

Recombinant adeno-associated virus encoding Epstein-Barr virus latent membrane proteins fused with heat shock protein as a potential vaccine for nasopharyngeal carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is a common cancer in Southern China and EBV is the most important pathogenesis. In this study, we explore the potential that a recombinant adeno-associated virus (rAAV) carrying a fusing gene containing heat shock protein as an adjuvant, EBV latent membrane proteins (LMP1 and LMP2) CTL epitope DNA as a vaccine prevents NPC. The tumor vaccine was devised by constructing a chimeric gene which contained EBV LMPs CTL epitope DNA fused with the heat shock protein gene as a tumor vaccine delivered via rAAV. Our results show that this vaccine can eliminate tumors in syngeneic animals and induce CTL activity *in vitro*. Taken together, the data suggest that this chimeric gene delivered by rAAV has potential as a NPC vaccine for prevention and therapy. [Mol Cancer Ther 2009;8(9):2754–61]

Introduction

Nasopharyngeal carcinoma (NPC), a tumor endemic to southern China, has three unique etiologic factors including genetic susceptibility, chemical carcinogens, and association with EBV infection (1–4).

EBV, also referred to as the human herpesvirus-4, is a double-stranded DNA γ -herpesvirus with a 172 kb genome (5). EBV infection occurs worldwide and most people become infected during their lifetime. Infection with EBV usually

occurs at a very early age, particularly in developing countries, and is strongly associated with NPC, Hodgkin's disease, Burkitt's lymphoma, and gastric carcinomas (6–8). It has been argued that the expansion strategies used for generating EBV-specific CTLs in these studies were dominated by a T-cell repertoire specific for EBV nuclear antigens, which are not expressed in the malignant cells of NPC. Hence, there is considerable interest in the possibility of targeting the virus-specific immune response to the viral antigens, which are expressed in these malignancies. These antigens include EBNA1, BARF0, and latent membrane proteins (LMP1 and LMP2). Of these, LMP1 and LMP2 are the only potential targets because EBNA1 and BARF0 are poorly processed and presented by virus-infected cells through the MHC class I pathway (9). Previous studies have shown a low CTL precursor frequency to epitopes within LMP1 in healthy virus carriers, suggesting that reconstitution of both LMP1-specific and LMP2-specific CTL responses may be necessary for a long-term therapeutic benefit in patients with NPC (10).

Recombinant adeno-associated virus (rAAV), a single-stranded virus, has been studied as a vector for gene therapy (11). Classified as a defective human parvovirus, rAAV has many natural features that are attractive for a human gene therapy vector, such as nonpathogenicity, targeted integrating capability, and a broad host range (human, simian, murine, canine, and avian). In sharp contrast to other viral vectors that have been used in vaccination, such as vaccinia virus or adenovirus, rAAV vectors do not express any viral genes. The only viral DNA that must be included in an rAAV vector is the 145-bp inverted terminal repeat. Because naked DNA is used for immunization, the only gene expressed by rAAV vectors is that for the antigen itself.

Because LMP1 is a transforming oncoprotein (12), dangerous side effects such as transformation are not anticipated with a protein vaccine. Peptide vaccination with a CTL epitope to prevent the outgrowth of a tumor is a safe and effective immunotherapeutic method (13). However, a peptide vaccine combined with a toxic adjuvant such as incomplete Freund's adjuvant can sometimes lead to rapid tumor growth through specific T-cell tolerance induction (14). Recently, *Mycobacterium tuberculosis* heat shock protein 70 (hsp70) has been used as an adjuvant-free carrier to stimulate the humoral and cellular response to a full-length HIV p24 (15) that is covalently linked to hsp70. Mycobacterial hsp as an adjuvant has also been reported to enhance the induction of cellular immunity by an ovalbumin peptide vaccine (15).

In this study, we have accordingly taken advantage of the safety of a peptide vaccine with hsp as an adjuvant and rAAV as a gene delivery vector to devise a vaccine by constructing a chimeric gene containing the EBV LMP1/LMP2 CTL epitope and hsp70. The efficacy of protection against tumors was

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investigated through the use of the vector rAAV against syngeneic tumors. Results show that i.m. vaccination with this chimeric gene via rAAV vector could efficiently eliminate tumor cells, indicating that vaccination with this gene could be a therapeutic treatment for NPC containing EBV *LMP1/2*.

Materials and Methods

Mice and Cell Lines

BALB/c (*H-2^d*) mice were housed in a 12 h light/12 h dark cycle in a pathogen-free environment and allowed *ad libitum* access to food and water. All animal studies were approved by the Animal Research Committee of Tongji Medical College and were done according to guidelines set forth by the NIH. The WT SP2/0 cells (a cell line with a BALB/c background) were grown in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum and 4 mmol/L of glutamine. In addition, the HEK 293 cell line was maintained in DMEM containing 10% fetal bovine serum.

