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Immune response of mice to a latency membrane protein 2 multiepitope antigen of Epstein-Barr virus applied as DNA vaccine and/or peptide vaccine

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Summary. – To evaluate immune responses of mice to a latent membrane protein 2 (LMP2) multiepitope antigen of Epstein-Barr virus (EBV), four kinds of prime-boost strategies were applied. In group 1, mice were primed and boosted with DNA vaccine delivered by human papillomavirus (HPV) major capsid protein L1 on weeks 0, 2, 4, and 6. Mice in group 2 were primed with DNA vaccine on weeks 0 and 2, and boosted with peptide vaccine on weeks 4 and 6. In group 3, mice were primed with peptide vaccine on weeks 0 and 2, and boosted with DNA vaccine on weeks 4 and 6. Mice in group 4 were primed and boosted with DNA vaccine together with peptide vaccine on weeks 0, 2, 4 and 6. EBV-LMP2-specific IgG, IgG1, IgG2a, and IgA antibodies, and HPV L1-specific IgG antibodies were determined by ELISA. Cytotoxic T-lymphocytes (CTL) activity was measured by LDH release assay. The results of this study show that priming with DNA vaccine, and boosting with peptide vaccine (group 2) induced a significant humoral immune response, and also an effective CTL activity, which could be regarded as an optimal prime-boost strategy for improving the immune effects of EBV-LMP2 multiepitope antigen.

Keywords: human papillomavirus 6; major capsid protein L1; Epstein-Barr virus; latent membrane protein 2; multiepitope antigen; virus-like particles; DNA vaccine; peptide vaccine; prime-boost strategy

Introduction

Epstein-Barr virus (EBV) is a human oncogenic herpesvirus associating with a spectrum of chronic and malignant diseases (Young and Rickinson, 2004). There is now compelling evidence to suggest that EBV is a causal agent of nasopharyngeal carcinoma (NPC), which is a common cancer among Southern Chinese (Wong *et al*., 2005). EBV mainly affects B lymphocytes, and the latent membrane protein 2 (LMP2) of EBV is the only viral gene product expressed on the B cell surface during the period of EBV latent infection (Rechsteiner *et al*., 2008). The sequence of LMP2 is extremely conserved, and contains multiple high-frequency HLA allele restricted cytotoxic T lymphocyte (CTL) epitopes with high incidence in Chinese NPC patients (Whitney *et al*., 2002). Thus, LMP2 is considered to be an ideal candidate for NPC immunotherapy. However, it is hard to cover broad populations for single CTL epitope vaccine because of HLA restriction. In our previous work we have designed CTL-, Th-, and B-cell epitope-rich peptide clusters (multiepitope) from EBV LMP2 in order to induce specific cellular and humoral immune response in broad populations (Zhu *et al*., 2010).

The major capsid protein L1 of human papilloma virus (HPV) expressed *in vitro* and *in vivo* can self-assemble into virus-like particles (VLPs), which are highly immunogenic and able to induce anti-viral humoral and cellular immune responses. Deletion or replacement of 60 aa at C-terminal of HPV L1 did not disturb the formation of VLPs and immunogenicity, and conformation of foreign protein could still be retained (Freyschmidt *et al*., 2004; Paz De la Rosa *et al*., 2009; Liu *et al*., 2000; Peng *et al*., 1998; Slupetzky *et al*., 2001). Therefore, we selected the mammalian codon optimized HPV-6 type L1 (HPV L1) as the vehicle for EBV LMP2 multiepitope delivery, in order to increase immunogenicity and keep conformation of multiepitope. In previous study we had

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Abbreviations: CTL = cytotoxic T lymphocytes; EBV = Epstein-Barr virus; E:T = effector/target cells ratio; HPV = human papillomavirus; LMP2 = latent membrane protein 2; NPC = nasopharyngeal carcinoma; VLPs = virus-like particles

constructed recombinant plasmid pcDNA3.1(+) containing mammalian codon optimized HPV L1 gene and EBV LMP2 multiepitope (pcHPV L1-EBV LMP2). We have confirmed that HPV L1 as a vehicle successfully delivered EBV LMP2 multiepitope by MHC class I and class II pathways to be presented to immunocytes and induced comprehensive immunological effect (Zhu *et al*., 2010).

Now, many researchers believe that vaccine for infectious pathogen needs to induce both sustained humoral immune response and strong cell-mediated response (Smith *et al*., 2010; Gomez-Roman *et al*., 2006; Wei *et al*., 2010; Kuo-Haller *et al*., 2010). Besides immunogenicity of antigen, immunization strategy is also an important factor to induce effective immune response. Therefore, a heterologous prime-boost immunization is considered to be a promising strategy: a DNA or vector vaccine to elicit cytotoxic T cells that destroy infected cells followed by a subunit vaccine to induce effective antibodies. The aim of this study was to find out an effective prime-boost strategy for improving the immune effect of EBV LMP2 multiepitope antigen in mice administrated by DNA vaccine and/or peptide vaccine.

