

Immune Responses in Wild-type Mice Against Prion Proteins Induced Using a DNA Prime-Protein Boost Strategy*

HAN YanLing^{1,2}, LI Yuan¹, SONG Juan^{1,2}, WANG Ying^{1,2}, SHI Qi¹, CHEN Cao¹, ZHANG BaoYun¹, GUO Yan¹, LI ChaoPing^{2,#}, HAN Jun^{1,#}, and DONG XiaoPing^{1,#}

1. State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 2. Medical School, Anhui University of Science and Technology, Huainan 231001, China

Abstract

Objective To break immune tolerance to prion (PrP) proteins using DNA vaccines.

Methods Four different human prion DNA vaccine candidates were constructed based on the pcDNA3.1 vector: PrP-WT expressing wild-type PrP, Ubiqu-PrP expressing PrP fused to ubiquitin, PrP-LII expressing PrP fused to the lysosomal integral membrane protein type II lysosome-targeting signal, and PrP-ER expressing PrP locating the ER. Using a prime-boost strategy, three-doses of DNA vaccine were injected intramuscularly into Balb/c mice, followed by two doses of PrP protein. Two weeks after the last immunization, sera and spleens were collected and PrP-specific humoral and cellular immune responses evaluated by ELISA and ELISPOT tests.

Results Higher levels of serum PrP antibodies were detected in mice vaccinated using the strategy of DNA priming followed by protein boosting. Of these, WT-PrP, Ubiqu-PrP, and PrP-LII induced significantly higher humoral responses. ELISPOT tests showed markedly increased numbers of IFN- γ -secreting T cells in mice vaccinated using the strategy of DNA priming followed by protein boosting after stimulation with recombinant PrP23-90 and PrP23-231. PrP-ER induced the strongest T-cell response.

Conclusion Prion vaccines can break tolerance to PrP proteins and induce PrP-specific humoral and cellular immune responses.

Key words: Prion; DNA vaccine; Humoral response; T-cell response; Prime-boosting regime.

Biomed Environ Sci, 2011; 24(5):523-529 doi:10.3967/0895-3988.2011.05.011 ISSN:0895-3988

www.besjournal.com/full_text

CN:11-2816/Q

Copyright © 2011 by China CDC

INTRODUCTION

Prion diseases are a group of fatal, transmissible, neurodegenerative disorders, which include Creutzfeldt-Jakob disease (CJD) in humans, and scrapie and bovine spongiform encephalopathy (BSE) in animals. BSE spreads

through the bovine food chain and the transmission of bovine prions to humans has given rise to variant CJD (vCJD). Although the incidence of BSE and vCJD may be stabilizing, or even declining, the steep rise in the incidence of chronic wasting disease (CWD) underlines the fact that prion diseases of farm and wild animals still represent a major threat. Thus,

*This work was supported by Chinese National Natural Science Foundation Grants 30771914 and 30800975, Institution Technique R&D Grant (2008EG150300), National Basic Research Program of China (973 Program) (2007CB310505), China Mega-Project for Infectious Disease (2009ZX10004-101, 2008ZX10004-001, and 2008ZX10004-002) and the SKLID Development Grant (2008SKLID102 and 2008SKLID202).

#Correspondence should be addressed to: Dr. DONG XiaoPing, Tel & Fax: 86-10-58900235, E-mail: dongxp238@sina.com; or Dr. HAN Jun, Tel & Fax: 86-10-58900680, E-mail: hanjun_sci@yahoo.com.cn; and LI ChaoPing, E-mail: cpli005@126.com

Biographical note of the first author: HAN YanLing, female, born in 1984, Master.

Received: September 27, 2010;

Accepted: May 13, 2011

there is an urgent and growing need for efficient prophylactic and therapeutic measures against prion diseases^[1-2].

Although prions are thought to gain access to the brain via immunocytes and lymphoreticular cells^[3], several reports indicate that humoral immune responses to PrP can antagonize prion infections^[4]. Such phenomena can occur even when the immune response is directed primarily against PrP^C, and does not selectively target PrP^{Sc}^[4]. Monoclonal antibodies (mAbs) and their F(ab) fragments that recognize PrP prevent scrapie infection, and prevent infection by PrP^{Sc} and prions in chronic scrapie-infected neuroblastoma cells^[3,5]. Furthermore, transgenic expression of anti-PrP antibodies in mice arrests the pathogenesis of peripheral scrapie^[6]. Injection of several types of anti PrP antibody into wild-type (WT) mice prevents the development of prion disease upon peripheral challenge^[5-6].

