Article

EBV LMP2A-specific T Cell Immune Responses Elicited by Dendritic Cells Loaded with LMP2A Protein

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Type II Epstein-Barr virus (EBV) associated malignancies such as nasopharyngeal carcinoma and non-Hodgkin's lymphomas consistently express latent membrane 2A (LMP2A) proteins, which have been suggested to be an ideal target for immunotherapy. In previous studies we have demonstrated that using LMP2A protein loaded dendritic cells, the most powerful antigen processing cells in the body can elicit specific and robust anti-tumor cellular immune response *in vitro*. In this paper, we further investigated the T cell profile of the anti-tumor immune response. We found that LMP2A specific $CD4^+$ and $CD8^+$ T cells could be stimulated by LMP2A protein loaded dendritic cells (DCs). The Th1 type immune response is dominant in the immune response mediated by LMP2A specific $CD4^+$ T cells. The $CD8^+$ cytotoxic T cells can lyse LMP2A bearing cells effectively and specifically. The $CD8^+$ cytotoxic T cells can also secrete high level of intracellular IFN-γ, which indicates these cells are EBV-LMP2A specific cytotoxic T cells. Altogether, our studies proved that LMP2A protein loaded DCs can elicit anti-tumor cellular immune responses efficiently. This study provides a rationale for the DC-based immunotherapy against EBV-LMP2A expressing malignancies. *Cellular & Molecular Immunology*. 2009;6(4):269-276.

Key Words: Epstein-Barr virus, latent membrane 2, dendritic cell, cytotoxicity

Introduction

Tumor specific $CD4^+$ and $CD8^+$ T cells have been emphasized to be critical components in anti-virus and anti-tumor immunity (1, 2). A successful tumor immunotherapy should include an efficient strategy to induce both $CD4^+$ and $CD8^+$ T cells immune responses against tumor cells (3-6). Dendritic cells are most powerful antigen-presenting cells (APCs) and capable of initiating and modulating the T cell responses including $CD4^+$ and $CD8^+$ T cell responses (7, 8). Protein loaded DCs can cross-present both MHC-I class and MHC-II class epitopes to T cells and initiate $CD4^+$ and $CD8^+$ T cell responses (9). It has been shown that protein-loaded DCs contain tumor specific antigens and have antigen presentating capability that makes them become a potentially attractive vector for triggering T-cell antitumor responses (11-15).

EBV is associated with all undifferentiated naso-pharyngeal carcinoma (NPC), about 40% of Hodgkin's lymphoma, and 20-100% of non-Hodgkin's lymphomas (NHL). EBV antigens expressing in all the lymphomas restricted to EBNA1, LMP1, and LMP2 (16-20). It has been shown LMP2 is the ideal tumor antigen to be chosen as target for EBV associated malignancies in immunotherapy (16, 21-25). Our previous study has shown that by using purified LMP2A protein loaded Daces, strong EBV-LMP2A specific catatonic T lymphocytes (CTL) responses can be elicited (26). However, whether these loaded Daces can elicit TH1 immune response and the subgroup of $CD8^+$ cells which can participate in anti-tumor immune response by secreting IFN-γ and TNF-α remains to be determined. In this study, we used LMP2A protein loaded Daces as Apes to stimulate T cells *in vitro*. We found besides a robust CTL response, LMP2A loaded Daces can also elicit specific Th1 type $CD4^+$ T cell responses and IFN-γ secreting CTLs as well. This finding proves the concept that protein loaded DCs are potential vaccinating strategy for LMP2A positive (type II latency) EBV associated malignancies treatments.

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Materials and Methods

Plasmids and cells

Retrovirus vectors pGEZ-Term, pHIT456 and pHIT60 were given by Dr. Xueguang Zhang from Soochow University. B95.8, a primate B cell line transformed by EBV, was given by Dr. Wenhan Wu in the University of Hong Kong. B lymphoblastoid cell lines (LCL) were produced by transforming healthy volunteers' peripheral blood monocytes (PBMCs) by B95.8 cell culture supernatant supplemented with cyclosporine (Sigma, USA). K562 cells were derived from chronic myelogenous leukemia cells and were obtained from The Institute of Cell Biology (Shanghai, China). Both cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, USA). PBMCs were separated from healthy volunteers by Ficoll gradient centrifuge. The PBMCs have been tested against HBV, HCV, HIV and syphilis.