The SP2/0 cell line was transfected with plasmid pCDNA3.1-*LMP2* via LipofectAMINE 2000 Reagent (Invitrogen) to allow stable *LMP2* expression, and the positive cell clones were selected by G418 (400 µg/mL) and named SP2/0-*LMP2* cell line.

Production of Antibodies against LMP2

LMP2 amino acids 228 to 242 synthetic peptide (GGLQGIYVLMVLL) was synthesized by Beijing AuGCT Biotechnology, Co., Ltd. The peptide and adjuvant were mixed thoroughly to form a stable emulsion that was injected beneath the skin of two New Zealand white rabbits (provided by Experimental Animal Center, Tongji Medical College, Wuhan, PR China). After 6 wk, the rabbits were sacrificed, and their blood was collected and purified to acquire rabbit anti-*LMP2* polyclonal antibodies (IgG).

Construction of Recombinant cDNA for Fused Proteins

The DNA fragment containing the EBV *LMP2* amino acids 131 to 461 coding sequence was synthesized by PCR using the plasmid EBV *LMP2*/pMD18-T as a template. This amplified nucleic acid sequence of the fragment encoded a Kozak consensus sequence, with restriction sites of a *Hind*III and *Apa*I at both ends, a methionine start codon, and *LMP2* sequence. The fragment was digested with *Hind*III and *Apa*I, cloned into pAAV-D(+) green fluorescent protein (GFP) vector, and named pAAV-*LMP2*-GFP.

The DNA fragment containing the EBV *LMP1* amino acid 51 to 159 coding sequence was synthesized by PCR using the plasmid EBV *LMP1*/pMD18-T as a template, and then subcloned into pAAV-*LMP2*-GFP vector (downstream of *LMP2*), and named pAAV-*LMP2/1*-GFP.

The DNA fragment containing the *hsp70* amino acids 1 to 620 coding sequence was synthesized by PCR using the plasmid *hsp70*/pMD18-T as a template. This amplified *hsp70* fragment was cloned into the pAAV-*LMP2/1*-GFP plasmid downstream of the *LMP1* sequence by digestion with *Bam*HI and *Xba*I to remove the GFP cDNA, and the newly formed plasmid was named pAAV-*LMP2/1-hsp* (Fig. 1A). The primers used for these cDNA clones were synthesized by Beijing AuGCT Biotechnology and their

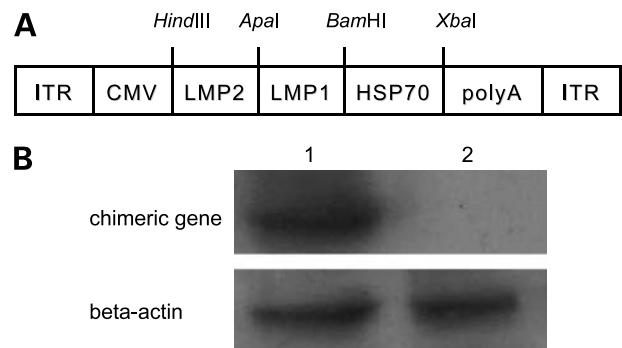


Figure 1. Construction and expression of *LMP2/1-hsp* chimeric gene. **A**, scheme of the construction of a rAAV with *LMP2/1-hsp* chimeric gene that expresses a sequence encoding for a polypeptide protein that contains 13 HLA class I-restricted *LMP1* and *LMP2* epitopes. **B**, representative Western blot. Samples of 293 cells were prepared 24 h after infection with rAAV-*LMP2/1* CTL-*hsp* or rAAV-GFP and results showed that *LMP2/1-hsp* chimeric gene (110 kDa) expression in infected cells (lane 1) but not in the control cells (lane 2).

sequences included 5'-GCCCaagcttGCCACCATGAATC-CAGTATGCCTGCC-3' (forward for *LMP2*), 5'-TAG-CAGggcccGAGGAAAATCAGGAATCCTGC-3' (reverse for *LMP2*), 5'-TATGCgggcccGCCCTCCTTGCTCTATTCC-3' (forward for *LMP1*), 5'-GCACTAggatccTAGAGTCCAC-CAGTTTTGTTGTAAG-3' (reverse for *LMP1*), 5'-GCACTAggatc-cATGGCTCGTGGGTCGGGATC-3' (forward for *hsp70*), and 5'-CTGCGtctagaTTATCACTTGGCCTCCCGGCCGTC-3' (reverse for *hsp70*).

Generation of rAAV Containing *LMP2/1-hsp* Fusing Gene

rAAV-*LMP2/1-hsp* viral vector was prepared, with modifications, by cotransfection according to published protocols (16). Briefly, a total of 49 mg of plasmid DNA (16 mg of pAAV-*LMP2/1-hsp* plasmid plus 8 mg of pXX2, which encoded Rep and Cap proteins, and 25 mg of pXX6, which encoded adenovirus gene products) was used to transfect 293 cells in each 15 cm diameter dish using a modified calcium phosphate precipitation method. Cells from 80 dishes were harvested 48 h posttransfection, then cell mixtures were homogenated, and CsCl was added to a final density of 1.37 g/mL. The virus particles were then purified by CsCl density gradient centrifugation as previously published (17). Titers of rAAV-*LMP2/1-hsp* were determined by slot blot analysis to calculate the relative concentration of viral particles. The vector titers were in the range of 1×10^{11} /mL to 1×10^{12} /mL viral particles. rAAV-GFP was also prepared as a control.

