Immune responses of a designed HIV-1 DNA vaccine on rhesus monkeys

ZHANG Lishu¹, JIN Ningyi¹, SONG Yingjin², SUN Yansong³, WANG Hong⁴, ZHAN Dawei³, MA Hewen⁵, SHANG Yupu³, JIN Hongtao¹, HONG Baoqing³ & LI Chang³

1. The 11th Institute, Academy of Military Medical Sciences, Changchun 130062, China;
2. College of Agricultural and Biological Engineer, Tianjin University, Tianjin 300072, China;
3. Laboratory Animal Center, Academy of Military Medical Sciences, Beijing 100071, China;
4. College of Life Science, Jinan University, Guangzhou 510632, China;
5. Agricultural Sciences Center, Jilin University, Changchun 130062, China

Correspondence should be addressed to Jin Ningyi (email: ningyij@yahoo.com)

Received December 28, 2005; accepted March 15, 2006

Abstract An effective HIV-1 vaccine will be the ultimate solution for the prevention of HIV/AIDS, though HAART plays important roles in treating the disease. In this study, a large-scale recombinant DNA plasmid containing a designed HIV-1 multi-epitope-p24 chimeric gene was prepared and purified. Rhesus monkeys were then inoculated muscularily with the plasmid for four times in week 0, 4, 8 and 18. Whole blood was collected two weeks after the third and fourth inoculation, followed by serum and peripheral blood mononuclear cell (PBMC) separation. The CTL activity and proliferation of PBMCs stimulated by macaque MHC-I-restricted HIV-1 CTL epitope peptide were analyzed by MTT and LDH release assay, respectively. Th1 cytokines in supernatant of cultured PBMC stimulated by HIV-1 CTL epitope peptide and anti-HIV-1 antibody in serum were assayed by ELISA. The results showed that increased CTL target-killing activity, higher secretion of Th1 cytokines (IFN-γ and IL-2) and promoted proliferative reaction of monkey PBMCs stimulated by HIV-1 CTL epitope peptide were detected in the immunization group inoculated by the recombinant DNA vaccine for three times, which were further enhanced by the fourth inoculation. At the same time, HIV-1 specific antibody in serum of immunized monkeys was higher than that in controls. We concluded that the designed HIV-1 DNA vaccine may induce HIV-1 specific cellular and humoral immunity on monkeys.

Keywords: HIV-1, designed, DNA vaccine, immune response, rhesus monkey.

According to the UNAIDS/WHO report (http://www.unaids.org/Epi2005/doc/report.html), there are about 40.3 million HIV/AIDS survivors, and the new HIV infection number amounts to about 4.9 million, while the death number is some 3.1 million in the world in 2005. The HIV-infected population in China is estimated to be about 840000 and the infection ratio in the population is 0.07% currently. There were 29623 more newly infected persons from the end of 2003 to September of 2004 in China⁵. It will be over 10 millions of HIV infections by the year of 2010 in China if there are no interventions to control the spread of HIV/AIDS, and great loss in economy and lots of social problems would appear consequently. As estimated by ref. [1], at least 3.1 billion RMB were spent in the HIV/AIDS prevention and treatment only in the year of 2004 in China.

It is of great importance to find out an effective way to restrain the epidemic of HIV/AIDS. Fortunately, more and more work has been done recently. Highly active antiretroviral therapy (HAART) is playing great roles in prolonging the life of AIDS patients²,³ especially in the short term clinic treatment⁴. However, their benefits are limited by problems of both long-term usage which causes resistance to the drugs and expensive price which a majority of infected patients find hard to pay. These realities urge scientists develop and test alternative forms of treatment. Several live-attenuated vaccines aiming at HIV have been tested on animals⁵–⁸, but the risk of virus mutant or virulent regaining makes them undesirable. DNA vaccines, possessing the advantages of safety, low cost of production and easiness of use in field conditions, have become a hot spot in the field of HIV vaccines development in recent years. Singh and his colleagues⁹ constructed a DNA vaccine by deleting the reverse transcriptase gene from SHIV genome. The results of immunization on six macaques showed that three out of four immunized animals survived, while the two controls died. It is suggested that DNA vaccine could prevent AIDS and could be used alone in large-scale immunization programs without the need for boosting with viral proteins.