Materials and Methods

Mice. 6-to 8-week-old female BALB/c mice (H2-Kd) were purchased from National Science Academy of Center for Laboratory Animals, Shanghai, China, and were used according to protocols approved by Animals Committee of Wenzhou Medical College.

Cells and reagents. EBV-positive B-cell lines B95-8 and mouse myeloma cells P815 were purchased from the American Type Culture Collection. pcDNA3.1(+)/HPV-6 L1 plasmid with optimized codon usage was kindly provided by Dr. Kong-Nan Zhao (University of Queensland, Queensland, Australia). Horseradish peroxidase (HRP)-conjugated anti-mouse-IgG, anti-mouse-IgG1, anti-mouse-IgG2a, and anti-mouse-IgA antibodies were purchased from NeoMarkers (Fremont, USA). HPV-6 L1 were expressed in baculovirus expression system and purified by CsCl gradient centrifugation to obtain VLPs to be preserved in our lab for use.

EBV LMP2 multiepitope peptide. The EBV LMP2 multiepitope containing CTL, Th, and B cell epitopes was preserved in our lab, of which design process had been described in previous work (Zhu *et al*., 2010). The EBV LMP2 multiepitope peptide was synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., and coupled with BSA for use.

Recombinant plasmid pcHPV L1-EBV LMP2. Eukaryotic codon-optimized DNA sequence of EBV LMP2 multiepitope had been determined (Zhu *et al*., 2010), and the sequence of sense and antisense oligonucleotides were introduced by *Bam*HI and *Eco*RI sites respectively. Sense strand of the EBV LMP2 multiepitope peptide: 5′-*GGATCC*ATGTGCCTGACATGGCGGA TCGAAGACCCTCCTTTCAATTCCCTGTTGTTCGCACTGC TCGCCGCCGCGGGTGGCCTCCAAGGTATCTATGTGCTCGTT ATGCTGGTACTCCTGATTACCTATGGACCTGTTTTTATGT GCCTGGGCGGCCTCTTGACCATGGTTGCCGGCTAAGGA TCC-3′ and antisense strand: 5′-*GAATTC*TTAGCCGGCAACCA TGGTCAAGAGGCCGCCCAGGCACATAAAAACA GGTCCATAGGTAATCAGGAGTACCAGCA TAACGAGCACATAGATACCTTGGAGGCCACCCGCGGC GGCGAGCAGTGCGAACAACAGGGAATTGAAAGGAGGG TCTTCGATCCGCCATGTCAGGCACATAAGCTT-3′. *BamH*I and *EcoR*I sites are underlined. These two pairs of complementary oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., annealed at 95°C for 5 min, and slowly cooled to RT. Target gene of LMP2 multiepitope was obtained, and the gene fragment digested by *Bam*HI/*Eco*RI was inserted into pcDNA3.1(+)/HPV L1 plasmid to generate the chimeric HPV L1 plasmid: pcDNA3.1(+)/HPV L1-EBV LMP2 multiepitope (pcHPV L1-EBV LMP2). Then pcHPV L1-EBV LMP2 was transformed into *Escherichia coli* DH5α (Stratagene, USA) and purified with an endotoxin-free plasmid DNA purification kit (Qiagen, Germany) following manufacturer′s instruction.

Table 1. Strategies of prime-boost immunization of mice with EBV LMP2 multiepitope antigen applied as DNA and/or peptide vaccine

Group of mice	Vaccine	
	Priming (week 0, 2)	Boosting (week 4, 6)
1	DNA vaccine (100 μg of pcHPV L1/EBV LMP2)	DNA vaccine (100 μg of pcHPV L1/EBV LMP2)
2	DNA vaccine (100 μg of pcHPV L1/EBV LMP2)	Peptide vaccine (10 μg of EBV LMP2 multiepitope peptide)
3	Peptide vaccine (10 μg of EBV LMP2 multiepitope peptide)	DNA vaccine (100 μg of pcHPV L1/EBV LMP2)
4	DNA vaccine (50 μg of pcHPV L1/EBV LMP2) + peptide vaccine (5 μg of EBV LMP2 multiepitope peptide)	DNA vaccine (50 μg of pcHPV L1/EBV LMP2) + peptide vaccine (5 μg of EBV LMP2 multiepitope peptide)
5	DNA vaccine control (100 μg of plasmid pcDNA3.1(+))	DNA vaccine control (100 μg of plasmid cDNA3.1(+))
6	Peptide vaccine control (10 μg of unrelated peptide coupled with BSA)	Peptide vaccine control (10 μg of unrelated peptide coupled with BSA)
Collecting blood samples for IgG, IgA at weeks 0,1, 3, 5, 7, 9, 12, 24 (n = 7)		
Collecting blood samples for IgG1,IgG2a at week 9 (n = 7)		
Collecting spleen samples for CTL at week 9 (n = 3)		