Western Blot Analysis of Chimeric *LMP* Gene Expression

To determine whether the chimerically constructed *LMP2/1CTL-hsp* gene can be expressed via rAAV delivered to cells, we infected 293 cells using 10^9 viral particles of rAAV-*LMP2/1CTL-hsp*. Two days later, the cellular protein was harvested and the expression of the *LMP2/1-hsp* chimeric gene was analyzed by Western blotting with rabbit anti-*LMP2* polyclonal antibodies.

Tumor Challenge

Two different vaccination strategies were used to assess the efficacy of the rAAV vaccine. In the first set of experiments, BALB/c mice were immunized with either rAAV-LMP2/1-hsp or rAAV-GFP (5×10^{10} viral particles/mouse) into the hind leg tibialis anterior muscles. Three weeks after the immunization, these mice were challenged s.c. with live SP2/0-LMP2 or SP2/0 cells (10^6 cells/mouse, $n = 6$ for each group). After challenge, these animals were regularly monitored for 30 d, and the tumor size was measured by calipers.

In the second set of experiments, BALB/c mice were first challenged with SP2/0-LMP2 or SP2/0 (10^6 cells/mouse, $n = 6$ for each group) tumor cells. Ten days after the challenge, when the tumor size was 0.2 cm in diameter, mice were immunized i.m. with either rAAV-LMP2/1-hsp or rAAV-GFP (5×10^{10} viral particles/mouse). The therapeutic efficacy of the rAAV vaccine was assessed by regular monitoring of tumor regression.

In vitro Cytotoxicity Assays

To assess the cellular immune response of the rAAV vaccine, BALB/c mice were vaccinated with 5×10^{10} viral particles of recombinant virus encoding LMP2/1, rAAV2 GFP, or normal saline (mock) into the hind leg tibialis anterior muscles ($n = 6$ for each group). After 3 wk, mice were sacrificed, and spleen cells were isolated with a nylon fiber column to enrich the T cells. Target cells were SP2/0-LMP2 or WT SP2/0, and effector cells were spleen cells. Target cells were washed thrice with PBS, resuspended in RPMI 1640, and mixed with effector cells at a 10:1, 20:1, 40:1, or 80:1

effector-to-target (E/T) ratio. After 4 h of incubation, lactate dehydrogenase release was measured using the Cytotox 96 cytotoxicity assay from Promega.

$$\text{Specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100\%$$

T-Cell Proliferation Assays

BALB/c mice were vaccinated with 5×10^{10} viral particles of rAAV-LMP2/1-hsp, rAAV-GFP, or normal saline (mock) into the hind leg tibialis anterior muscles ($n = 6$ for each group). After 3 wk, mice were sacrificed, and spleen cells prepared from animal spleens were pulsed *in vitro* with or without LMP2 amino acids 131 to 139 synthetic peptide (PYLFWLAAI), a CTL epitope, or pulsed with nonspecific peptide (LLAYPLKIFP) in peptide-coated microtiter plates for a 4-d proliferation assay. Proliferation was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Stimulation index was defined as the ratio of mean absorbance values for cells with synthetic peptide to mean absorbance values for cells without any added antigen.

In order to determine the attribution of LMP2₁₃₁₋₁₃₉-stimulated cell proliferation to CD4+ or CD8+ T cells, carboxyfluorescein succinimidyl ester staining and T-cell proliferation assays were done. Briefly, BALB/c mice were vaccinated with rAAV-LMP2/1-hsp (5×10^{10} viral particles for each). After 3 wk, the spleens were collected. Spleen cells were separated and cultured and labeled with carboxyfluorescein succinimidyl ester as described previously (18), followed by treatment with the synthetic peptide at a concentration