In China, multi-epitope DNA vaccines targeting at bird flu¹⁰ or SARS¹¹ have been explored, and the
ARTICLES

researches on the vaccine of HIV multi-neutralizing epitopes have also been carried out\[12\]. All of these studies showed that animal models have good immunities, suggesting a promising prospect of multi-epitope DNA vaccine. We previously designed a multi-epitope gene based on HIV-1 sequence, and constructed a DNA plasmid containing a chimeric gene of the designed multi-epitope gene and p24 gene of HIV-1, which showed a good immunogenicity on mice. In this study, the designed DNA vaccine was inoculated intramuscularly on macaques, and immune responses were observed.

1 Materials and methods

1.1 Materials

pVAX1-MEGp24, a recombinant DNA plasmid, containing a chimeric gene of a designed HIV-1 multi-epitope gene and HIV-1 p24 gene; HIV-1 antibody ELISA kit, purchased from Beijing Kinghark Pharmaceutical Co., Ltd; CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit, purchased from Promega Co.; Monkey lymphocytes separation medium, purchased from Institute of Biomedical Engineering, the Chinese Academy of Medical Sciences; Synthesis peptides of P1: RGPGRAFVTI (HIV-1 Env 311—320 aa), macaque MHC-I-restricted HIV-1 CTL epitope peptide, P3: ELDKWA (HIV-1 Env 662—667 aa), HIV-1 antibody epitope peptide, and P6: AGCKNFFWKTFTSC, unrelated control peptide, synthesized by AC Scientific Inc (Xi’an), with a purity greater than 95%; Monkey IFN-γ and IL-2 kit, purchased from TBI Co.; MTT, analytical reagent grade, 5 mg/mL; LLC-MK2, a cell line derived from macaque kidney, provided by Shaihai Institute of Cell Biology, the Chinese Academy of Sciences; Macaca mulatta, 12—20 months of age, both female and male, no signs of clinical diseases, provided by Laboratory Animal Center, the Academy of Military Medical Sciences.

1.2 Large-scale preparation and purification of the recombinant plasmid

Proceeded as described previously\[13\]. The purity of recombinant plasmid DNA was determined by agar electrophoresis and the ratio of $A_{260} - A_{280}$.

1.3 Grouping and immunization

11 macaques were randomly divided into three groups (3—5 macaques per group), including PBS control, pVAX1 plasmid control and pVAX1-MEGp24 recombinant plasmid group. Monkeys were inoculated intramuscularly with the plasmid DNA (1 mg each, dissolved in 800 μL sterile ddH2O) or PBS (800 μL each) for four times at week 0, 4, 8, and 18. 6 mL of circular blood was collected from the groin vein of macaques at week 10 and 20 for separation of serum and peripheral blood mononuclear cell (PBMC). Serum was used to detect the HIV-1-specific antibody, while PBMC was applied to analyzing the specific proliferation, CTL activity and content of cytokines.

1.4 Detection of HIV-1-specific antibody in serum

Kit assay was carried out under the guide of the manual. The synthesis peptide P3 was diluted to a final concentration of 0.1 mg/mL with coating solution. Each well of the polyethylene plate was coated with 100 μL of diluted P3 and incubated overnight at 4°C. Coating solution was removed and rinsed well. Each well was blocked with 100 μL of 5% skimmed milk and incubated at 37°C for 1 h. Blocking solution was removed and rinsed. 100 μL of serum isolated from immunized or control macaques (diluted by 50 times) was added to each well and incubated at 37°C for 30 min. The primary antibody solution was removed and rinsed. 100 μL of 1:1000 anti-monkey IgG antibody conjugated to HRP was added to each well and incubated at 37°C for 30 min. The secondary antibody solution was removed and rinsed. 100 μL of TMB substrate solution was added and incubated at 37°C for 10 min. 50 μL of 2 mol/L H2SO4 was added to stop the reaction of color development. The absorption was detected at 450 nm in an ELISA reader.

