healthy HLA-A2 positive donors were separated using standard Ficoll-Hypaque (Sigma) gradient density centrifugation. These PBMCs were used as responder cells (3×10^6 cells/well) and co-cultured with the HLA-A2-LMP2-bearing aAPCs (3×10^5 cells/well) in 24-well plates in RPMI 1640 medium supplemented with 10% FBS (1 ml/well). IL-7 (10 ng/ml) was added on day 1. IL-2 (50 u/ml; R&D Systems, Abingdon, UK) was added on day 4. IL-2 and IL-7 were replenished twice a week. On day 7 and weekly thereafter, responder cells were collected, counted and re-plated at 3×10^6 cells per well, together with 3×10^5 aAPCs per well in RPMI 1640 medium with 10% FBS, 10 ng/ml IL-7 and 50 u/ml IL-2. After a total of four rounds of stimulation *in vitro*, responder cells were tested for tetramer staining and cytotoxicity assay.

Tetramer staining

Tetramer staining was performed as previously described [19,20]. In brief, 1×10^6 cells were incubated in 100 μl fluorescence activated cell sorter (FACS) staining buffer (PBS supplemented with 1% BSA and 0.05% NaN_3) with 20 μg/ml HLA-A2-peptide tetramer at 37 ºC for 30 min. Cells were washed with PBS and subsequently incubated with PE-Cy5 labeled anti-CD8 antibody (BD PharMingen, San Diego, USA) at 4 ºC for 30 min. All cells were washed with PBS twice after being stained, then fixed in 1% formaldhyde. Stained cells were analyzed with FACScalibur (Becton Dickinson).

Cytotoxicity assay

The colorimetric CytoTox 96 assay (Promega, Madison, USA) was used to quantify the release of lactate dehydrogenase (LDH), a cytosolic enzyme, in the target cell lysis. Target cells T2pLMP2 (the pLMP2-pulsed T2 cells), T2pHIV (the pHIV-pulsed T2 cells) and T2pTyr (the pTyr-pulsed T2 cells) were prepared by incubating T2 cells with pLMP2 (50 μg/ml), pHIV (50 μg/ml) and pTyr (50 μg/ml) for 3 h at 37 ºC. T2 cells pulsed without a peptide were used as the negative control. The constant target cells (1×10^4 cells/well) were added to serial dilution of various effectors for 4 h incubation at 37 ºC in 100 μl. After centrifugation, 50 μl supernatant was assayed for LDH content. To correct for spontaneous LDH release from effector cells, LDH levels were measured for each individual effector cell (effector spontaneous). Target cell spontaneous LDH release (target spontaneous) and maximum target cell LDH release (target maximum) were also measured. Triplicate wells were averaged and the percentage of specific lysis was calculated as follows: (experimental effector spontaneous–target spontaneous)/(target maximum–target spontaneous).

Inhibition of the cytotoxicity with HLA class I-specific monoclonal antibody

T2pLMP2 target cells were incubated with anti-HLA class I monoclonal antibody W6/32 (ATCC, USA; http://www.atcc.org/) [21] and a control isotypic monoclonal antibody (mAb) of irrelevant specificity (immunoglobulin G; BD PharMingen, USA) at a final concentration of 30 μg/ml for 40 min at 4 ºC before cytotoxicity assay. After incubation, the target cells were mixed with effector cells for the LDH release assay.

Statistical analysis

All data in this study were analyzed using SPSS version 10.0 software (SPSS, Chicago, USA). $P<0.05$ was considered as statistically significant.

Results

Growth kinetics of the co-culture bulk and the phenotype of the induced T cells

Following the first stimulation using HLA-A2-pLMP2-bearing aAPCs, PBMCs were expanded continuously. After 4 weeks of co-culture with aAPCs, an approximately 40-fold increase of cell number in the culture bulk was achieved. The phenotype of the expanded cells was measured by flow cytometry. The percentage of $CD4^+$ cells gradually decreased while the $CD8^+$ cells increased with the progression of the co-culture with aAPCs. The phenotypes of the CTLs for LMP2 were $CD8^+$, $CD4^-$, $CD3^+$, $CD16^-$ and $CD56^-$.