Reagents

Cytokines IL-4, GM-CSF, TNF-α and IL-2 were purchased from Peprotech, England. Heparin (12,500 U/vial) was purchased from Wan Bang Pharr. Lipofectamine 2000 and Zeocine were purchased from Invitrogen, USA. Ficoll gradient solutions were purchased from Institute of Hematology, China Academy of Medical Sciences. ^{3}H-TdR was purchased from The Institute of Atomic energy, China Academy of Medical Science. Rabbit anti-LMP2A antibody was kindly given by Dr. Midderdrop, Netherland. Rabbit anti-6×His-Tag antibody was purchased from ABR, USA. FITC labeled goat anti-human IgG antibody was purchased from Booster, China. The primers for amplifying and cloning LMP2A were synthesized from Bioasia, China. *Pst* I and *EcoR* I sites were added in the upstream and downstream primers respectively. The sequences: PN1, 5'-AAC TGC AGC ACC ATG TCT GAT AAT GGA CCC CAA TCA A-3'; PN2, 5'-GAA TTC TTA CAC CAC CAC CAC CAC CAC TGC CTG AGT TGA ATC AGC AGA A-3'.

Blood sample

Total five peripheral blood samples from EBV positive healthy adults (male, average age 27-31) were detected for the level of anti-EBV antibodies including EBV-capsid antigen (CA) IgG, EBV-CA IgM, EBV-EA IgG, EBNA IgG by indirect immunofluorescence assay kit (EUROIMMUN, Germany), according to the instructions of EU EBV-antibodies detection kit.

Immunofluorescence

In EU EBV-antibodies detection kit, biochips coated with different EVB antigens are incubated with a diluted serum sample. If the sample is positive, specific antibodies of classes IgA, IgG and IgM attach to the viral antigens. First, 25 μl of different diluted serum was applied to each reaction field, and then incubated for 60 min at room temperature. The slides was rinsed for at least 5 min. Second, apply 20 μl PBS Tween to fields A to D, and to field E 20 μl of freshly reconstituted complement for a stronger complement reaction, then incubate for 30 min at room temperature. Rinse the slides. Third, apply 20 μl of fluorescein-labelled anti-human globulin (conjugate) to each reaction field then incubate for 30 min at room temperature. Rinse the slides. Last, apply embedding medium to cover glasses. Read the fluorescence with the microscope.

Micro-bead sorting immune $CD4^+$ T and $CD8^+$ T cells

$CD4^+$ and $CD8^+$ T cells in the PBMCs separated from the blood samples were isolated by CD4 Dynabeads or CD8 Dynabeads, (Daynal, USA) respectively, according to the manufacturer's instruction. The isolated $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry with anti-CD4-FITC or anti-CD8-FITC antibodies (BD PharMingen, USA).

^{3}H-Thymidine Uptake Assay

The functions of LMP2A protein unloaded and loaded DC to stimulate naïve $CD4^+$ and $CD8^+$ T cells proliferation were evaluated by [^{3}H]-thymidine uptake assays. Briefly, $CD4^+$ or $CD8^+$ T cells were seeded at 5×10^5 cells per well and DCs were added at various DC: T ratios. The cells were maintained 10% human AB serum RPMI-16 medium supplemented with TNF-α (200 U/ml) and IL-2 (10 U/ml) for 5 days followed by adding 0.5 μCi/well [^{3}H]-thymidine. After 16 h, the cells were harvested onto filtered glass filter strips and measured the incorporated [^{3}H]-thymidine. Before harvesting cells, the supernatant medium were collected for further ELISA assays.

ELISA

Secreted IFN-γ and IL-10 levels in the culture medium of $CD4^+$ T cells stimulated with LMP2A protein unloaded and loaded DCs were measured by commercial ELISA kits (Jingmei Bio. Inc., China) according to the instruction.

Induction of LMP2A specific CTL

DCs were induced by IL-4 and GM-CSF cocktail as described (26). Immature DCs (1×10^5) were loaded with 40 μg/ml LMP2A protein for 1 h and mixed with 1×10^6 $CD8^+$ T cells at the ratio of 1:10. The cells were cultured with TNF-α for 4 days at 37°C, 5% CO_2. IL-2 (10 U/ml) was added at day 4 and the same amount of loaded DCs were added at day 7 and day 10. The cytotoxicity or CTL was measured at day 14 and day 21 as described (26).