Immunization. Mice were randomly divided into six groups (10 mice per group). Mice in the group 1 were immunized with 100 μg pcHPV L1-EBV LMP2 (100 μg/mouse/time), by injecting into the left (50 μg) and the right (50 μg) quadriceps muscle bundles at weeks 0, 2, 4, and 6. Group 2 was primed with pcHPV L1-EBV LMP2 (100 μg/mouse/time), by injecting into the left (50 μg) and the right (50 μg) quadriceps muscle bundles at weeks 0 and 2, and boosted with EBV LMP2 multiepitope peptide (10 μg/mouse/time) at weeks 4 and 6. In the group 3, mice were primed with EBV LMP2 multiepitope peptide (10 μg /mouse/time) at weeks 0 and 2, and boosted with pcHPV L1-EBV LMP2 at weeks 4 and 6. Mice in the group 4, were co-immunized with pcHPV L1-EBV LMP2 (50 μg/mouse/time, 25 μg respectively into the left and the right quadriceps muscle bundles) and EBV LMP2 multiepitope peptide (5 μg/mouse/time) at weeks 0, 2, 4 and 6. Group 5 was immunized with 100 μg pcDNA3.1(+) without target gene inserts as DNA vaccine control. Group 6 was immunized with 10 μg of irrelevant peptide (multiepitope peptide from *Chlamydia trachomatis* preserved in our lab) coupled with BSA as peptide vaccine control. Intramuscular injection immunization was operated for control DNA vaccine, and subcutaneous injection immunization for control peptide vaccine. The immunization schedule was repeated 4 times at 2 week intervals. Prime-boost vaccine programs and immune response detection are shown in Table 1.

ELISA. For antibody detection, serum samples were separated from blood collected from the tail vein of mice. EBV-specific IgG, IgA, and HPV L1-specific IgG were detected using ELISA at weeks 0, 1, 3, 5, 7, 9, 12, and 24. EBV-specific IgG1 and IgG2a were detected at week 9. 100 μl (10 μg/ml) of membrane protein extraction from B95-8 cells and 100 μl (10 μg/ml) of purified HPV L1 VLPs were coated onto 96 flat-bottom well micro-titer plates (Corning, Lowell, USA) at 4 °C overnight. The coated plates were blocked for 1 hr at 37°C with blocking buffer (5% nonfat dry milk, 0.05% Tween 20 in PBS), then incubated for 1 hr at 37°C with serum samples (1:100 dilution for IgG, IgA, and 1:40 dilution for IgG1, IgG2a). After washing with 0.05% Tween 20 in PBS, the plates were incubated for 1 hr at 37°C with HRP-anti-mouse IgG, anti-mouse IgA, anti-mouse IgG1, or anti-mouse IgG2a at dilution 1:3,000. The plates were washed 5 times with 0.05% Tween 20 in PBS. After adding OPD and H_2O_2, color development was determined at A_{492} using an automated ELISA reader (Bio-Tek ELx800, USA). All samples were run in triplicate.

