SCIENCE CHINA
Life Sciences

• RESEARCH PAPERS •

March 2011 Vol.54 No.3: 263–266
doi: 10.1007/s11427-011-4147-0

Specific cellular immune responses in mice immunized with DNA, adeno-associated virus and adenoviral vaccines of Epstein-Barr virus-LMP2 alone or in combination

WANG Zhan[1†], YANG SongMei[1†], ZHOU Ling[1*], DU HaiJun[1], MO WuNing[2] & ZENG Yi[1*]

[1]*State Key Laboratory for Infectious Disease Prevention and Control, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China;*
[2]*Laboratory Department, The First Affiliated Hospital, Guangxi Medical University, Nanning 530021, China*

Received January 18, 2010; accepted May 12, 2010

Cellular immune responses, particularly those associated with $CD3^+CD8^+$ cytotoxic T lymphocytes (CTL), are critical factors in controlling viral infection. Nasopharyngeal carcinoma (NPC) is closely associated with persistent Epstein-Barr virus (EBV) infection. NPC vaccine studies have focused on enhancing specific antiviral CTL responses. In this study, three vaccines capable of expressing the EBV-latent membrane protein 2 (LMP2) (a DNA vector, an adeno-associated virus (AAV) vector, and a replication-defective adenovirus serotype 5 (Ad5) vector) were respectively used to immunize female Balb/c mice (4–6 weeks old) at weeks 0, 2 and 4, either alone or in combination. Our results suggest that combined immunization with DNA, AAV, and adenovirus vector vaccines induced specific cellular immunity more effectively than any of these vectors alone or a combination of two of the three, constituting a sound vaccine strategy for the prevention and treatment of NPC.

EBV-LMP2, DNA vaccine, recombinant adenovirus vaccine, recombinant adeno-associated virus vaccine, combinatorial immunization, specific cell-mediated immune responses

Citation: Wang Z, Yang S M, Zhou L, *et al*. Specific cellular immune responses in mice immunized with DNA, adeno-associated virus and adenoviral vaccines of Epstein-Barr virus-LMP2 alone or in combination. Sci China Life Sci, 2011, 54: 263−266, doi: 10.1007/s11427-011-4147-0

Epstein-Barr virus (EBV) is associated with a number of human malignancies. One of the most widely spread EBV-associated malignancies, nasopharyngeal carcinoma (NPC), has a high incidence rate of 10–50 cases per 100000 people per year in the southern regions of China [1–3]. Although the tumor is responsive to radiotherapy and a combined treatment of radiotherapy and chemotherapy, it is resistant to these therapies in about 30% of patients [3]. Therefore, it is necessary to develop novel treatments for NPC. EBV existing in the tumor tissue provides an ideal target for cytotoxic T lymphocyte (CTL)-based immunotherapy, and therefore immunotherapy may become a beneficial complementary approach to the conventional treatments of NPC. EBV infection in NPC cells exhibits a latency II pattern and the viral proteins EBNA1, LMP1 and LMP2 are expressed. LMP2 mRNAs are regularly detected in NPC tumor cells. Although the LMP2 protein has not been reproducibly detected with currently available techniques, antibodies to LMP2 are frequently detected in sera obtained from NPC patients [4]. Meanwhile, among all EBV proteins, LMP2 is most readily recognized by CTLs. Additionally, many HLAI restricted epitopes of LMP2 that are conserved across different populations have been identified [5–8]. Therefore, LMP2 is an ideal target for NPC immunotherapy.