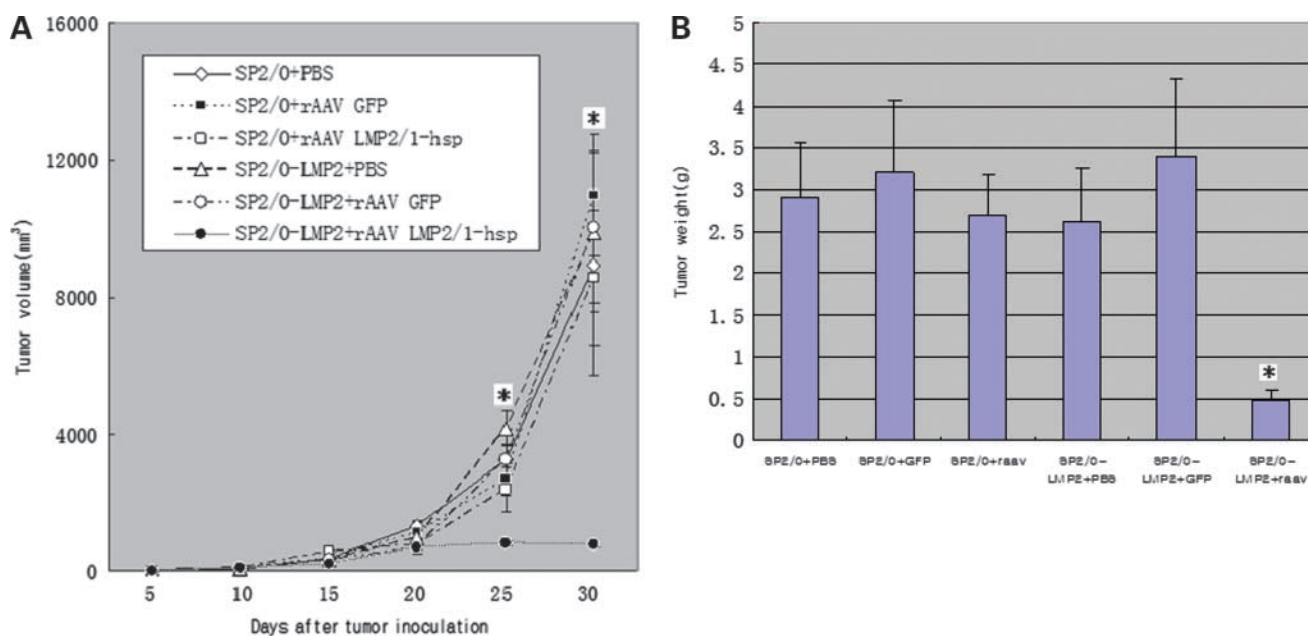


Figure 2. *In vivo* tumor elimination assay. Mice were immunized with 5×10^{10} viral particles of rAAV-LMP2/1-hsp or rAAV-GFP or PBS (mock), and after 3 wk, were injected s.c. with 1×10^6 SP2/0-LMP2 or SP2/0 tumor cells. *A*, the tumor volume was monitored once every 5 d. *B*, the tumor load was weighted by 35 d. Columns, mean; bars, SE; *, $P < 0.001$ versus SP2/0-LMP2+rAAV-LMP2/1-hsp group. Every experiment was repeated thrice.

of 200 ng/mL for 72 h. Spleen cells were then stained with monoclonal antibodies to cell surface antigens (CD4 and CD8) and evaluated by flow cytometry for the fraction of proliferating cells in different T-cell subsets.

ELISA for the Detection of Antibodies to EBV LMP2

BALB/c mice were vaccinated with rAAV-LMP2/1-hsp (5×10^{10} viral particles for each), rAAV-GFP, or normal saline (mock) into the hind leg tibialis anterior muscles ($n = 6$ for each group). After 3 wk, mice were sacrificed, and serum was collected. Serum antibodies to EBV LMP2 were measured by ELISA. Ninety-six-well microtiter plates were coated with LMP2_{228–242} peptide (10 mg/L) in 100 μ L of 0.1 mol/L carbonate-bicarbonate buffer (pH 9.6) at 4°C for 24 h. After the wells were blocked with 1% bovine serum albumin for 1 h, blocking buffer–diluted serum samples (1:500) were added to the wells and incubated for 2 h at 37°C, followed by the addition of horseradish peroxidase–conjugated goat anti-mouse IgG (1:1,000), and the plates were incubated for another 2 h. After the plate was washed, 100 μ L of 3,3',5,5'-Tetramethylbenzidine were added to each well and then the reaction was stopped by adding 50 μ L of 0.5% hydrofluoric acid. The absorbance values were measured at 450 nm on an ELISA plate reader (Bio-Rad).

Statistical Analysis

Continuous data were expressed as mean \pm SE. Comparisons between groups were done by a Student's paired two-tailed *t* test. $P < 0.05$ were considered statistically significant.

Results

rAAV Gene Expression

Western blots showed that \sim 110 kDa of protein was detected by anti-LMP2 antibodies in rAAV-LMP2/1-hsp-infected cells but not in the control cells infected with rAAV-GFP (Fig. 1B), which suggests that the chimeric LMP2/1-hsp gene (lane 1) can be efficiently expressed via rAAV-mediated delivery.