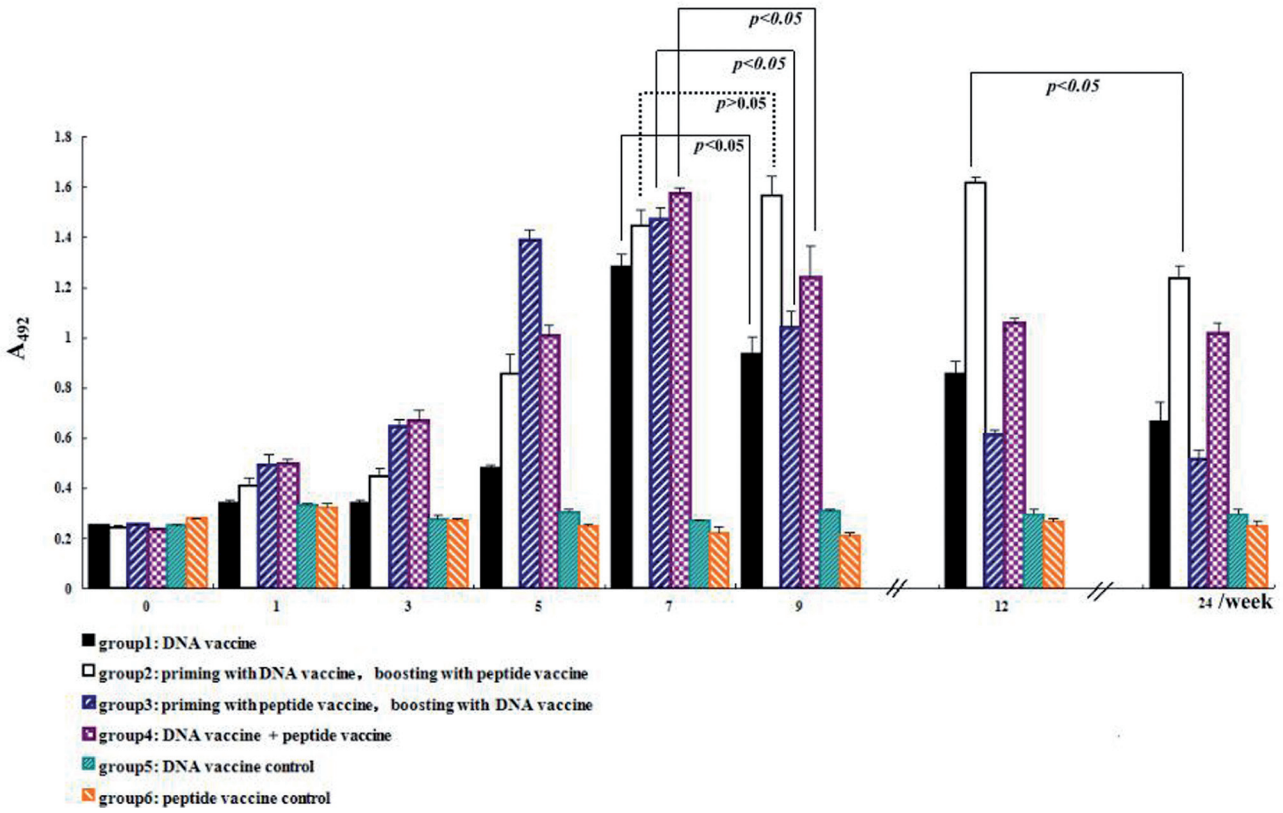

Fig. 1

EBV-LMP2-specific IgG antibodies in vaccinated mice

Antibodies in serum of mice vaccinated by different prime-boost strategies (groups 1–6) were assayed at weeks 0–24 by ELISA.

Cytotoxicity assay. Single lymphocyte suspensions from spleen were prepared for cytotoxic assay (Kodama *et al.*, 2008). The cytotoxic activity of T lymphocytes was determined by lactate dehydrogenase (LDH) release assay using CytoTox 96w non-radioactive cytotoxicity assay (Promega, USA). P815 cells with the same H2-Kd haplotype as BALB/c mice were loaded with one CTL peptide within EBV LMP2 multiepitope (TYGPVFMCL, H2-Kd-restricted) (10 mg/ml) at 37°C for 2 hrs to be used as target cells. Primed T-cells from spleen of mice were used as effector cells, and incubated in 50 ml of complete medium, and then 150 ml of peptide-pulsed target P815 cell suspension (1×10^4 cells) was added to triplicate wells on 96-well plate. After incubation at 37°C for 4 hrs, 50 ml of culture supernatant was harvested and LDH amount was quantified by reading at A_{492} on automated ELISA reader. The percentage of lysis of each well was calculated by the following formula: cytotoxicity (%) = [(experimental release-spontaneous release) / (maximal release-spontaneous release)] × 100. In control group of mice, the target cells were incubated either in culture medium for spontaneous release or in a mixture of 2% Triton X-100 for maximum LDH release.

Statistical analysis. All data were presented as means ± SD. The statistical significance of differences was tested by One-Way ANOVA test using SPSS statistical software (SPSS inc., 1989–1999, USA). Difference with $P \leq 0.05$ was considered significant.