1.5 Separation of PBMC

Fresh monkey blood containing anticoagulant was diluted with an equal volume of Hank’s medium. The diluted blood was slowly and carefully layered over equal volume of lymphocytes separation medium, and centrifuged for 20 min at 1300 rpm at room temperature. The lymphocytes at the interface of blood and separation medium were carefully poured into a new tube, diluted with 4 mL of Hank’s medium, and centrifuged for 10 min at 1700 rpm. Supernatant was discarded and 4 mL of red blood cell lysis solution was added. The lysis solution and the precipitation were mixed well and incubated for 15 min at room temperature, followed by centrifugation for 10 min at 1700 rpm. Supernatant was discarded and 1 mL of RPMI 1640
medium with 10% NBS was added to the precipitation. The lymphocytes in the solution was counted and adjusted to $5 \times 10^6$ mL$^{-1}$ for the determination of the specific proliferation, CTL activity and content of cytokines.

1.6 Analysis of proliferative response of PBMCs with lymphocyte transformation (LT) assay

$2 \times 10^6$ of PBMC from immunized macaques in 1 mL of medium was cultured alone, or co-cultured with HIV-1 CTL epitope peptide P1 (10 mg/L) or control peptide P6 (10 mg/L) in a 24-well plate for 72 h at 37°C in 5% CO$_2$. 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h in 5% CO$_2$. The suspension containing cells was collected and centrifuged for 5 min at 1000 rpm. 150 μL of DMSO was added and shaken for 10 min to dissolve the crystals of MTT. The absorption was detected at 490 nm in an ELISA reader. The intensity of cell proliferation was denoted by stimulation index (SI), which was calculated as follows: $SI = \frac{\text{Mean experimental } A_{490}}{\text{Mean control } A_{490}}$.

1.7 Analysis of HIV-1-specific CTL activity

Macaque MHC-I-restricted HIV-1 CTL epitope peptide RGPGRAFVTI (P1, 10 mg/L) was added to cultured LLC-MK2 cells and incubated for 2 h at 37°C in 5% CO$_2$. These peptide-labeled cells would be served as target cells. PBMCs from non-immunized macaques were co-incubated with synthesis peptide P1 and P6 (as in vitro stimulator) for 2 h at 37°C in 5% CO$_2$. Mitomycin C was added to a final concentration of 40 mg/L, and incubated for 2 h. Cells void of mitomycin C were collected by 4 times of washing with PBS, which would be used as stimulator cells. PBMCs from immunized macaques were co-cultured with stimulator cells in RPMI 1640 medium with 10% NBS for 24 h at 37°C in 5% CO$_2$. Human IL-2 was added to a final concentration of 100 U/mL, and cultured for another 5 d. Cells were collected by 5 min of centrifuging at 2000 rpm, followed by re-suspended in RPMI 1640 medium with 5% NBS and adjusted to a final concentration of $10^7$ per milliliter, which would be used as effector cells. Determination of HIV-1-specific CD8$^+$ CTL activity was carried out as described in the manual. The ratio of effector cells to target cells (E/T) was 50:1, 25:1 or 12.5:1. The absorption was detected at 490 nm in an ELISA reader, and the target-killing activity of CTL was calculated as follows: % Cytotoxicity = (Experimental $A_{490}$ – Effector spontaneous $A_{490}$ – Target spontaneous $A_{490}$)/(Target maximum $A_{490}$ – Target spontaneous $A_{490}$) × 100.

1.8 Determination of Th1 cytokines

$2 \times 10^6$ of PBMCs from immunized macaques in 1 mL of medium was cultured alone, or co-cultured with HIV-1 CTL epitope peptide P1 (10 mg/L) or control peptide P6 (10 mg/L) in a 24-well plate for 72 h at 37°C in 5% CO$_2$. Supernatants were collected to determine the content of Th1 cytokines such as IFN-γ and IL-2.

1.9 Statistical analysis

Data were represented as $\Delta \bar{X} \pm SD$, and $t$-test was employed to analyze the statistical significance.

2 Results

2.1 The designed antigen gene of HIV-1 DNA vaccine

A sketch of the multi-epitope antigen gene MEG$^{[14]}$ of HIV-1 DNA vaccine is shown in Fig. 1(a), and that of chimeric gene MEGp24 used in this study is demonstrated in Fig. 1(b).

2.2 Purity of the recombinant plasmid DNA

The electrophoresis of the purified plasmid pVAX1-MEGp24 on 1% agar showed that no RNA existed in the plasmid. The value of $A_{260}/A_{280}$ of the plasmid was 1.85, suggesting that the plasmid was not contaminated by proteins or phenol. Thus the plasmid was desirable for immunization on animals.