Preparation of target cells

Recombinant retrovirus (pGEZ-LMP2A) containing LMP2A gene was produced as described (26). PBMCs were resuspended to a density of 1×10^6 cells/ml in 10% human AB serum and seeded into 24-well plate to let the cells attach. After 2 h, the nonattached cells were removed by gently rinsing the cells. Macrophages (Mϕ) were isolated by adherence and cultured in 37°C, 5% CO_2 for 24 h. On DAY 2, adherent cells were incubated with 1ml infectious recombinant or empty retroviral s/n supplemented with 1000 U/ml GM-CSF and 8 μg/ml polybrene. This procedure was repeated on each of the following two days. On day 7 identification of LMP2A expression of infected macrophages

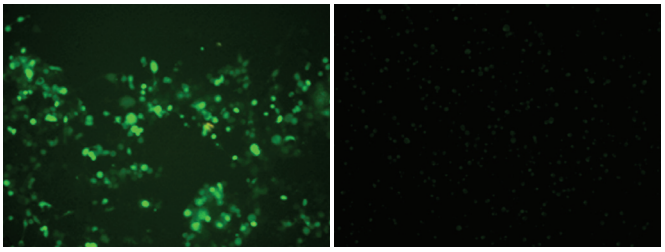

Figure 1. EBV infection status of donors at the time of peripheral blood collection for CTL generation. The index of EBV-CA IgG reflected whether volunteers were previously infected with EBV or not. As shown in the left picture, positive results obtained from IIF analysis of EBV-CA IgG antibody in serum of all volunteers, observed by fluorescent microscope. The right picture represented negative control group.

Table 1. The titers of anti-EBV antibodies from EBV healthy carriers

EBV healthy carriers	EBV associated antibody assay			
	EBV-CA IgG	EBV-CA IgM	EBV-EA IgG	EBNA IgG
1	+/++	—	+/++	—
2	++	—	—	—
3	++	—	+/++	—
4	+/++	+	—	—
5	++	—	+/++	—

The antibody titer can be evaluated from the fluorescence results obtained using different sample dilutions. The dilutions of 1 : 10, 1 : 32 and 1 : 100 is defined as "+", "++" and "+++", respectively. The results indicated these five volunteers were all EBV healthy carriers.

by flow cytometric analysis began. The harvesting cells were then used as target cells. Another three groups of targeting cells are K562 and cells of autologous or mismatched LCL induced by EBV as mentioned before.

Cytotoxicity assay（LDH Cytotoxicity Detection）

To compare the cytotoxic specificity of CTL induced by LMP2A loaded or unloaded DC, LDH release detection assays (BD, USA) were performed. CTL suspended in 1% FBS RPMI 1640 medium were seeded into 96-well plate at 2×10^5 cells/well, 1×10^5 cells/well and 5×10^4 cells/well in triplicate. The target cells mentioned above were added into the corresponding wells at 1×10^4 cells/well to make the effector/target ratios equal 20:1, 10:1 and 5:1. At the same time background control (1% FCS RPMI1640 200 μl), minimum cytotoxicity control (target cells in 1% FCS RPMI 1640), maximum cytotoxicity control (2% NP-40 and target cells) and effector cells control (CTL in 1% FCS RPMI 1640) were set up. The plates were spinned at 1000 rpm for 10 min and then incubated at 37°C, 5% CO_2 for 12h. After incubation, the plates were spinned again at 1000 rpm for 20 min and 100 μl supernatant were transferred into new 96-well plates. 100 μl fresh-made reaction mixtures were added into each well, and then the plates were incubated at room temperature for 30 min, protected from light. The absorbance (A) of the samples were measured at 490/492 nm with a reference wavelength of 600 nm. The cytotoxicity rate is calculated according to the following equation: Cytotoxicity (%) = 100% × [(Triplicate - effector cells control - minimum cytotoxicity control) / (Maximum cytotoxicity control - minimum cytotoxicity control)]