Vaccination with rAAV-LMP2/1-hsp Generates Tumor Elimination

To test whether the rAAV-LMP2/1-hsp vaccine-induced T-cell responses can afford protection against LMP2-expressing tumor cells, BALB/c mice (six mice in each group) were first immunized with rAAV-LMP2/1-hsp or rAAV-GFP, and then challenged with SP2/0 or SP2/0-LMP2 cells. These mice were regularly checked for tumor outgrowth. Although both groups of animals developed tumors, the tumor outgrowth in rAAV-GFP was highly aggressive (Fig. 2A). In contrast, these tumors grew much less aggressively in animals immunized with rAAV-LMP2/1-hsp, and outgrowth was completely resolved in 90% of the animals by the end of the observation period in mice with the load of SP2/0-LMP2 cells. By day 35, the average tumor load in rAAV-LMP2/1-hsp-immunized mice was 5-fold lower ($P < 0.001$) when compared with rAAV-GFP-immunized mice (Fig. 2B). It is important to mention here that animals immunized with rAAV-LMP2/1-hsp or rAAV-GFP showed no protection against challenge with SP2/0

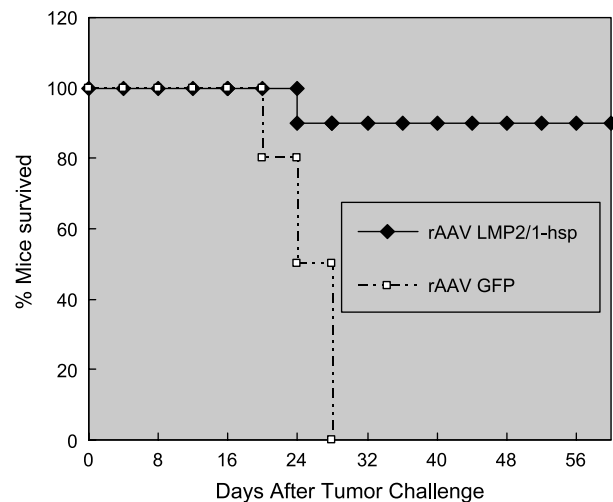


Figure 3. Survival time assay. BALB/c mice were challenged with 10^6 SP2/0-LMP2 tumor cells. Eight days after the challenge, when the tumor size was 0.2 cm in diameter, these mice were immunized with either rAAV-LMP2/1-hsp or rAAV-GFP (5×10^{10} viral particles/mouse) and then survival time was recorded. The data were the averages of 10 vaccinated mice from each group and presented as a percentage of mice surviving after immunization with rAAV-LMP2/1-hsp or rAAV-GFP.

cells, indicating that the epitope-specific immune response was critical for this protection.

Vaccination with rAAV-LMP2/1-hsp Extends Survival Time

In the second set of experiments, following immunization, the tumor size progressively increased in almost all animals injected with rAAV-GFP, and by day 20 after immunization (day 30 after tumor challenge), 100% of the mice were dead. In contrast, a dramatic reduction in the tumor load was observed in those mice immunized with rAAV-LMP2/1-hsp, and showed long-term protection (Fig. 3). Moreover, the average tumor load in rAAV-LMP2/1-hsp mice was significantly lower when compared with the rAAV-GFP-vaccinated mice (data not shown).

Cellular Immune Response in Mice Immunized with rAAV-LMP2/1-hsp

To elucidate the mechanism of protection against SP2/0-LMP2 tumors, we determined whether a CTL response was induced in mice immunized with rAAV-LMP2/1-hsp. T cells from BALB/c mice immunized with rAAV-LMP2/1-hsp, rAAV-GFP, or PBS were isolated and stimulated *in vitro* with LMP2 amino acids 131 to 139 synthetic peptide (PYLFWLAAI). These stimulated T-cells were then tested for recognition and lyses of target cells, i.e., SP2/0-LMP2, WT SP2/0, WT SP2/0 pulsed with LMP2 peptides 131 to 139, and WT SP2/0 pulsed with nonspecific peptide (LLAYPLKIFP), results showed that SP2/0-LMP2 and WT SP2/0 cells pulsed with LMP2 peptides 131 to 139 were lysed (Figs. 4A and C), whereas WT SP2/0 cells alone or WT SP2/0 cells pulsed with nonspecific peptide were not lysed (Figs. 4C and D). The CTL response to SP2/0-LMP2 cells was significantly higher than that to WT SP2/0 cells in the rAAV-LMP2/1-hsp-vaccinated mice by Student's *t* test ($P < 0.01$), and the

CTL lysis activity for LMP2 peptide-pulsed SP2/0 cells was also higher than that for nonspecific peptide-pulsed cells ($P < 0.05$).

Moreover, we assayed for stimulation of LMP2 peptides 131 to 139-specific MHC class I-restricted proliferation response. Results showed that T cells from mice vaccinated with rAAV-LMP2/1-hsp could be stimulated by LMP2 peptides 131 to 139, rather than the cells from rAAV-GFP-vaccinated and mock-vaccinated mice (~3.5-fold of control) and the T cells from mice vaccinated with rAAV-LMP2/1-hsp could not be stimulated by nonspecific peptides. Taken together, these results suggest that vaccination with rAAV-en-

coding LMP2/1-hsp may induce CTL response to retard tumor growth (Fig. 5A).