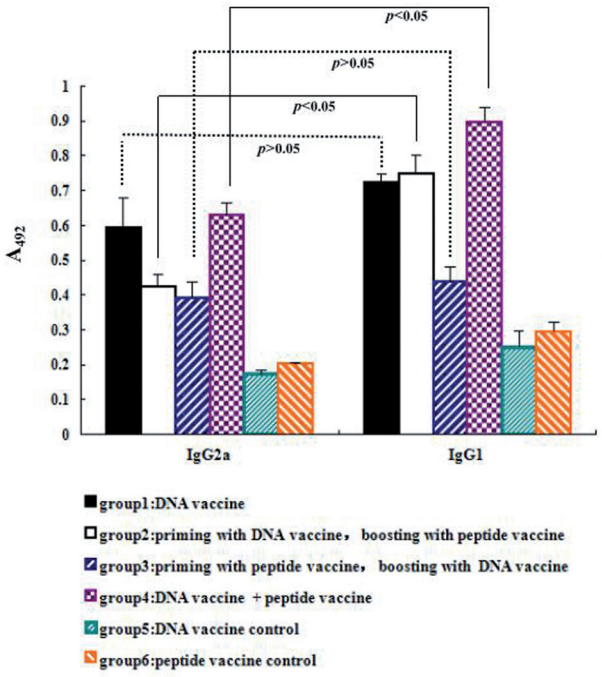

Fig. 2

EBV-LMP2-specific IgG1 and IgG2a antibodies in vaccinated mice

Antibodies in serum of mice vaccinated by different prime-boost strategies (groups 1–6) were assayed at week 9 by ELISA.

Results

EBV-LMP2-specific antibodies

Except control groups 5 and 6, EBV-LMP2-specific IgG in groups 1, 2, 3, and 4 gradually increased after immunization. A high IgG production in group 1 was measured at week 7 and significantly decreased at week 9 ($F = 52.342$, $P < 0.05$). The IgG production in group 2 also rose to a high level at week 7 and still kept an approximate level at week 9 and 12, and then significantly decreased at week 24 ($F = 143.219$, $P < 0.05$). The IgG production in group 3 rose to high level at week 5 and 7, and significantly decreased at week 9 ($F = 84.889$, $P < 0.05$). The IgG production in group 4 reached a peak level at week 7 and significantly decreased at week 9 ($F = 21.291$, $P < 0.05$) (Fig. 1).

EBV-specific IgG subclasses (IgG1, IgG2a) were detected at week 9 (Fig. 2). There was no significant statistical difference ($P > 0.05$) between IgG1 and IgG2a in groups 1 and 3. A significant predominance of IgG1 over IgG2a antibodies was observed in groups 2 and 4 ($P < 0.05$).

Except control groups 5 and 6, EBV-LMP2-specific IgA also gradually increased after immunization. The highest level of IgA in groups 1 and 3 was detected at week 5, then decreased at week 7 ($F = 17.991$, $P < 0.05$), and the same level was maintained until week 24. The IgA production in group 2 reached the highest level at week 5, and then was maintained till week 24. The IgA production in group 4 reached the highest level at week 7, slightly decreased at week 9 and maintained the IgA level until week 24 (Fig. 3).

HPV L1-specific IgG antibodies

Control groups 5 and 6 didn't produce HPV L1-specific IgG antibodies. Production of HPV L1-specific IgG in groups 1, 2, and 4 reached the highest level at week 5, and began to decrease at week 7, and then decreased to the lowest level at week 24. As for group 3, there was no significant production of HPV L1-specific IgG (Fig. 4).

Cytotoxic activity of T-lymphocytes

As shown in Fig. 5, in any case of effector/target cell (E:T) ratios for groups 1, 2, 3, and 4, there were significant statistical differences of cytotoxicity in comparison with control groups ($P < 0.05$). T lymphocytes from group 3 exhibited significantly higher cytotoxicity than group 1 at E:T ratio of 40, 20, and 10 ($F = 9.702$, 9.774, and 14.359 respectively, $P < 0.05$), while, group 4 exhibited significantly higher cytotoxicity than group 1 only at E:T ratio of 20 and 10 ($F = 7.783$ and 1.559 respectively, $P < 0.05$). As for group 2, there were no significant differences compared to groups 3 and 4 at any of E:T ratios ($P > 0.05$).