2.3 HIV-1-specific target-killing activity of CD8$^+$ CTL in PBMCs

For three kinds of E/T ratio, the recombinant DNA plasmid pVAX1-MEGp24 induced HIV-1-specific target-killing activity of CD8$^+$ CTL in PBMCs of immunized macaques stimulated by macaque MHC-I-restricted HIV-1 CTL epitope peptide P1 after the third immunization (compared with the control groups, $p<0.01$). The CTL activity was further enhanced by the fourth immunization (for 50:1 and 25:1 of E/T ratio, compared with the third immunization group, $p<0.05$), which was over 35 % for the high E/T ratio and close to 10 % for the low E/T ratio (Fig. 2).

2.4 Level of Th1 cytokines in supernatant of cultured PBMCs

Based on the standard curves, the lineal regression
equation for the calculation of IFN-γ content is: \( y = 351.4x - 47.96, r = 0.9933 \), and that for IL-2 is: \( y = 719.92x - 25.44, r = 0.9982 \).

High level of IFN-γ was observed in the supernatant of cultured PBMC stimulated by HIV-1 CTL epitope peptide P1 from macaques immunized with the recombinant plasmid (compared with the control groups, \( p < 0.01 \)). The fourth immunization further increased the proliferative response of PBMCs (compared with the third immunization group, \( p < 0.05 \)).

### Table 1: Content of Th1 cytokines in the supernatant of cultured PBMC in immunized macaques

|                | IFN-γ Content (pg/mL) | IL-2 Content (pg/mL) |
|----------------|-----------------------|----------------------|
|                | 10 w                  | 20 w                 | 10 w                  | 20 w                  |
| pVAX1-MEGp24   | 82.7 ± 16.8a          | 125.2 ± 25.1a        | 75.9 ± 14.4a          | 109.6 ± 21.1a         |
| pVAX1 control  | 21.7 ± 7.7            | 32.4 ± 8.7           | 46.7 ± 9.1            | 59.2 ± 11.8           |
| PBS control    | 13.6 ± 6.9            | 23.9 ± 7.2           | 41.8 ± 8.5            | 53.4 ± 10.2           |

a) Compared with PBS and pVAX1 controls, \( p < 0.01 \).

The level of HIV-1 antibody in serum was detected by kit assay and peptide-coating assay. The results (Table 3) showed that the level of HIV-1 antibody in serum from macaques immunized with the recombinant plasmid was significantly higher than that in PBS and pVAX1 controls (\( p < 0.01 \)). The HIV-1 antibody level was statistically higher than the control groups (\( p < 0.01 \)). The statistical analysis demonstrated a similar change in IL-2 content.

### 2.5 HIV-1 specific proliferation of PBMCs

The HIV-1 specific proliferation of PBMCs from immunized macaques determined by lymphocyte transformation assay is shown in Table 2. The proliferative response of cultured PBMCs under the stimulation of HIV-1 CTL epitope peptide was observed in the group immunized with the recombinant plasmid pVAX1-MEGp24 (compared with the control groups, \( p < 0.01 \)).

### 2.6 Level of HIV-1 antibody in serum

The level of HIV-1 antibody in serum from immunized macaques was detected by kit assay and peptide-coating assay. The results (Table 3) showed that the level of HIV-1 antibody in serum from macaques immunized with the recombinant plasmid was significantly higher than that in PBS and pVAX1 controls (\( p < 0.01 \)). The HIV-1 antibody level was statistically higher than the control groups (\( p < 0.01 \)). The statistical analysis demonstrated a similar change in IL-2 content.
Table 2  HIV-1 specific proliferative response of PBMCs in immunized macaques

|                        | 10 w       | 20 w       |
|------------------------|------------|------------|
| pVAX1-MEGp24           | 1.91 ± 0.38 \( \text{a)} \) | 2.51 ± 0.40 \( \text{a)} \) |
| pVAX1 control          | 1.08 ± 0.15 | 1.12 ± 0.13 |
| PBS control            | 1.06 ± 0.13 | 0.92 ± 0.14 |

\( \text{a)} \) Compared with PBS and pVAX1 controls, \( p<0.01 \).