Carboxyfluorescein succinimidyl ester staining assay showed that the proportion of CD8+ T cells was increased from $5.43 \pm 0.82\%$ to $35.67 \pm 5.76\%$ after peptide treatment (Fig. 5B). The proliferation index of CD8+ T cells was 2.16 ± 0.35 , and the index of CD4+ T cells was 1.04 ± 0.02 (Fig. 5C). These results showed that the LMP2₁₃₁₋₁₃₉-stimulated T-cell proliferation response was attributed to CD8+ T cells.

In order to prove the effect of the purification, the ratio of T cells was detected by flow cytometry, and results showed that the ratio of T cells increased by ~10% (data not shown).

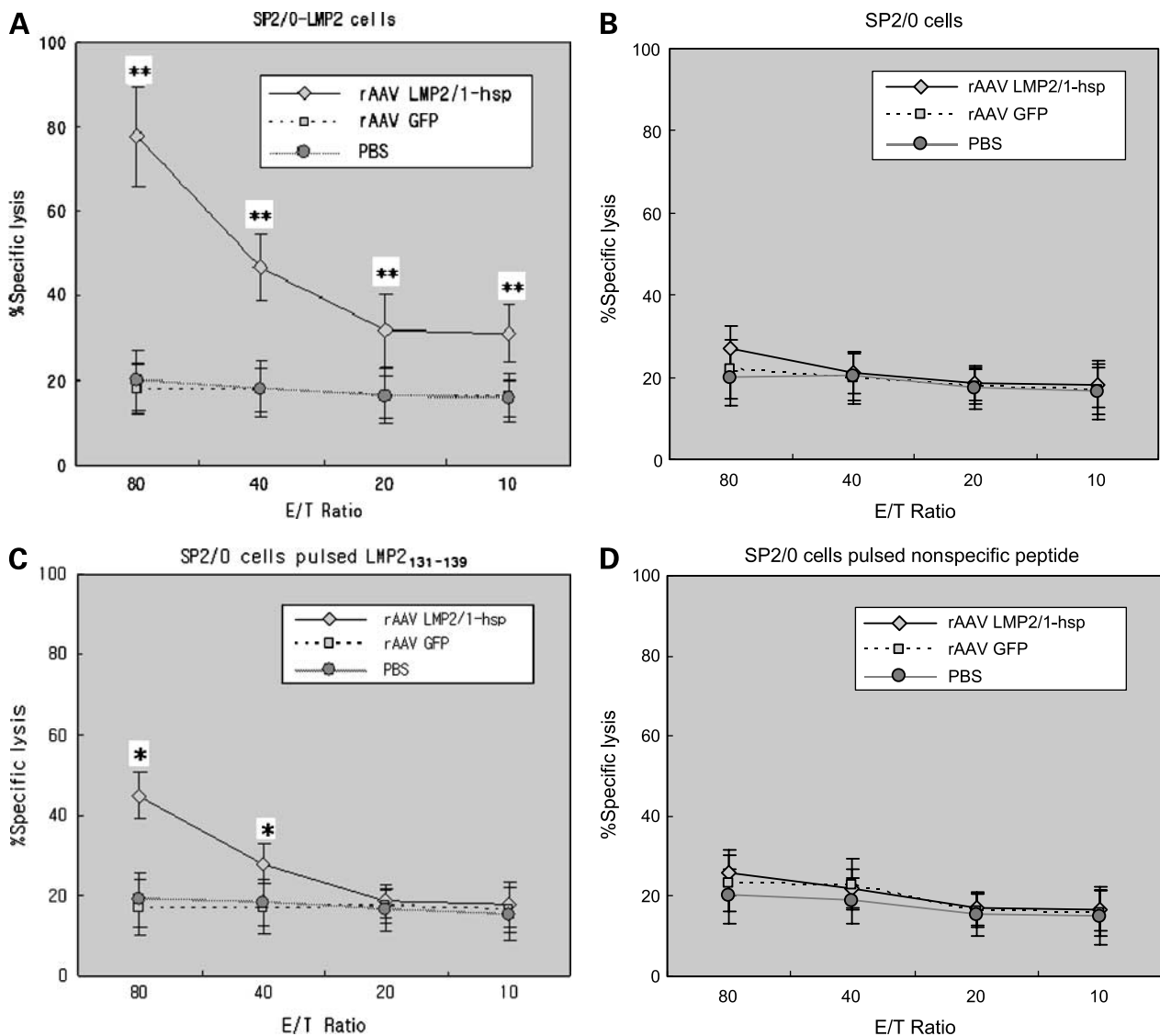


Figure 4. LMP2 peptide₁₃₁₋₁₃₉ responses induced by immunization with rAAV-LMP2/1CTL-hsp. BALB/c mice were immunized i.m. with rAAV-LMP2/1CTL-hsp. Four weeks after the vaccination, the T cells were collected and analyzed for *in vitro* CTL assay. **A**, SP2/0-LMP2 cells; **B**, SP2/0 cells; **C**, SP2/0 cells pulsed with LMP2 peptide₁₃₁₋₁₃₉; and **D**, SP2/0 cells pulsed with nonspecific peptide. The data were the averages of six vaccinated mice. E/T, effector/target ratio; *, $P < 0.05$ versus controls and **, $P < 0.01$ versus controls.