Frequency of LMP2-specific CTLs increased by co-culture with aAPCs as determined by tetramer staining

Flowcytometric analysis of PBMCs was performed before or after co-culture with the aAPCs bearing HLA-A2-pLMP2. Before the four rounds of stimulation using aAPCs, the frequency of $CD8^+$ T cells stained with HLA-A2-LMP2 tetramers was 0.07%. However, after stimulation, FACS analysis revealed that 13.9% of $CD8^+$ T cells were stained with HLA-A2-LMP2 tetramers, which was not observed when staining with the control tetramers (HLA-A2-pHIV tetramer and HLA-A2-pTyr tetramer) (**Fig. 1**). LMP2-specific CTLs expanded by aAPCs from the five donors showed similar results (**Table 1**).

Cytotoxicity of the aAPC-induced pLMP2-specific CTLs

The cytotoxic activity of the aAPC-induced CTLs against various target cells (T2pLMP2, T2pHIV, T2pTyr and T2 cells without a pulsed peptide) was tested using the LDH-releasing assay. The CTLs exhibited approximately 60% specific lysis against the T2pLMP2 at an effector:target ratio of 50:1. However, the CTLs showed an approximately 10% cytolysis against the T2pHIV, T2pTyr and the T2 without a pulsed peptide at the same effector:target ratio (**Fig. 2**). The specific killing activity of the CTLs induced by the aAPCs against T2pLMP2 target cells was much more obvious than in any other group ($P<0.05$). Specific CTLs for LMP2 induced by the aAPCs from the five donors showed similar specific lysis (**Table 1**). This result shows

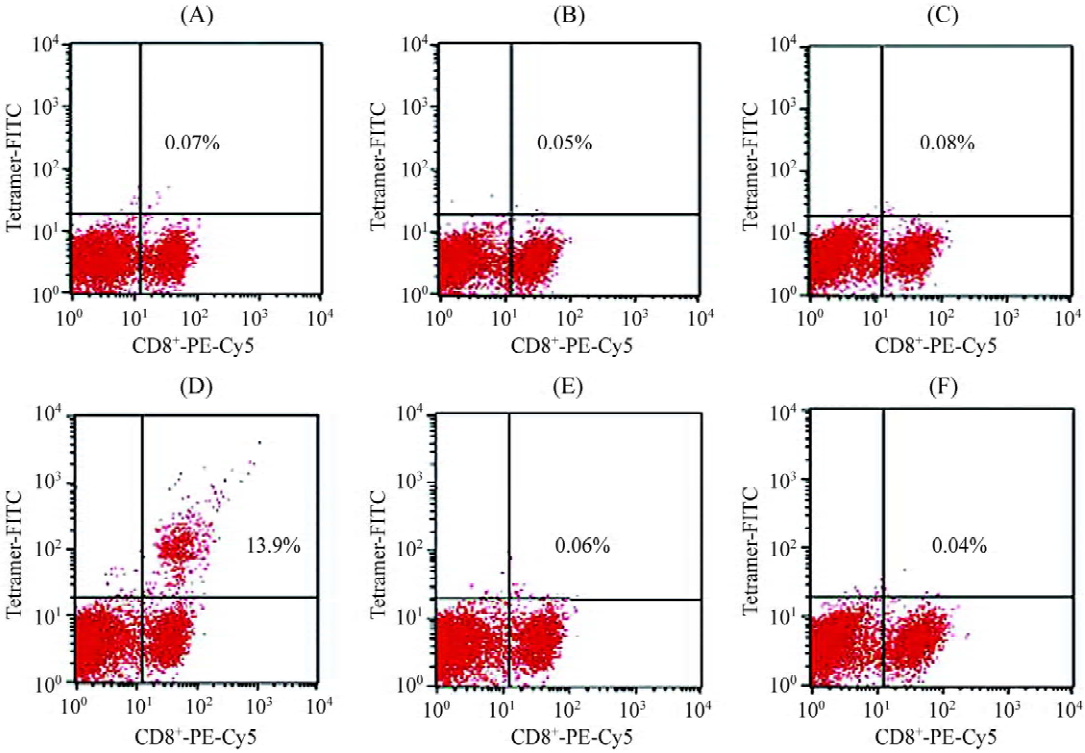

Fig. 1 Identification of the artificial antigen-presenting cell (aAPC)-induced specific cytotoxic T lymphocytes by human leukocyte antigen (HLA)-A2-peptide tetramer staining