MHC class I-restricted CTLs are a key factor in controlling the status of EBV infection. EBV-specific CTLs are present at high levels in the blood during the initial stages of

†Contributed equally to this work
*Corresponding author (email: zhouling@ht.rol.cn.net; zengy@public.bta.net.cn)

infection and persist for the entire period of the viral infection. When the level of CTL responses is reduced, as seen in patients receiving organ transplantation or in HIV-infected individuals, EBV-induced lymphocyte proliferation will occur. However, when immunosuppression is alleviated or autologous EBV-specific CTLs expanded *in vitro* are infused back to the patients, the cellular immune responses will recover, and the disease will regress [9]. These observations suggest that the EBV-specific immune responses might be useful in the treatment of EBV-associated human tumors. To develop a therapeutic vaccine for NPC, Zuo JianMin *et al.* [10] from our laboratory constructed rAd-LMP2, a recombinant adenovirus containing the full-length cDNA of EBV-LMP2. The adenoviral vector has a broad host-range, its transduction efficiencies for cells in both quiescent and dividing phases are high, and it can express high levels of target protein in a short time [11]. *In vivo* animal studies of AIDS vaccines showed that priming with a viral vector or DNA vaccine followed by boosting with another vector induced stronger immune responses in comparison to vaccination with a single vaccine [12]. Therefore, two additional types of vaccine, a DNA vaccine and an adeno-associated virus (AAV) vaccine were also included in our experiments. Injection of a DNA vaccine into an animal can directly transfect animal cells with the DNA and the antigenic proteins expressed can induce CTL responses to a greater degree [13]. Recently, AAV has been proposed as a promising vehicle for gene therapy due to its broad host range, excellent safety profile, and stable expression of genes in the host system. A major advantage of AAV is that in comparison to other viral vectors, they exhibit an extremely low immunogenicity [14]. The aim of this study was to investigate the immunologic effects of combined immunization with DNA, AAV and adenoviral vaccines of EBV-LMP2, in comparison to individual immunization with each vaccine.

1 Materials and methods

1.1 Vaccine vectors

1.1.1 DNA vaccine

The vector pVR-LMP2 was constructed by inserting the EBV-LMP2 gene into the VR plasmid under the control of the human cytomegalovirus (CMV) intron A promoter and bovine growth hormone terminator. Plasmid pVR-LMP2 was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg mL^{-1}.

1.1.2 AAV vaccine

The same EBV-LMP2 gene was used in the construction of the AAV-1 vector. AAV-1 is a pseudotyped AAV vector containing ITRs and the Rep gene from AAV-2, and the Cap protein from AAV-1. The EBV-LMP2 gene was cloned into pSNAV-1, an expression plasmid of recombinant AAV, to construct the recombinant plasmid pSNAV-LMP2. BHK cells were transfected with pSNAV-LMP2 to establish BHK-LMP2, an LMP2-expressing stable cell line; BHK-LMP2 cells were infected with HSV1-rc/ΔUL2, and the recombinant virus, rAAV2/1-LMP2, was generated and purified.

1.1.3 Adenovirus vector vaccine

The rAd-LMP2, a recombinant replication defective adenovirus type 5 containing the full-length cDNA of EBV-LMP2 was constructed in our laboratory.

1.2 Immunization

Female Balb/c mice (4–6 weeks old) were purchased from the Institute of Laboratory Animal Sciences (Chinese Academy of Medical Sciences). Seven groups of five mice were immunized by intramuscular injection at week 0, and boosted twice at weeks 2 and 4. The immunization program is shown in Table 1. All mice remained healthy throughout the experiments. The immunization doses of PBS, pVR-LMP2, rAd-LMP2 and rAAV2/1-LMP2 used were 100 μL, 100 μg, 5×10^{10} and 1×10^{11} viral particles (vp), respectively.

1.3 ELISPOT assay

The level of LMP2-specific IFN-γ-secreting cells in mice was determined using an ELISPOT assay kit (BD™ ELISPOT Mouse IFN-γ ELISPOT set; BD Biosciences) following the manufacturer's protocol. Mouse spleen lymphocytes (5×10^5) were stimulated with an EBV-LMP2 peptide pool (4 μg mL^{-1} final concentration) and incubated at 37°C/5% CO_2 for 24 h. Untreated cells were used as the background control. Cells were removed after 24 h and 200 μL deionized water was added to each well. Subsequently, the plate was washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and incubated with pre-diluted biotinylated anti-IFN-γ antibody assay solution (100 μL/well) for 2 h at room temperature. The antibody solution was discarded and the plate was washed three times with PBS-T. A 100 μL volume of a streptavidin-horseradish peroxidase conjugated anti-biotin antibody was added to each well and the plate was incubated for 1 h at room temperature. The