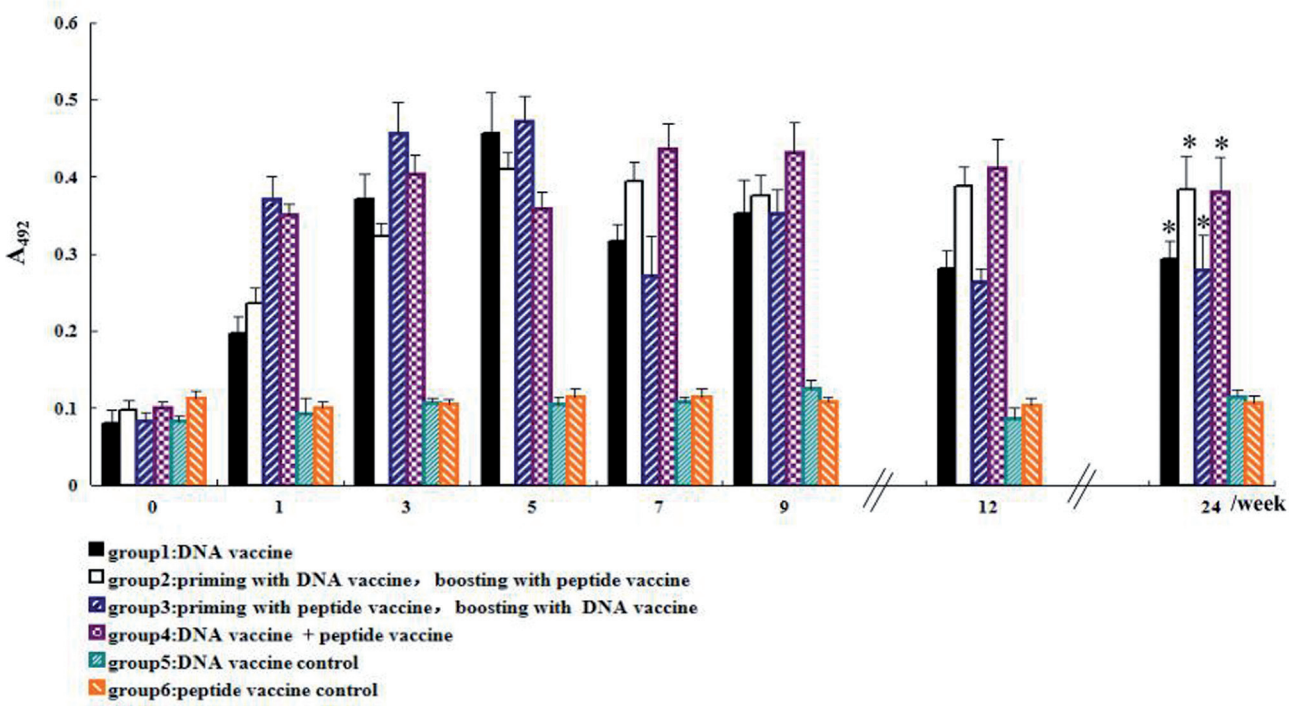

Fig. 3

EBV-LMP2-specific IgA antibodies in vaccinated mice

Antibodies in serum of mice vaccinated by different prime-boost strategies (groups 1–6) were assayed at weeks 0–24 by ELISA. *P >0.05, compared with week 12, 9, and 7, respectively.