The upper panel indicates the frequency of HLA-A2-peptide tetramer-binding $CD8^+$ T lymphocytes before stimulation with aAPCs. The following HLA-A2-restricted peptides were used: the latent membrane protein 2-derived peptide CLGGLLTMV (pLMP2); the tyrosinase peptide YMDGTMSQV (pTyr) [16]; and the HIV-Gag peptide SLYNTVATL (pHIV). (A) HLA-A2-pLMP2 tetramer-binding $CD8^+$ T lymphocytes (0.07%). (B) HLA-A2-pHIV tetramer-binding $CD8^+$ T lymphocytes (0.05%). (C) HLA-A2-pTyr tetramer-binding $CD8^+$ T lymphocytes (0.08%). The lower panel shows the frequency of HLA-A2-peptide tetramer-binding $CD8^+$ T lymphocytes after four rounds of stimulation with aAPCs. (D) HLA-A2-pLMP2 tetramer-binding $CD8^+$ T lymphocytes (13.9%). (E) HLA-A2-pHIV tetramer-binding $CD8^+$ T lymphocytes (0.06%). (F) HLA-A2-pTyr tetramer-binding $CD8^+$ T lymphocytes (0.04%). These results are representative of many experiments, and demonstrate that the co-culture of aAPC and HLA-A2 positive peripheral blood mononuclear cells can expand the pLMP2-specific CTLs. FITC, fluorescein-isothiocyanate.

Table 1 Induction of cytotoxic T lymphocytes (CTLs) specific for latent membrane protein 2 (LMP2) in five human leukocyte antigen (HLA)-A2 positive healthy donors

Donor	Tetramer staining (%) [a]		Lysis (%) [b]	
	HLApLMP2	HLApHIV	T2pLMP2	T2pHIV
1	10.9	0.07	56.9	10.4
2	12.5	0.10	60.5	8.5
3	11.3	0.05	61.4	11.1
4	13.1	0.09	54.7	7.8
5	12.8	0.16	50.1	9.7

The CTLs induced by the artificial antigen-presenting cells for 28 d were tested for tetramer staining and cytotoxic activity. [a] indicate the percentage of viable $CD8^+$ HLA-A2-pLMP2 tetramer-positive or $CD8^+$ HLA-A2-pHIV tetramer-positive lymphocytes in the total population of viable lymphocytes; [b] indicates the percent lysis of LMP2-derived peptide-pulsed T2 cells (T2pLMP2) or pHIV-pulsed T2 cells (T2pHIV). Results are shown for an effector:target ratio of 50:1. These results show the coordination of the HLA-A2-pLMP2 tetramer staining and the specific lysis of T2pLMP2. HLApLMP2, HLA-A2-pLMP2; HLApHIV, HLA-A2-pHIV.

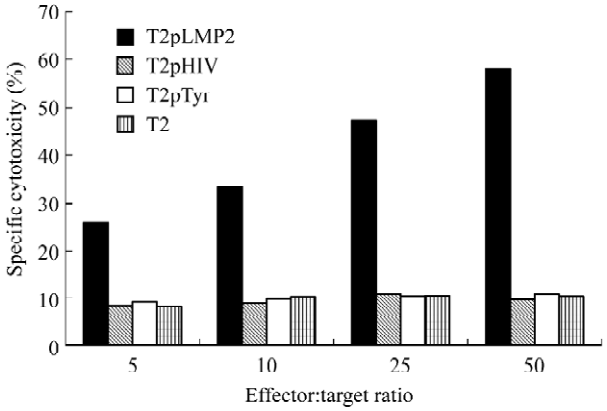

Fig. 2 Lysis of various target cells by artificial antigen-presenting cell (aAPC)-induced cytotoxic T lymphocytes (CTLs)