Intracellular cytokines assay

The CTL induced by LMP2A loaded or unloaded DCs were stimulated in 6-well plate by autologous PBMCs for 3 times. After 3-week culture, the CTL were mixed with macrophages infected by retrovirus pGEZ-LMP2A and incubated at 37°C, in 5% CO_2 for 16 h. The cells were harvested into tube and incubated with 5 μl of anti-CD3-FITC and 5 μl anti-CD8-PE antibiodies (all from BD PharMingen) for 20 min at room temperature, protected from light. The cells with antibodies were fixed by incubating with 100 μl of fixation for 20 min at room temperature. After the fixed cells were washed by 3 ml of PBS for 3 times. For the intracellular IFN-γ assay, CTL were treated with 100 μl permeabilization and 5 μl of anti-IFN-γ-PE antibody (BD PharMingen) and incubated for 15 min at room temperature, protected from light. The cells were washed as before. All the measurements were performed on FACSCalibur. The gates for CD8 positive cells were set to calculate $CD8^+$, $CD3^+$ and IFN-γ^+ cells.

Statistical tests

Values are presented as mean ± SD. Results were analyzed by the Student *t* test for independent samples (2-tailed). Statistical significance for comparisons was assigned at $p < 0.05$.

Results

The infection status of EBV healthy carriers

To know the infection status of EBV healthy carriers, we measured the titers of anti-EBV antibodies (Figure1). The titers were shown in Table 1. All of the carriers were EBV-CA IgG positive, which indicated the healthy carriers were previously infected with EBV. EBV-CA-IgM and EBV-EA IgG, indicate current infection of EBV, are all negative in the carriers except No.4 carrier. EBNA IgG, a diagnostic antibody in determining acute and convalescent stages of infectious mononucleosis, is all negative in the healthy carriers.

In vitro stimulation $CD4^+$, $CD8^+$ T cells of EBV healthy carriers with LMP2A loaded DCs

To measure the proliferative activity of LMP2A loaded DC -stimulated T cells, we used ^{3}H-thymidine uptake assay to compare LMP2A loaded and unloaded DCs' abilities to stimulate proliferation of autologous naïve T cells. When mixed with $CD4^+$, or $CD8^+$ T cells (both purities >99%, as shown in Figure 2A), loaded DCs could stimulate proliferation of both $CD4^+$ and $CD8^+$ T cells comparing with unloaded DCs (Figure 2B). The ability of stimulation increased with the increase of DC : T ratio. Even at 1 : 500

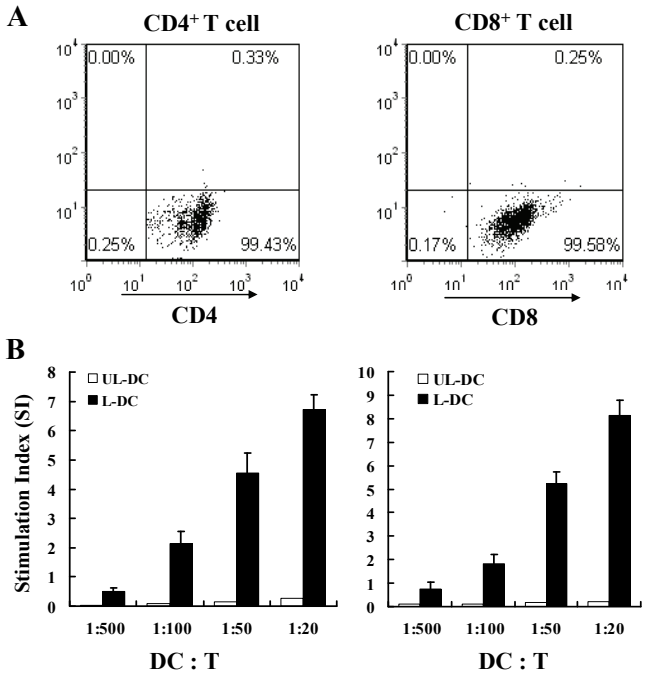

Figure 2. Proliferation of $CD4^+$ T and $CD8^+$ T Cells after stimulation with autologous LMP2A loaded or unloaded DCs. (A) T cells were purified from the donors by positive selection with anti-$CD4^+$ or $CD8^+$-coupled Dynabeads. Median purity of the $CD4^+$ T and $CD8^+$ cell preparations were determined by flow cytometry analysis and were 99.43%, 99.58% respectively. (B) $CD4^+$ T and $CD8^+$ T cells proliferated strongly in response to stimulation with autologous LMP2A loaded DCs at graded doses (from 1 : 500 to 1 : 20). Purified T cells from donors were co cultured with autologous irradiated loaded or unloaded DCs, and proliferation was recorded by ^{3}H-thymidine uptake after 4 days of culture. Each determination was from 6-fold replicate microwells. Results are presented as mean values from five independent experiments. L-DC, LMP2A loaded DC; UL-DC, LMP2A unloaded DC.