Table 1 Immunization program for the different groups of mice

Group No.	Priming (0 week)	Boosting (2nd week)	Boosting (4th week)
1	PBS	PBS	PBS
2	pVR-LMP2	pVR-LMP2	pVR-LMP2
3	pVR-LMP2	pVR-LMP2	rAd-LMP2
4	pVR-LMP2	rAd-LMP2	rAd-LMP2
5	rAd-LMP2	rAd-LMP2	rAd-LMP2
6	rAAV2/1-LMP2	rAAV2/1-LMP2	rAAV2/1-LMP2
7	rAAV2/1-LMP2	pVR-LMP2	rAd-LMP2

plate was washed four times with PBS-T, twice with PBS, and then 100 μL of AEC color development solution was added to each well. The plate was incubated in the dark at room temperature. When clear spot formation was observed, deionized water was added to the wells and the reactions were terminated with two washes. After the plate was air-dried at room temperature, the spots were counted in a computer-assisted video image analyzer. The results were expressed as the number of spot-forming cells (SFC) per million cells.

1.4 Adenovirus-specific neutralizing antibody assay

Mouse sera taken from Groups 1, 3, 4 and 5 mice at week 5 post-immunization (diluted 1∶50) were incubated with an equal volume of serially diluted Ad5 vector (10^{10}, 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 viral particles) at 37°C for 1 h. The mixture was incubated with Hek 293 cells in a 96-well plate at 37°C for an additional 96 h. The cytopathic effect was analyzed after 96 h incubation. The neutralizing titer was expressed as the number of neutralized Ad5 particles at a 1∶50 dilution.

1.5 Data analysis

All results were expressed as mean±SD. Statistical analyses of the experimental data and control were conducted by one-way factorial analysis of variance (ANOVA), with statistical significance defined as $P<0.05$.

2 Results

2.1 Specific cellular immunity

Seven groups of five mice were respectively immunized intramuscularly with pVR-LMP2, rAAV2/1-LMP2, Ad-LMP2, or a combination of these vaccines, at weeks 0, 2 and 4 according to the program shown in Table 1. The levels of EBV-LMP2-specific T cells were determined by Elispot assay as described in Materials and methods. The mean values obtained for the groups are listed in Table 2. One-way factorial analysis of variance showed that the values for Groups 3 (pVR-LMP2, pVR-LMP2, rAd-LMP2), 4 (pVR-LMP2, rAd-LMP2, rAd-LMP2), 5 (rAd-LMP2, rAd-LMP2, rAd-LMP2), and 7 (rAAV2/1-LMP2, pVR-LMP2, rAd-LMP2) were significantly higher than that of Group 1 (PBS control; $P<0.01$ for all) and the values for Groups 3, 4 and 7 were significantly higher than that of Group 5 ($P<0.01$; Figure 1).

These results indicate that the magnitude of vaccine-induced T-cell responses was related to the immunization strategies. Individually, rAd-LMP2 (Group 5) induced higher specific cellular immune response than pVR-LMP2 (Group 2) and rAAV2/1-LMP2 (Group 6). However, the highest response was induced when the mice were immunized with all three vaccines (Group 7). The second highest response was induced by priming with pVR-LMP2 twice followed by boosting with rAd-LMP2 (Group 3) whereas priming with pVR-LMP2 once and boosting with rAd-LMP2 twice was not as effective.

2.2 Adenovirus-specific neutralizing antibody assay

Titers of anti-adenovirus neutralizing antibodies in the sera of Groups 1, 3, 4 and 5 mice were assayed. The neutralization titers were expressed as the number of Ad5 particles neutralized at a 1∶50 dilution of the serum sample. Repeated administration of Ad-LMP2 induced an increase in the titer of anti-adenovirus neutralizing antibodies (Table 3).