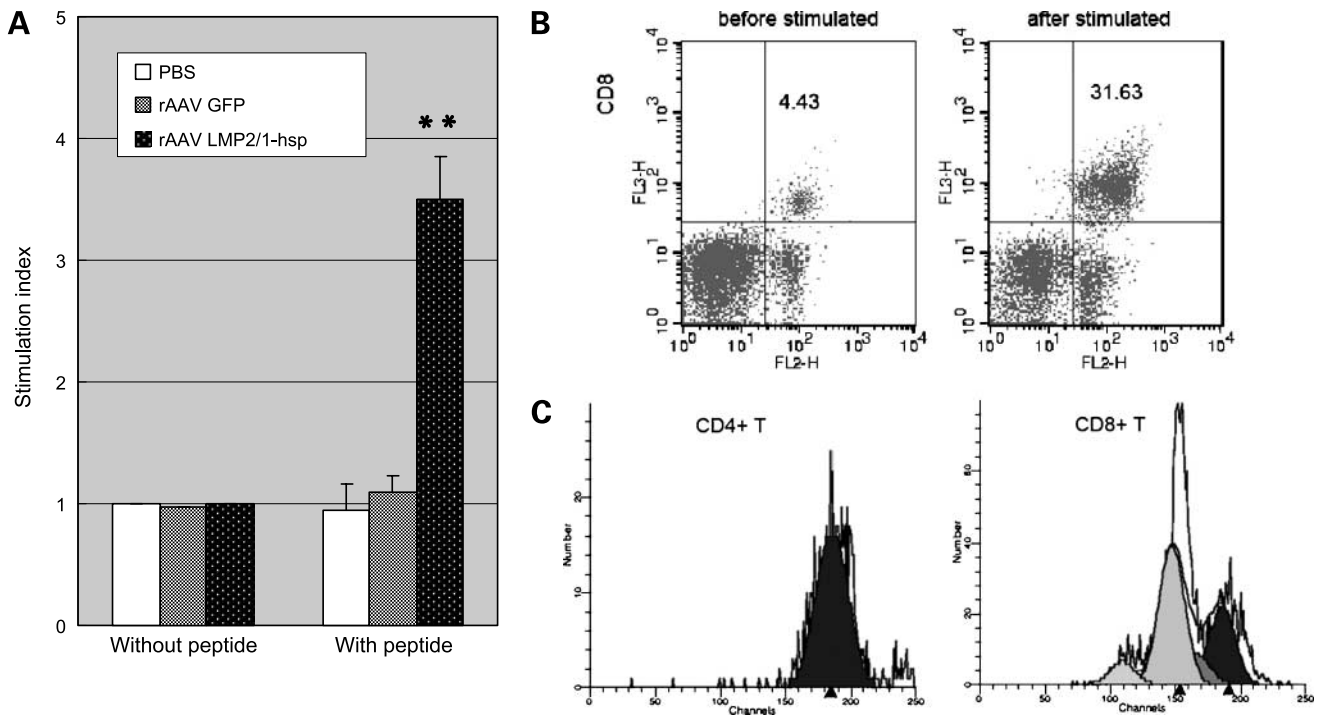


Figure 5. Proliferation assays of rAAV-LMP2/1-hsp-vaccinated lymphocytes in response to LMP2 peptide 131 to 139. **A**, BALB/c mice were immunized i.m. with rAAV-LMP2/1-hsp, rAAV-GFP, or mock. Four weeks after the vaccination, the spleens were collected and analyzed for T-cell proliferation as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data were the averages of six vaccinated mice of each group. Bars, SEs. Stimulation index was defined as the ratio of mean absorbance values for cells with antigen (LMP2 peptide 131–139) to mean absorbance values for cells without any added antigen; **, $P < 0.001$ versus controls. **B**, representative flow cytograms of T-cell subsets after LMP2 peptide_{131–139} stimulation for 72 h. **C**, proliferation kinetics model of T subsets after peptide stimulation.

Humoral Immune Response in Mice Immunized with rAAV-LMP2/1-hsp

rAAV-LMP2/1-hsp-vaccinated mice presented IgG levels against LMP2 significantly higher than all other groups

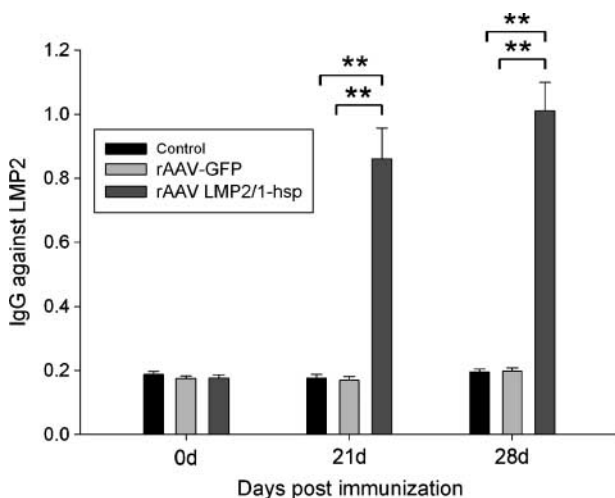


Figure 6. Humoral immune responses in mice. Serum samples ($n = 6$) were collected at various time points. Antibodies against EBV LMP2 were detected with ELISA using a single dilution (1:500). Columns, mean (absorbance values); bars, SD; **, $P < 0.01$ versus the indicated groups.

from 21 to 28 days after immunization (Fig. 6). These results suggest that vaccination with rAAV encoding LMP2/1-hsp induce humoral immune responses.