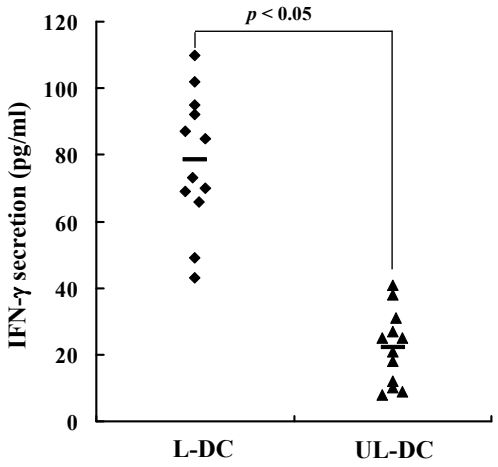

Figure 3. Secretion of IFN-γ is enhanced when purified $CD4^+$ T from five donors are cultured with irradiated LMP2A loaded autologous DCs respectively. Cytokine production was measured by analysis of culture supernatants by sandwich ELISA specific for IFN-γ and IL-10 as described in Materials and Methods. Data are individual level of IFN-γ from $CD4^+$ T cells cultured with autologous loaded DCs of five donors (n = 12) or unloaded DCs (n = 12). The bar represents the inter-group mean level of the IFN-γ ($p < 0.05$). IL-10 values were below the limit of detection. L-DC, LMP2A loaded DC; UL-DC, LMP2A unloaded DC.

ratios, loaded DCs still could stimulate naïve $CD4^+$ and $CD8^+$ T cells proliferation significantly. These data suggest mature DCs loaded with LMP2A could present antigens to both $CD4^+$ and CD8 T cells and express co-stimulatory molecules to initiate proliferation of the immune cells.

EBV-LMP2 specific $CD4^+$ T cells secrete IFN-γ

To determine whether the $CD4^+$ T cells are functional, we measured IFN-γ and IL-10 secretion of $CD4^+$ T cell stimulated by LMP2A loaded and unloaded. As shown in Figure 3, when stimulated with LMP2A loaded DCs, autologous $CD4^+$ T cells could secrete more IFN-γ into the supernatant than the control group ($p = 0.031$). That means those $CD4^+$ T cells stimulated by LMP2A loaded DCs were functional and specific. We also tried to detect the IL-10 of each group. However using our ELISA method, we could not detect the IL-10 levels in the supernatants.

Determination of the expression level of transduced LMP2A in infected macrophages

To evaluate the generated CTL that recognize endogenously processed LMP2A antigen, macrophages infected with recombinant or empty retrovirus (pGEZ/LMP2A or pGEZ) were used as target cells. Transduced LMP2A will be quantified at the protein level and correlated to retroviral transduction efficiency, i.e. the proviral copy member. Flow cytometry of intact infected macrophages with rabbit anti-LMP2A antibody will be used to document surface expression of transduced LMP2A. For retroviral vector pGEZ containing GFP report gene, goat anti-rabbit conjugated with PE were then used as second Abs. GFP report gene expressed in unscreened infected macrophage cells under fluorescent microscope. As shown in Figure 4, flow cytometry analysis results revealed 56.4% of the whole infected cells expressing LMP2A protein. Unlike peptide-loaded target cells, HLA genotyping of donors can not be considered by using the infected autologous cells as targets.