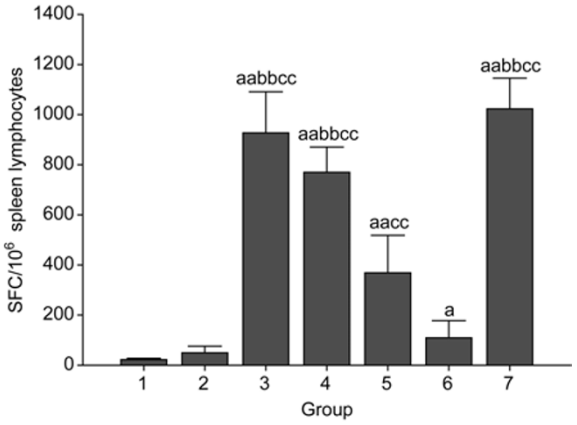

Figure 1 IFN-γ-ELISPOT histogram of spleen lymphocytes from mice treated with various vaccine strategies (1×10^6 spleen lymphocytes). a, significantly higher than Group 1 ($P<0.05$); aa, much higher than Group 1 ($P<0.01$); bb, much higher than Group 2 ($P<0.01$); cc, much higher than Group 5 ($P<0.01$).

Table 2 Cellular immune responses induced in different groups of mice

Group No.	Mean SFC	STD	*N*
1	22.4000	5.54977	5
2	49.6000	26.43483	5
3	927.2000	164.00976	5
4	770.0000	100.55844	5
5	368.8000	149.36265	5
6	109.2000	68.53612	5
7	1023.2000	122.60995	5

Table 3 Titers of anti-adenovirus neutralizing antibody in rAd-LMP2 immunized mice[a)]

Test	Group 1 (vp)	Group 3 (vp)	Group 4 (vp)	Group 5 (vp)
1	–	1×10^6	1×10^8	1×10^9
2	–	1×10^5	1×10^8	1×10^9
3	–	1×10^6	1×10^8	1×10^9
4	–	1×10^5	1×10^8	1×10^9
5	–	1×10^5	1×10^8	1×10^9
Mean	–	$(6.40\pm4.93)\times10^5$	$(1.00\pm0.00)\times10^{8*}$	$(1.00\pm0.00)\times10^{9*}$

a) *, the value was significantly higher than that of Group 3 ($P<0.05$).

3 Discussion

There is increasing interest in the use of viral and non-viral systems as vectors to elicit anti-EBV-LMP2 immune responses for the treatment of certain tumors. Comparative studies on the immunogenicity of the vectors and entire systems in non-human primates have provided experimental evidence for the clinical application of these vectors. It has been found in animal experiments that priming with a viral vector or nucleic acid vaccine followed by boosting with either another vector or a subunit/polypeptide vaccine induced stronger immune responses compared with vaccination with either vaccine alone [12,15]. Over the past decade, several prime-boost strategies have been tested for an HIV vaccine and the results have been promising [16,17].

In this work, we evaluated the cellular immune responses to EBV-LMP2 induced by DNA, AAV and adenovirus vectors. Our results showed that the combined immunization with DNA, AAV, and adenovirus vectors was promising in inducing specific anti-LMP2 CTL responses. A combination of DNA and adenovirus vectors also elicited robust LMP2-specific CTL responses in mice. The DNA vectors had very low immunogenicity. We also found that immunization with the AAV vector alone elicited low cellular immune responses. Because similar observation has been reported previously [18], we did not further pursue the immunization strategy with AAV alone in this study. Our study focused on LMP2-specific CTL responses elicited by the strategy of AAV-DNA-rAd5 immunization.

Based on common adenovirus serotypes, the immunogenicity of a vaccine may be reduced by any pre-existing immunity against the viral vector. The results of the present study showed that the levels of EBV-LMP2-specific T cells tended to decrease with increasing time of immunization with the adenovirus type 5 vector. Increased doses and times of administration will increase the titer of neutralizing antibodies, and will subsequently reduce the efficiency of gene delivery [19].