Discussion

NPC is one of the most common cancers in Southern China and Southeast Asia. The highest rates are noted among the Cantonese who inhabit the central region of Guangdong Province in Southern China, of which Hong Kong is a part.

Radiotherapy is the standard treatment for NPC. Unfortunately, it can produce undesirable complications after treatment because of the location of the tumor at the base of the skull, closely surrounded by and in close proximity to radiation dose-limiting organs, including the brain stem, spinal cord, pituitary-hypothalamic axis, temporal lobes, eyes, middle and inner ears, and parotid glands. Because NPCs tend to infiltrate and spread towards these dose-limiting organs, they are even more difficult to protect. Several studies in the past two decades have reported the results of the use of chemotherapy in combination with radiotherapy for the management of locoregional advanced cases of NPC. Nevertheless, the classic principles of chemoradiotherapy timing have not been borne out by the study results (19). Despite the use of concurrent chemoradiotherapy,

distant metastases remains the major cause of treatment failure, and the outlook for stage IV patients remains poor (20).

In addition to the novel treatment approaches that are generally applicable to cancers at other sites, the close association between EBV and NPC gives further opportunities for novel treatment. Strategies targeted at EBV include gene therapy and immune therapy, and proof-of-principle studies have been done in laboratories.

A recombinant vaccinia virus-based *LMP1* polyepitope vaccine was successfully used to immunize mice, demonstrating the potency of these formulations in inducing therapeutic immunity against *LMP1*-expressing tumors (21). An adenoviral vaccine also was successfully used to reverse the outgrowth of *LMP1*-expressing tumors (22). However, for gene therapy, AAV vector may be superior to other viral vectors that have been used in vaccination, such as vaccinia virus and adenovirus because AAV vectors do not express viral genes. As with immunization with naked DNA, the only expressed gene carried by AAV vectors is the cloned gene itself. Using vaccinia virus or adenovirus as a vector to deliver vaccine may offer some advantages in the stimulation of the immune system; however, the advantages are probably outweighed by the significant risks associated with virus infection, especially for immunodeficient patients (23).

In addition, it was previously reported that AAV vector does not induce strong cellular immune responses to the transduced cells, allowing the persistence of gene expression. The gene expression in skeletal muscle transduced by AAV has been shown to persist for more than 1.5 years (11). Although AAV vector can elicit a humoral immune response which results in neutralizing activity after a second administration, repeated dosing does not seem to be necessary given that the originally transduced cells can escape a CTL response and persist in the long-term. AAV may thus be useful for the viral immunization of humans. Up to now, for vaccination, AAV vectors represent the combination of the best properties of viral and nonviral vectors. Recently, a study of vaccine development targeted at herpes simplex virus infection has shown that AAV-mediated immunization could prime specific CTL and antibody responses (24).

As described above, vaccination with EBV LMP by rAAV can inhibit tumor growth. The response to tumor inhibition might be mediated by CTLs. The reason may be that *M. tuberculosis* hsp70 is an especially powerful antigen containing multiple B-cell and T-cell epitopes (25). Previously, *M. tuberculosis* hsp70 had been used as an adjuvant-free carrier to stimulate the humoral and cellular response to a full-length HIV p24 protein or ovalbumin peptide (15) that is covalently linked to the hsp. The mechanisms by which hsp70 enables covalently linked polypeptide fusion partners to enter into the MHC class I and II antigen-presenting pathway and to elicit CD8 CTLs have been proposed to be (a) the ability of hsp70 to assist in protein folding (26, 27) and to facilitate the translocation of proteins into subcellular compartments (28), (b) the ability of hsp to facilitate the breakdown of intracellular proteins (29), and (c) the high frequency of T cells directed against mycobacterial hsp70. Our study further supports the idea that hsp may act as an adjuvant to facilitate

LMP2/1 peptide antigen inducement of CTL responses and humoral immune responses to EBV-induced tumors.

Our study shows that AAV vector, which has all viral coding sequences (96% of the genome) removed (17), represents a promising alternative for delivering tumor vaccine (24). The study provides a potential vaccine for EBV-induced tumors. We successfully developed rAAV encoding EBV *LMP2/1* CTL peptide DNA fused with hsp DNA as a tumor vaccine. It is a potential vaccine for NPC treatment using hsp as a carrier protein and delivery by rAAV vector.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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