The CTLs were induced by aAPCs for 28 d. The cytotoxic activity of one individual's CTLs is shown. The cytotoxic activity of the CTLs was assessed against latent membrane protein 2-derived peptide-pulsed T2 cells (T2pLMP2), pHIV-pulsed T2 cells (T2pHIV), pTyr-pulsed T2 cells (T2pTyr) and T2 cells pulsed without a peptide at various effector:target ratios. These results show that the cytotoxicity of the aAPC-induced CTLs is pLMP2-specific.

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Inhibition of the cytotoxicity of the aAPC-induced T cells by HLA class I specific mAb (W6/32)

To determine whether the induced CTLs could recognize the specific target cells in an HLA class I-restricted manner, anti-HLA class I mAb W6/32 was utilized to block the cytotoxicity of the aAPC-induced CTLs. The cytotoxic activity against the T2pLMP2 was significantly eliminated by W6/32. As shown in **Fig. 3**, incubation of T2pLMP2 target cells with W6/32 led to the inhibition of the targeted cells lysis, whereas mouse immunoglobulin G, used as an isotype control, showed no effect. These results suggested that the aAPC-induced CTLs lysed the specific targets in an HLA class I-restricted manner.

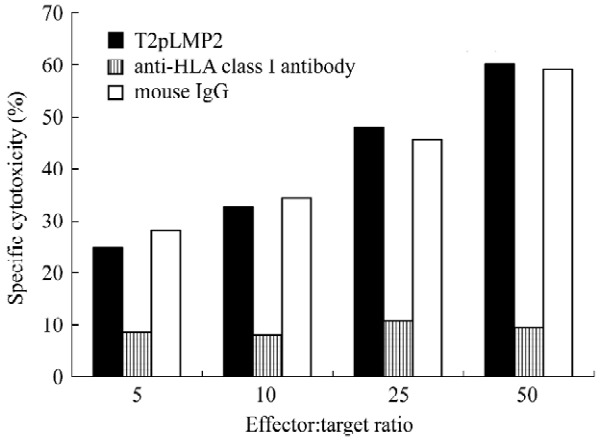

Fig. 3 Inhibition of the cytotoxic activity of effector cells, artificial antigen-presenting cell (aAPC)-induced cytotoxic T lymphocytes (CTLs), against specific target cells, latent membrane protein 2-derived peptide-pulsed T2 cells (T2pLMP2), by anti-human leukocyte antigen (HLA) class I monoclonal antibodies

Anti-HLA class I antibody or a control isotypic antibody (mouse IgG) were added to the specific target cell T2pLMP2 and incubated for 40 min at 4 °C. After the incubation, the target cells were mixed with effector cells for lactate dehydrogenase release assay. The CTL assays were performed at various effector:target ratios. The data of one individual is shown as the mean from triplicate wells. The results indicate the pLMP2-specific cytotoxicity of the aAPC-induced CTLs is mediated by the binding of the T cell receptor (TCR) to the HLA-A2-pLMP2 complex expressed on the surface of the T2 cell.

Discussion

Adoptive immunotherapy holds promise as a treatment for cancer. However, nonspecific T cell therapy is not considered efficient for clinical applications at present because of its low killing activity, lack of specificity and side-effects. Tumor-specific CD8-positive CTLs constitute the most important effector cells for antitumor responses [22]. CTLs recognize "processed" peptides that are derived from endogenous proteins and presented on the cell surface in association with MHC class I molecules [22,23]. Peptides that bind to a given MHC class I molecule have been shown to share common amino acid motifs, which are called major anchor motifs [23]. Hence, tumor-specific CTLs can recognize and select the antigenic peptides by scanning peptide sequences, then kill tumor cells in an antigenic peptide-specific fashion. EBV-encoded LMP2 is the target antigen available for therapeutic augmentation of CTL responses in patients with EBV-associated malignancies [5,6].