Table 3  Level of HIV-1 antibody in serum of immunized macaques

|                        | Kit assay  | Peptide-coating assay |
|------------------------|------------|-----------------------|
|                        | 10 w       | 20 w                  | 10 w       | 20 w                  |
| pVAX1-MEGp24           | 0.406 ± 0.039 \( \text{a)} \) | 0.481 ± 0.044 \( \text{a)} \) | 0.522 ± 0.047 \( \text{a)} \) | 0.607 ± 0.057 \( \text{a)} \) |
| pVAX1 control          | 0.064 ± 0.008 | 0.062 ± 0.007 | 0.086 ± 0.008 | 0.094 ± 0.008 |
| PBS control            | 0.066 ± 0.007 | 0.073 ± 0.006 | 0.069 ± 0.006 | 0.060 ± 0.007 |

\( \text{a)} \) Compared with PBS and pVAX1 controls, \( p<0.01 \).

3 Discussion

People face many obstacles in developing a vaccine which can be used universally throughout the world, although the need for an effective vaccine against HIV-1 to ensure the public health is well recognized and accepted. One of the most significant problems in vaccine design strategies is how to solve the genetic variation of HIV-1 isolates\([15-17]\).

Among current vaccine design strategies, vaccines based on CTL epitopes may induce effective cellular immunity, mainly because multiple epitopes can be selected and constructed into one vaccine vector, thus inducing broad immune responses to different epitopes. Epitope selection can be biased toward those that are most highly conserved among viral types and subtypes, as well as dominant or subdominant epitopes from numerous viral products. Strategies in HIV vaccine design\([18-20]\) were specifically concentrated on consensus sequences of prevalent HIV-1 types either within specific populations or the local target population. In a former study\([14]\), we first scanned the whole sequence of HIV-1 and picked out several highly conserved and predominant epitopes. We further optimized the codons and the arrangement of these epitope genes and constructed the designed gene to a DNA vaccine vector. The results of the immunization with the recombinant plasmid on mice showed that the antigen expressed from the designed gene had a good immunogenicity.

Infection with HIV-1 caused a disease state characterized by progressive immune dysfunction, ultimately resulting in AIDS in a majority of infected patients. Studies on acute HIV-1 infections\([21,22]\) showed that early elevation of CD8\(^{+}\) CTL specific for HIV-1 can be observed several weeks after infection, corresponding to the initial decline of HIV-1 plasma viraemia, thus suggesting that CD8\(^{+}\) CTL plays important roles in controlling the virus replication in initial period of HIV-1 infection. Similarly, detections on clinical long-term nonprogressors\([23-26]\) infected by HIV-1 demonstrated the presence of low viral loads, slower decrease in CD4\(^{+}\) T lymphocyte counts and broad CTL immune responses, supporting the notion that CTL responses may help to curb viral replication in these chronically HIV-1-infected individuals. Virus-specific CTL responses induced by epitope vaccines can constrain SIV replication and prevent disease progression either under the SIV infection or in pathogenesis model with rhesus monkeys or pathogenic strains of the hybrid simian HIV-1\([27-30]\). The effectiveness of CTL responses on the control of virus replication and disease progression was further clarified by the findings\([31-34]\) that SIV virus mutated to dominant CTL epitopes could escape CTL recognition, resulting in an increase in viral replication and acceleration of disease progression. We determined the target-killing activity of macaques immunized with the designed DNA vaccine by LDH assay. The results showed that the target-killing activity of CD8\(^{+}\) CTL in PBMCs of immunized macaques, under the stimulation of macaque MHC-I-restricted CTL epitope peptide, approached 35% and 10% in the high and low ratio of effector cell to target cell respectively, suggesting that the designed DNA vaccine may induce the production of HIV-1-specific CTL in macaques.

It has been demonstrated\([35-37]\) that Th1 responses represented by secretion of Th1 cytokines, such as IL-2
and IFN-γ, may enhance the resistance of host to microbe infection, especially to virus and intracellular pathogens, while Th2 responses represented by secretion of Th2 cytokines, such as IL-4 and IL-10, may be conducive to the progression, permanence and chronicization of infections. Studies on highly risk population for HIV\(^{38-40}\) showed that those whose HIV antibody and RNA were detected to be negative bore strong Th1 responses. Further observations on HIV-infected patients without clinical symptoms\(^{38-40}\) demonstrated that those who did not progress to AIDS possessed permanent and dominant Th1 responses, while those who progressed to AIDS exhibited a gradually decreased production of IFN-γ and IL-2 but a gradually increased production of IL-4 and IL-10. These researches indicated that Th1 responses may play key roles in either the prevention of HIV infection or delaying the progression of HIV infections. In this study, the designed DNA plasmid was observed to induce significantly high production of IFN-γ and IL-2 from cultured PBMCs under the stimulation of HIV-1 CTL epitope peptide, thus suggesting that the designed vaccine can enhance Th1 responses on macaques.