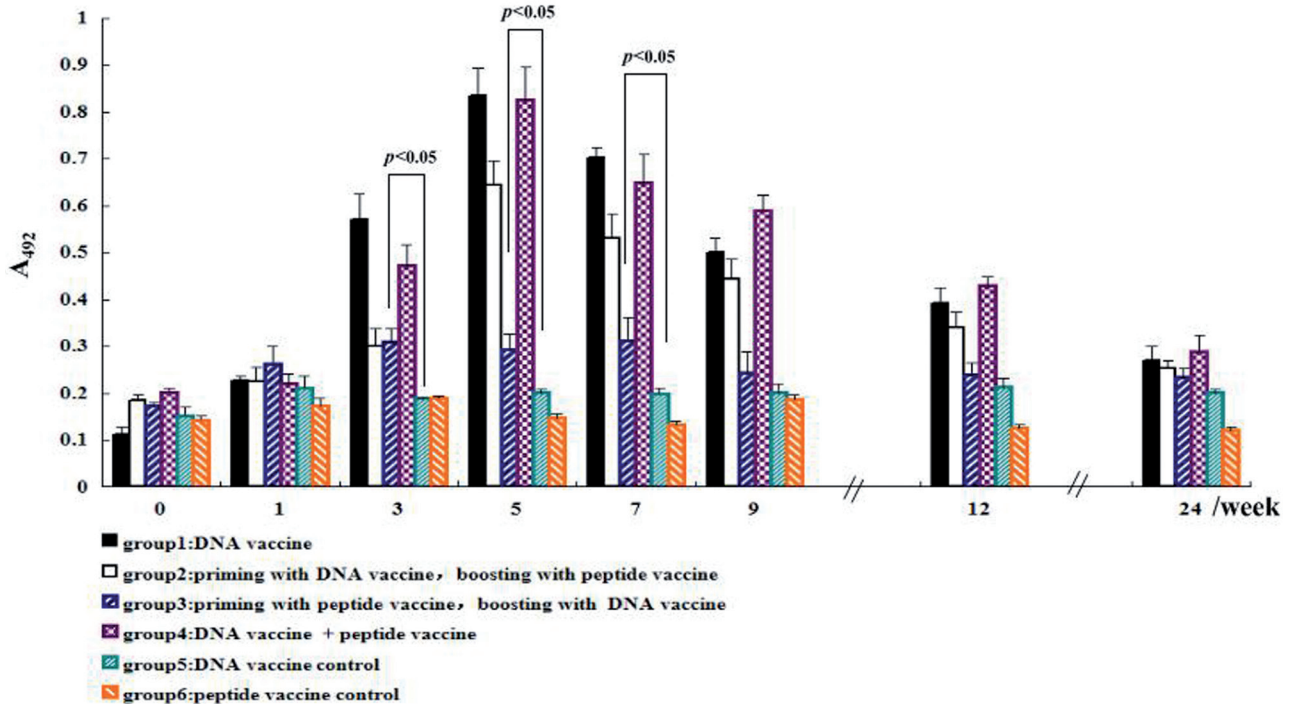

Fig. 4

HPV L1-specific IgG antibodies in vaccinated mice

Antibodies in serum of mice vaccinated by different prime-boost strategies (groups 1–6) were assayed at weeks 0–24 by ELISA.

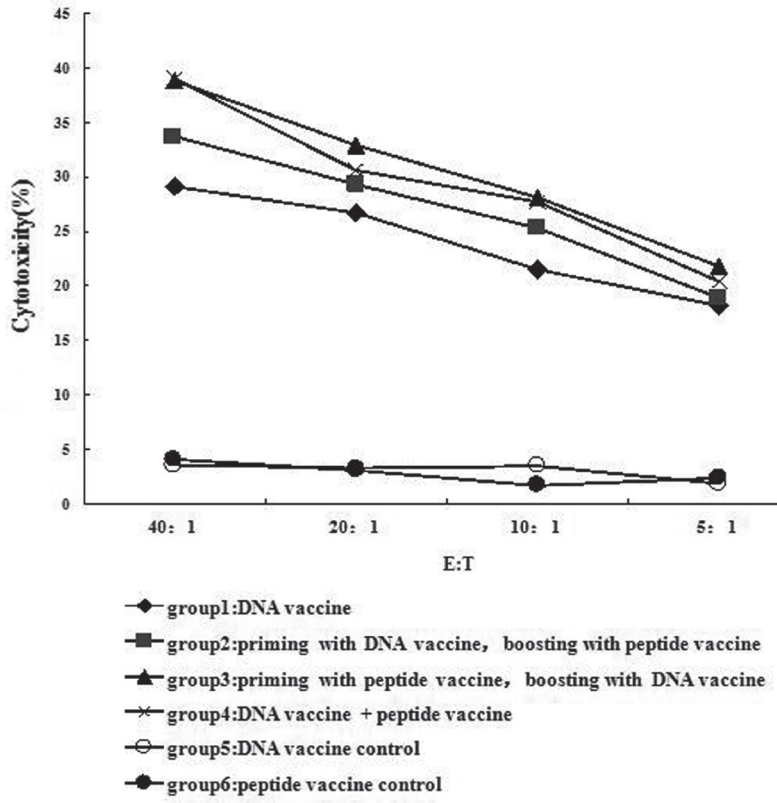

Fig. 5

CTL activity in vaccinated mice

CTL activities in mice vaccinated by different prime-boost strategies (groups 1–6) were determined by LDH release assay. E:T refer to effector/target cells ratio.

Discussion

EBV is an oncogenic virus, which mainly affects B lymphocytes, and is significantly associated with NPC in southern China population and other malignancies. It is well known that tumor immunotherapy is to eliminate tumor cells by activating immune system, while EBV-associated NPC traditional immunotherapy is based on the function of autologous CTL activity (Chua *et al.*, 2001; Leen *et al.*, 2009). On the other hand, B cell mediated humoral immune response also plays an indispensable role, such as neutralization, opsonization and ADCC effects (Chao *et al.*, 2010; Rhodes *et al.*, 2010; Markman *et al.*, 2010). So it would be a good approach to improve the efficacy of a candidate therapeutic vaccine to induce specific cellular and humoral immune co-response against EBV associated malignancies.

LMP2 is considered to be a potential target for an immunotherapy to EBV-associated malignancies because it can induce autologous cytotoxic T lymphocytes *in vitro* (Lalonde *et al.*, 2007; Wang *et al.*, 2009; Pan *et al.*, 2006; Duraiswamy *et al.*, 2004). Straathof *et al.* (2005) synthesized an overlapping 15-mer peptide covering the whole LMP2 sequence, and 25 LMP2-specific CTL cell lines were obtained by screening these peptides by cytotoxicity assay. Another study reported that immunization with a replication-incompetent adenoviral vaccine, which encodes multiple HLA class I-restricted CTL epitopes from LMP1 and LMP2 as a multiepitope, consistently generated strong LMP-specific CTL response in HLA A2/K(b) mice, and a human CTL response to LMP antigens which could be rapidly expanded after stimulation with this recombinant multiepitope vector (Duraiswamy *et al.*, 2004). So in our previous study, LMP2 multiepitope (T- and B-cell epitope-rich peptide clusters) was designed as immunogen in order to induce a cellular and humoral immune synergetic effect.