It has been shown previously that *in vitro* specific CTLs can be generated using peptide-pulsed autologous DCs as APCs [24,25]. However, CTL expansion to clinically relevant amounts requires multiple leukophoreses to obtain enough autologous DCs. Variability in both quantity and quality of obtained DCs, which presumably relates to underlying diseases and the pre-treatment of the patients, also significantly impacts on the viability of DC-based therapeutics. For these reasons, use of DCs has been a limiting step in *ex vivo* expansion of T cells [14]. Strategy of aAPC offers a promising way to overcome the disadvantages of natural APCs. CTLs generated by artificial stimulation protocols can kill both peptide-loaded and natural target cells [14,26,27]. The presented data showed that CTLs specific for LMP2 could be induced *in vitro* with aAPCs coating HLA-A2-pLMP2, anti-CD28 antibody and CD54 molecule to cell-sized latex beads. The HLA-A2 positive PBMCs were induced to generate pLMP2-specific CTLs *in vitro* by co-culture with aAPCs, and specificity of the aAPC-induced CTLs was confirmed by both their binding to HLA-A2-pLMP2 tetramers and killing activity against HLA-A2-pLMP2-bearing cells. In order to test for the specificity of the CTL cytotoxicity, T2 cells were used as the target in this study. The T2 cell is an HLA-A*0201 human lymphoid cell line, which is defective in endogenous antigen processing and presenting, but can effectively present exogenously supplied peptides [28,29]. The specific killing activity of CTLs induced by the aAPCs against specific target cells T2pLMP2 was much more effective than that of any other control group. The results indicated the cytotoxicity of the aAPC-induced CTLs is antigen-specific, that is, against the target cells bearing the corresponding HLA-A2-pLMP2 complex. The aAPC-mediated stimulation was better than T2pLMP2 stimula-

tion in *in vitro* expansion of antigen-specific CTLs, because of the advantages of aAPCs (refer to the following description).

For clinical studies, HLA tetramer-based aAPCs have several distinct advantages over cellular APCs, including DCs. One of these is ease of preparation, which is not required for sterile cell culture and cytokines, thereby reducing both the variability and expense associated with *ex vivo* expansion. The variability is particularly important when considering therapies for cancer as there have been reported defects in DCs obtained from patients with malignancies [30]. Another of the advantages is the good stability of aAPCs, unlike the biologic variability and patient-to-patient variation when producing cellular APCs such as DCs. In addition, aAPCs can present defined combinations of MHC alleles and peptides, and be easily adapted using other MHC alleles and/or peptides, so that immunodominant or subdominant epitopes can be expanded preferentially. When cellular APCs are used, an array of MHC molecules is employed, and a broad but uncontrolled MHC restricted response is generated. The last advantage is the ability to control the combination of costimulatory complexes associated with aAPCs, unlike cellular APCs that may participate in the T cell-APC interaction in such a way to promote tolerance or anergy. For example, on T cell activation, B7 binding to CTLA-4 instead of CD28 would limit T cell expansion [31,32]. It is convenient to prepare aAPCs coated with anti-CD28, which binds specifically to CD28, avoiding the binding of B7 to CTLA-4. In this study, we used a combination of anti-CD28 and CD54 for the generation of a costimulatory signal, because CD54 molecule appears to augment cell expansion by limiting apoptosis when coupled to aAPCs [33].

The use of aAPCs represents the state-of-the-art in generation of antigen-specific CTLs for adoptive immunotherapy. Thus, HLA tetramer-based aAPCs coated with an HLA-peptide complex, anti-CD28 antibody and CD54 molecule could provide a useful tool for the reproducible expansion of specific CTLs *in vitro* and significantly advance the field of adoptive immunotherapy. aAPC might become useful reagents to enrich LMP2-specific CTLs for the treatment of patients with EBV-associated malignancies.

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