Furthermore, HIV-1-specific proliferative response of PBMCs under the stimulation of HIV-1 CTL epitope peptide and serum HIV-1 antibody were detected in immunized macaques by lymphocyte transformation assay and ELISA assay respectively, suggesting that the designed DNA vaccine did stimulate immune responses on monkeys. Let it be noted that further evaluation on humeral immunity of the designed DNA vaccine was confined for lack of data on neutralizing antibody level in immunized macaques by the limitation of experimental conditions. In addition, we observed enhanced immune responses induced by the fourth immunization, indicating that immune responses can be promoted by the same antigen based on the prime immunization with proper interval between immunizations.

In a word, our study indicated that the designed HIV-1 recombinant DNA vaccine may induce HIV-1-specific humoral and cellular immune responses, and a proper interval between prime and boost immunization may enhance the immunity of the same antigen in macaques. The designed DNA vaccine is worthy of clinical trial and has a promising prospect in clinic practice.

Acknowledgements We thank an anonymous referee for very helpful suggestions which improved the quality of the paper. This work was supported by the National 863 Foundation Project (Grant No. 2003AA219051).

References

1. State Council AIDS Working Committee Office and UN Theme Group on HIV/AIDS in China. A Joint Assessment of HIV/AIDS Prevention, Treatment and Care in China (2004). 2004: 4 – 10.

2. Palella F J, Delaney K M, Moorman A C, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. Outpatient Study Investigators. New Engl J Med, 1998, 338: 853 – 860.

3. Mocroft A, Vella S, Benfield T L, et al. Changing patterns of mortality across Europe in patients infected with HIV-1. Lancet, 1998, 352: 1725 – 1730.

4. Walsmley S, Bernstein B, King M, et al. Lopinavir: Ritonavir versus nelfinavir for the initial treatment of HIV infection. New Engl J Med, 2002, 346: 2039 – 2046.

5. Daniel M D, Kirchhoff F, Czajak S C, et al. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science, 1992, 258: 1938 – 1941.

6. Cranage M P, Hall G A, Dennis M J, et al. Macaques infected with live attenuated SIVmac are protected against superinfection via the rectal mucosa. Virology, 1997, 229(1): 143 – 154.

7. Smith S M, Khoreshev M, Marx P A, et al. Constitutively dead, conditionally live HIV-1 genomes. Ex vivo implications for a live virus vaccine. J Biol Chem, 2001, 276(34): 32184 – 32190.

8. Stebbing S, Berry N, Stott J, et al. Vaccination with live attenuated simian immunodeficiency virus for 21 days protects against superinfection. Virology, 2004, 330(1): 249 – 260.

9. Singh D K, Liu Z, Sheffer D, et al. A noninfectious simian/human immunodeficiency virus DNA vaccine that protects macaques against AIDS. J Virol, 2005, 79(6): 3419 – 3428.

10. Peng J M, Tong G Z, Wang Y F, et al. Multi-epitope DNA vaccines against avian influenza in chickens. Chin J Biotech (in Chinese). 2003, 19(5): 623 – 627.

11. Wang X H, Tong D Y, Xu H B, et al. Construction and immunogenicity of a multi-epitope sased SARS-CoV gene vaccine. J Shanghai Med Univ (in Chinese), 2005, 32(2): 130 – 133.

12. Ding J, He F, Lu Y, et al. Induction of multi-epitope-specific antibodies against HIV-1 by multi-epitope-vaccine. Chin Sci Bull, 2001, 46(22): 13 – 18.

13. Sambrook J, Fritsch E F, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989. 16 – 68.

14. Li Z J, Jin N Y, Jiang W Z, et al. A new type of multiple-epitope DNA vaccine against HIV. Chin J Microbiol Immunol (in Chinese), 2004, 24(11): 910 – 913.

15. Walker B D, Korber B T. Immune control of HIV: The obstacles of HLA and viral diversity. Nat Immun, 2001, 2: 473 – 475.