Cytotoxic specificity of CTL induced by LMP2A loaded DC

To determine if the CTLs generated by LMP2A loaded DCs had cytotoxic activity and the cytotoxicity is specific, CTLs were tested against a panel of targets in cytotoxicity assays. LMP2A-specific CTLs were generated from healthy, HLA-A*0201-positive, EBV-seropositive healthy donors by stimulating $CD8^+$ T cells with autologous LMP2A-expressed macrophages. At effector: target ratio from 20:1 to 5:1, CTLs generated by autologous LMP2A loaded DCs lysed autologous macrophages infected with retrovirus pGEZ/LMP2A (Figure 4, 63.8 ± 3.26%, 51.3 ± 1.19% and 37.9 ± 1.58%, respectively), where as autologous LCLs (34.1 ± 1.91%, 22.8

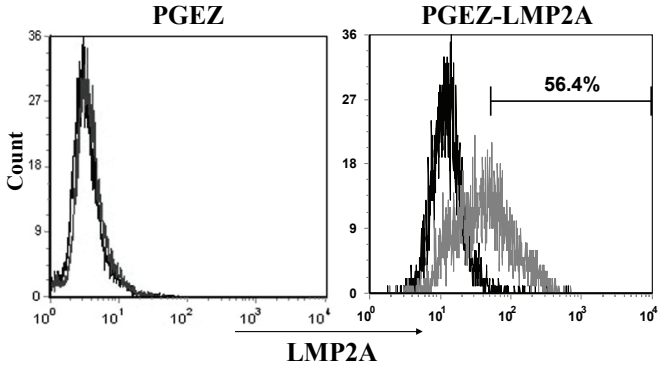

Figure 4. Macrophages infected by recombinant retrovirus pGEZ/LMP2A could express the LMP2A protein. Macrophages were infected with recombinant retrovirus three times to establish the target cells for CTL cytotoxity. FACS analysis showed about 56.4% of LMP2A expression in unscreened infected macrophages. These cells were treated with rabbit anti-LMP2A, and then incubated with a PE-conjugated secondary Ab. The figure shown is representive of five donors. No expression of LMP2A protein was detected in macrophages infected with empty pGEZ.

± 2.1 and 14.5 ± 1.82%, respectively), or mismatched LCLs (10.4 ± 0.94%, 8.2 ± 0.95 and 6.3 ± 0.87% respectively), macrophages infected with empty retrovirus (10.5 ± 0.98%, 9.3 ± 1.22 and 7.2 ± 0.65%, respectively) and MHC un-matched K562 cells (11.3 ± 1.26%, 6.8 ± 1.36 and 5.9 ± 0.51% respectively) were resistant to lyse. The cytotoxicity of the CTLs increased (79.7 ± 2.13%, 71.5 ± 3.04 and 53.7 ± 1.68% respectively) when stimulating time extended to 21 days whereas no increase found in groups using macrophage-pGEZ, autologous or mismatched LCL, K562 as target cells (Figure 5, $p < 0.05$).

Intracellular IFN-γ levels of EBV-LMP2A specific CD8$^+$ T cells in EBV healthy carriers

To determine the intracellular IFN-γ levels of EBV-LMP2A specific CD8$^+$ T cells in EBV healthy carriers, we used flow cytometry to analyze percentages of intracellular IFN-γ positive CD8$^+$ T cells in EBV healthy carriers. When stimulated with target cells, LMP2A loaded DCs stimulating group showed higher percentages of IFN-γ$^+$CD3$^+$CD8$^+$ cells (Figure 6, $p < 0.01$). The unloaded DCs stimulating group only has the same percentages of IFN-γ$^+$CD3$^+$CD8$^+$ cells with unstimulating group (data not shown). These indicate loaded DC can induce functional antigen specific CD8$^+$ T cells.

Discussion

CD4$^+$ and CD8$^+$ T cells play key roles in controlling the development of nasopharyngeal carcinoma and other EBV associated diseases (1, 6, 27, 28). CD4$^+$ T cells help B cells to produce antibodies and secrete cytokines to enhance anti-tumor immunity by stimulating NK cells, macrophages and CD8$^+$ cytotoxic T cells as well. CD8$^+$ T cells are the major effector of specific anti-tumor immunity. They recognize