All these results strongly suggest that the synergy observed in the three vaccines present a feasible strategy for circumventing the negative effects of generally existing high adenovirus type 5 immunity in human populations.

In conclusion, our results suggest a good prospect for the application of combined immunization with DNA, AAV, and adenovirus vectors in producing cellular immunity in human. Based on our study, Rhesus monkey trials have been designed. These trials will assess the tolerability and immunogenicity of DNA, AAV, and adenovirus type 5 vectors expressing the LMP2 protein, either alone or in combination.

This work was supported by the National High Technology Research and Development Program of China (Grant No. 2006AA02A229).

1 Chan A T, Toe P M, Johnson P J. Nasopharyngeal carcinoma. Ann Oncol, 2002, 13: 1007–1025
2 Yu M C. Nasopharyngeal carcinoma: Epidemiology and dietary factors. IARC Sci Publ, 1991, 105: 39–47
3 Taylor G S. T cell-based therapies for EBV-associated malignancies. Expert Opin Biol Ther, 2004, 4: 11–21
4 Lennette E T, Winberg G, Yadav M, *et al*. Antibodies to LMP2A/2B in EBV-carrying malignancies. Eur J Cancer, 1995, 31A: 1875–1878
5 Lee S P, Tierney R J, Thomas W A, *et al*. Conserved CTL epitopes within EBV latent membrane protein 2: A potential target for CTL-based tumor therapy. J Immunol, 1997, 158: 3325–3334
6 Lee S P, Chan A T, Cheung S T, *et al*. CTL control of EBV in nasopharyngeal carcinoma: EBV-specific CTL responses in the blood and tumors of NPC patients and the antigen-processing function of the tumor cells. J Immunol, 2000, 165: 573–582
7 Konishi K, Maruo S, Kato H, *et al*. Role of Epstein-Barr virus-encoded latent membrane protein 2A on virus-induced immortalization and virus activation. J Gen Virol, 2001, 82: 1451–1456
8 Straathof K C, Leen A M, Buza E L, *et al*. Characterization of latent membrane protein 2 specificity in CTL lines from patients with EBV-positive nasopharyngeal carcinoma and lymphoma. J Immunol, 2005, 175: 4137–4147
9 Rooney C M, Smith C A, Ng C Y, *et al*. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr virus related lympholiferation. Lancet, 1995, 345: 9–13
10 Zuo J M, Zhou L, Wang Q, *et al*. The *in vitro* and *in vivo* immunogenicity of recombination adenovirus vaccine containing EBV-latent membrane protein 2. Chin J Microbiol Immunol, 2003, 23: 446–449
11 Ghosh S S, Gopinath P, Ramesh A. Adenoviral vectors: A promising tool for gene therapy. Appl Biotechnol, 2006, 133: 9–29
12 Excler J L, Plotkin S. The prime-boost concept applied to HIV preventive vaccines. AIDS, 1997, 11: S127–137
13 Donnelly J J, Ulmer J B, Shiver J W. DNA vaccines. Annu Rev Immunol, 1997, 15: 617–648
14 Li C, Bowles D E, Dyke T V. Adeno-associated virus vectors: Potential applications for cancer gene therapy. Cancer Gene Ther, 2005, 12: 913–925
15 Excler J L. AIDS vaccine development: Perspectives, challenges & hopes. Indian J Med Res, 2005, 121: 568–581
16 Casimiro D R, Chen L, Fu T M. Comparative immunogenicity in rhesus monkeys of DNA plasmid, recombinant vaccinia virus, and replication-defective adenovirus vectors expressing a human immunodeficiency virus type 1 gag gene. J Virol, 2003, 77: 6305–6313
17 Xin K Q, Jounai N, Someya K. Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV. Gene Ther, 2005, 12: 1769–1777
18 Ponnazhagan S. Adenoassociated virus vectors for genetic immunization. Immunol Res, 2002, 26: 247–253
19 Nikitina E Y, Chada S, Muro-Cacho C. An effective immunization and cancer treatment with activated dendritic cells transduced with full-length wild-type p53. Gene Ther, 2002, 9: 345–352