Multiepitope-based vaccine represents a powerful approach to overcome immunodominance and simultaneously generate broad immune responses (Stevenson *et al.*, 2004; Ercolini *et al.*, 2003). However, multiepitope vaccine needs a suitable antigen delivery and release vehicle to induce effective immune response. HPV major capsid protein L1 is an important vehicle for the delivery of epitopes. HPV L1 fused with foreign epitopes or polypeptides at the C terminus region

can still form chimeric VLPs, which induce broad immune responses and lead to more powerful anti-tumor response by loading effective CTL-, Th-, and B-cell multiepitopes rather than single epitope (Mattil-Fritz *et al.*, 2008).

The chimeric pcHPV L1-EBV LMP2 multiepitope vaccine prepared by our previous work had been successful in expression of the chimeric HPV L1-EBV LMP2 multiepitope gene at the mRNA and protein levels in eukaryotic cells, and exhibited corresponding immunogenicity. In present paper, we further estimated the "prime-boost" immunization for influencing the immune responses by HPV L1-EBV LMP2.

From the results, we can see that peptide vaccine may contribute to stabilizing the level of EBV LMP2-specific IgG antibodies, because group 4 (co-immunization with DNA and peptide vaccine) could maintain same level of IgG from week 12 to 24, whereas in group 1 it decreased from week 12 to 24 (only DNA vaccine). Groups 2 and 3 showed a similar pattern. By priming the mice with peptide vaccine twice, group 3 produced antibodies faster, and reached the peak level at week 5, but then gradually decreased from week 9 to 24. This means that boosting by DNA vaccine failed to preserve antibody level. As for group 2, antibody production rose slowly and reached a peak at week 9, and could preserve the antibody level until week 24, which was the highest antibody level among of all groups. We assume that the strategy of priming with DNA vaccine and boosting with peptide vaccine has predominance to significantly enhance antibody production.

The results of EBV-IgG antibody subclass analysis showed that DNA vaccine (group 1), or DNA booster vaccine (group 3) generated a mixed Th1/Th2 response and no difference in Th1/Th2 response occurred. However, peptide booster vaccine (group 2), or peptide together with DNA vaccine (group 4) generated higher Th2 response than Th1 response.

Similarly, groups 2 and 4 where we used peptide boosting, or peptide together with DNA vaccine, a high level of EBV-specific IgA was maintained, whereas it decreased significantly in groups 1 and 3, where no subsequent peptide boosting was used. High production of HPV L1-specific IgG in groups 1 and 4 indicated that there was a valid expression of HPV L1 VLPs as vehicle. Although there was only one B cell epitope in multiepitope, groups 2 and 4 showed obvious production of EBV-specific IgG and IgA, which indicated that HPV L1 is a better vehicle to deliver multiepitope effectively. However, at week 7 after first immunization, there was significant decrease of HPV L1-IgG in group 3. We assume that boosting with DNA vaccine only twice, failed to keep sufficient level of HPV L1-specific antibody. Considering longer preservation of EBV LMP2-specific antibodies than HPV L1-specific antibodies, we assume that EBV LMP2 multiepitope is immunodominant.

EBV LMP2 multiepitope designed in the study not only contains HLA-A*0201, HLA-A*2402, HLA-DRB1*0401, HLA-DRB1*0301, and HLA-DRB1*1501 restricted T cell epitopes, but also contains mouse H2-Kd restricted CTL epitopes in order to study the immune effects on murine experimental model. In this experiment, all spleen lymphocytes from mice immunized with four kinds of prime-boost strategies had shown effective CTL activities, which demonstrated the effectiveness of used CTL epitopes and activity relation to E:T ratio. Much more effective CTL activity from strategy in groups 3 and 4 probably attributed to the immunization programs of DNA booster vaccine, or peptide combination with DNA vaccine.

The designed multiepitope in present study containing seven HLA-A*0201 (more than 50% genotype of Chinese population) CTL epitopes, five HLA-A*2402 CTL epitopes, HLA-5 DRB1*0301 Th cell epitope, five HLA-DRB1*1501 Th cell epitopes, two HLA-DRB1*0401 Th cell epitopes, and one immunodominant B epitope would be valuable for application, because of effective cellular and humoral immune response with broad population coverage. The prime-boosting strategy of HPV L1-EBV LMP2 multiepitope suggested that immunization program of priming by DNA vaccine and boosting with multiepitope peptide vaccine could induced significant humoral immune responses and also effective CTL activity, and could be a good candidate for EBV associated NPC immunotherapy.

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