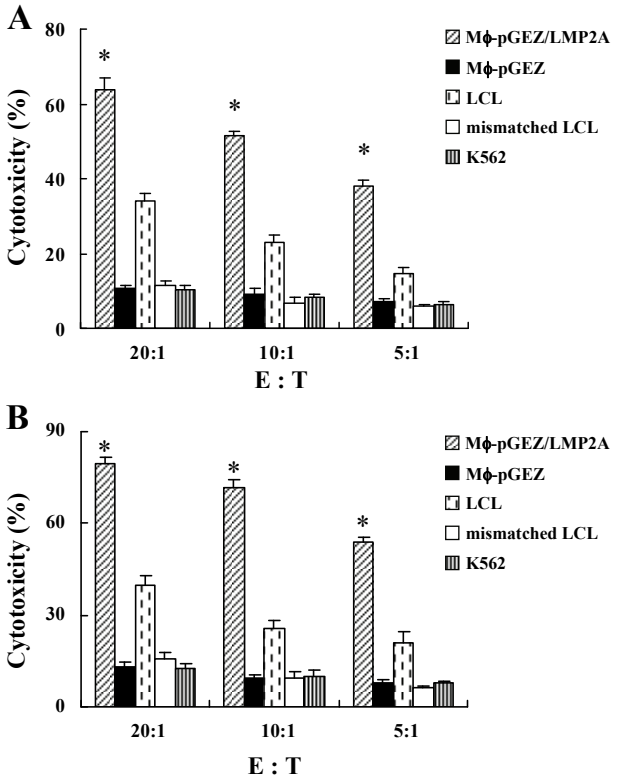

Figure 5. Cytotoxic assays of the effector cells. LMP2A loaded DCs-induced cytotoxic T lymphocytes, against specific target cells, autologous macrophages (Mφ) expressing LMP2A at a ratio from 20 : 1 to 5 : 1. Other panels of control targets are macrophages infected with empty pGEZ, LCL, mismatched LCL, and K562 cells. CD8$^+$ T cells from five donors collected 2 weeks (A) and 3 weeks (B) after stimulated once or twice with LMP2A loaded DCs, sorted, and detected for their cytolytic activity against a panel of the targets. The results showed that the cytotoxicity of the LMP2A loaded DCs-induced CTLs is LMP2A specific and also enhanced with the increased E: T cell ratio and loaded DCs stimulated times. Results are expressed as the mean of triplicate assays. *$p < 0.05$, compared with other panels of control targets.

tumor antigens by TCR and kill tumor cells directly (29-33). However, in EBV latency type II malignancy, LMP2 specific immune responses can be detected in 84% patients (24). That implies malignancies developed despite the patients have a competent immunity. This phenomenon indicates an insufficiency of endogenous T cells to stop tumor growth. Thus, *ex vivo* expansion of the tumor-specific T cells is a potential strategy to circumvent the situation of immunosuppression. In our previous studies, we have shown using protein loaded DCs did not result in DCs dysfunction and were successfully induced functional HLA restrictive CTL *in vitro* (26). Here, we further studied the subtypes of effector T cells and their immune responses towards targets cells. Our results indicate using LMP2A protein loaded DCs can elicit both CD4$^+$ and CD8$^+$ T cell mediated immunity towards LMP2A antigen *in vitro*.