16. Korber B, Gaschen B, Yusim K, et al. Evolutionary and immunological implications of contemporary HIV-1 variation. Br Med Bull, 2001, 58: 19 – 42.
17 Gaschen B, Taylor J, Yusim K, et al. Diversity considerations in HIV-1 vaccine selection. Science, 2002, 296: 2354—2360
18 Novitsky V, Smith U R, Gilbert P, et al. Human immunodeficiency virus type 1 subtype C molecular phylogeny: Consensus sequence for an AIDS vaccine design? J Virol, 2002, 76(11): 5435—5451
19 Williamson A L, Marais D, Passmore J A, et al. Human papillomavirus (HPV) infection in Southern Africa: Prevalence, immunity, and vaccine prospects. IUBMB Life, 2002, 53(4-5): 253—258
20 Mashishi T, Loubser S, Hide W, et al. Conserved domains of subtype C Nef from South African HIV type 1-infected individuals include cytotoxic T lymphocyte epitope-rich regions. AIDS Res Hum Retroviruses, 2001, 17(17): 1681—1687
21 Borrow P, Lewicki H, Hahn B H, et al. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol, 1994, 68(9): 6103—6110
22 Koup R A, Safrit J T, Cao Y, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol, 1994, 68(7): 4650—4655
23 Rosenberg E S, Billingsley J M, Caliendo A M, et al. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. Science, 1997, 278: 1447—1450
24 Ogg G S, Jin X, Bonhoeffer S, et al. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science, 1998, 279: 2103—2106
25 Gea-Banacloche J C, Migueles S A, Martino L, et al. Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. J Immunol, 2000, 165: 1082—1092
26 Chouquet C, Autran B, Gomard E, et al. Correlation between breadth of memory HIV-specific cytotoxic T cells, viral load and disease progression in HIV infection. AIDS, 2002, 16: 2399—2407
27 Barouch D H, Santra S, Kuroda M J, et al. Reduction of simian-human immunodeficiency virus 89. 6P viremia in rhesus monkeys by recombinant modified vaccinia virus Ankara vaccination. J Virol, 2001, 75(11): 5151—5158
28 Amara R R, Villinger F, Altman J D, et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science, 2001, 292: 69—74
29 Shiver J W, Fu T M, Chen L, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature, 2002, 415: 331—335
30 Horton H, Vogel T U, Carter D K, et al. Immunization of rhesus macaques with a DNA prime/modified vaccinia virus Ankara boost regimen induces broad simian immunodeficiency virus(SIV)-specific T-cell responses and reduces initial viral replication but does not prevent disease progression following challenge with pathogenic SIVmac239. J Virol, 2002, 76(14): 7187—7202
31 Allen T M, O’Connor D H, Jing P, et al. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature, 2000, 407: 386—390
32 Barouch D H, Kunstman J, Kuroda M J, et al. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature, 2002, 415: 335—339
33 Vogel T U, Friedrich T C, O’Connor D H, et al. Escape in one of two cytotoxic T-lymphocyte epitopes bound by a high-frequency major histocompatibility complex class I molecule, Mamu-A*02: A paradigm for virus evolution and persistence? J Virol, 2002, 76(22): 11623—11636
34 Nacsa J, Stanton J, Kunstman K J, et al. Emergence of cytotoxic T lymphocyte escape mutants following antiretroviral treatment suspension in rhesus macaques infected with SIVmac251. Virology, 2003, 305(1): 210—218
35 Romagnai S. Human Th1 and Th2 subsets: doubt no more. Immunol Today, 1991, 12: 256—257
36 Adorini L, Sinigaglia F. Pathogenesis and immunotherapy of autoimmune diseases. Immunol Today, 1997, 18: 209—211
37 Romagnani S. The Th1/Th2 paradigm. Immunol Today, 1997, 18: 263—266
38 Clerici M, Shearer G M. A Th1→Th2 switch is a critical step in the etiology of HIV infection. Immunol Today, 1993, 14: 107—111
39 Clerici M, Sarin A, Coffman R L, et al. Type 1/type 2 cytokine modulation of T-cell programmed cell death as a model for human immunodeficiency virus pathogenesis. Proc Natl Acad Sci USA, 1994, 91: 11811—11815
40 Clerici M, Lucey D R, Berzofsky J A, et al. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. Science, 1993, 262: 1721—1724