Immature DCs have the capacity to capture and process

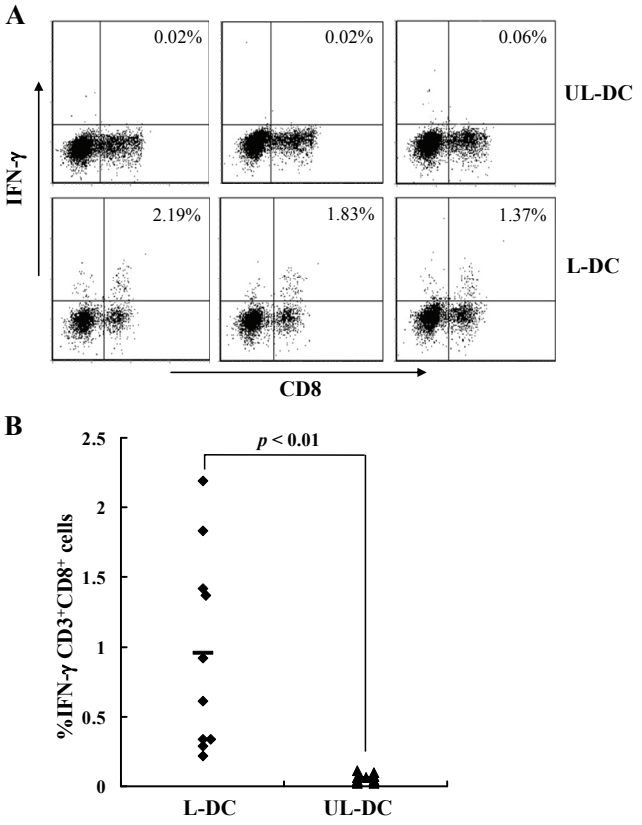

Figure 6. Flow cytometric analysis of percentages of CTL precursors (CTLp) and IFNγ-secreting lymphocytes obtained from LMP2A loaded or unloaded DCs-induced PBMCs in response to autologous macrophages expressing LMP2A. (A) Representative results of all five EBV healthy carriers using antibodies against CD3, CD8, and IFN-γ. The gates were set using the anti-CD3 antibody. Cells in the upper right quadrant were IFN-γ^+CD3$^+$CD8$^+$ T cells and were considered to be CTL precursors. A clear population of cells doubly stained for CD8 and intracellular IFN-γ^+ in all the donors. (B) The percentage of CD8$^+$/IFN-γ^+ cells in PBMCs (ranging from 0.22% to 2.19%) from 5 donors is shown in the figure, results expressed as the mean ± SD of three separate experiments. The frequency of CTLs induced by loaded DCs is higher than that from unloaded DCs ($p < 0.01$). L-DC, LMP2A loaded DC; UL-DC, LMP2A unloaded DC.

exogenous antigens. Once maturing, DCs can cross-present processed antigens with MHC class I and MHC class II molecules (34). DC vaccine has been considered as a novel therapeutic tool of cancer (35, 36). EBV-LMP2A has many potential MHC class I and II epitopes, which make LMP2A an ideal antigen for eliciting both CD4$^+$ and CD8$^+$ T cell mediated anti-tumor immunity (22-26, 37). In our study, we used purified EBV-LMP2A protein as an antigen to load DC directly. Using purified protein to load DCs has more advantages than other methods such as viral vector loading, peptides loading or liposome loading. First, full-length protein loading can keep all of the potential MHC class I and II epitopes for DCs processing and presenting. Second, protein loading appears much safer than using viral vector loading. Third, protein loading mimics physiological DCs capture and processing antigen in the body. It may maximize the capacity of DCs to present antigen and initiate immunity with minimized by side effects. Our study showed using LMP2A protein loading DCs can stimulate proliferation of both CD4$^+$ and CD8$^+$ T cells comparing with unloaded DCs. It suggests protein loading would not interfere with maturation and antigen presentation of DCs. And most importantly, DCs loaded with LMP2A can cross-present processed antigen *via* both MHC class I and II pathway, which is critical for anti-tumor immunity.

CTL are major effectors in anti-tumor immunity. Two mechanisms are involved in the cytotoxicity of CTL. One is *via* directly kills tumor cells through direct lysis or inducing apoptosis of tumor cells. Whether a tumor immunotherapy is successful depends mostly on its efficacy and efficiency to elicit tumor specific CTL (16, 25-28, 38). In our study, we found LMP2A protein-loaded DCs can induce CTL responses against LMP2A expressing target cells. The subgroups of the CTL contain CTL which lyses target cells directly and also participate anti-tumor immune response by secreting IFN-γ and TNF-α. The latter has been proved to be able to destroy tumor cells indirectly.

TH1 type CD4$^+$ T cells secret cytokines to activate other components of immune system. It is believed that a shift from TH2 to TH1 type immune response is critical for immune system to fight against tumor cells. This shift is determined by the cytokine profile of the antigen presenting cells (APCs) (39-41). DCs can secrete IL-12, a TH1 type cytokine, to induce Th0 T cells differentiate into TH1 type T cells when they present tumor antigen (34, 42). In this study, we found CD4$^+$ T cells induced by LMP2A-loaded DCs produced a large amount of IFN-γ, but no IL-10, which indicates they are Th1 type. These CD4$^+$ T cells can enhance anti-tumor immunity by activating NK cells, macrophages and CTL by producing IFN-γ and other cytokines.

In summary, this study showed that purified LMP2A protein loaded DCs efficiently stimulated both CD8$^+$ and CD4$^+$ T cells which have specific immune-function against EBV-LMP2A expressing target cells *in vitro*, therefore suggesting that LMP2A protein loaded DCs are promising polyvalent vaccine for immunotherapy targeting LMP2A-expressing EBV associated malignancy. Future studies will be conducted in the tumor models we have established (43), and the immune response types will be monitored carefully to evaluate the efficacy of the DC vaccine.

Acknowledgements

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