The efficacy of PrP antibodies against prion infection seems to be largely extraneural^[7]. However, passive transfer of PrP-specific IgG is often inefficient when performed after the onset of clinical signs, possibly due to the limited influx of immunoglobulin into the CNS and the high prion load of clinically symptomatic animals. Therefore, stable, sustained PrP-specific antibody levels induced by active immunization may be prophylactic for prion diseases.

Unlike other conventional infectious agents, a major obstacle to active immunization is host tolerance to endogenous PrP^C. DNA vaccines have some advantages over conventional vaccines and induce both humoral and cellular immune responses. In fact, nucleic acid immunization has been shown to break tolerance to host proteins^[4,8]. In the present study, specific immune responses to several PrP DNA vaccine candidates were evaluated in mice. These vaccines included a DNA vaccine, pcNDA3.1-Ubiq-PrP, encoding a human PrP fused to the cellular protein ubiquitin to enhance antigen presentation *via* major histocompatibility complex (MHC) class I; pcNDA3.1-PrP-LII encoding human PrP fused to the lysosome-targeting signal from the lysosomal integral membrane protein type II (LIMP II); and pcDNA3.1-PrP-ER encoding human PrP fused to the ER location signal KEDL to enhance MHC class II antigen presentation. pcNDA3.1-PrP, expressing an unmodified human PrP protein was used as control. Vaccinations were performed using a DNA priming/protein boosting regime. The results

showed that prion vaccines could break immune tolerance to PrP proteins and induce PrP-specific humoral or T-cell responses.

MATERIALS AND METHODS

Plasmid Construction

To construct a expressing full-length human PrP (aa 1-253) vector localized to the ER, a four amino-acid tag (Lys-Asp-Glu-Leu) as a signal peptide^[9] was fused to PrP at the C-terminus (PrP-KDEL) by PCR using the forward primer (5'-ggatccatggcgaaccttgctgctg-3'; *Bam*H I site underlined) and the reverse primer (5'-gccccgcctagagctcatcttctccgactgtacagctcgtcat-3'; *Not* I site underlined) with the recombinant plasmid pcDNA 3.1-huPrP1-253^[10] as the template, then cloned into pcDNA3.1 (zeo+). The human ubiquitin gene was obtained from peripheral blood monocytes by PCR with the forward primer (5'-CGAAGCTTCCACCA-TGCAGATCTTCGTGAAAACCC-3'; containing a Hind III site and a Kozak sequence) and the reverse primer (3'-cccGGATCCgccACCACGAAGTCTCAACACAAG-5', containing a BamHI site) and inserted into pcDNA 3.1-huPrP1-253. A sequence encoding the lysosome targeting signal peptide (KGQGSMDEGTADERAPLIRT) was obtained by synthesizing two complementary oligonucleotides: an LIMP-II sense-strand (ccgGAA-TTCAAAGGA CAGGGATCCATGGATGAG GGAACAGCG-GATGAAAGAGCACCCCTATTCGAACCTAACTCGAGcgg) and an antisense-strand (ccgCTCGAGTTAG GTTCG-AATGAGGGGTGCTCTTTCATCCGCTGTCCCTCATCCAT GGATCCCTGTCTTTGAATTCcgg), containing an *Eco*R I site at the 5' terminus and an *Xho* I site at 3' terminus. The two oligonucleotides were annealed and inserted into pcDNA3.1 to generate pcDNA3.1-LIMP. Human *PRNP* was then released from pcDNA3.1-huPrP1-253 and inserted into pcDNA3.1-LIMP to generate pcDNA3.1-PrP-LII.

Preparation of the DNA and Protein Vaccines

Various PrP DNA vaccines were prepared and purified using an Endofree Plasmid Giga Kit (QIAGEN, Germany), according to the manufacturer's instructions. Recombinant human PrP protein (PrP23-231) was expressed and purified from *E. coli* as previously described^[11] to yield the PrP protein vaccine.

Cell Transfection and Preparation of Cell Extracts

HeLa, COS7, and CHO cells were maintained in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were transiently transfected with the different recombinant plasmids using Lipofectamine 2000 Reagent (Invitrogen, USA) according to the manufacturer's protocol. Cells were harvested 24, 36 or 48 h after transfection, pelleted by centrifugation and suspended in lysis buffer (10 mmol/L Tris-HCl, pH 7.8, 0.5% sodiumdeoxycholate, 0.5% Nonidet P-40, 100 mmol/L NaCl, 10 mmol/L EDTA), supplemented with a complete proteasomal inhibitor cocktail. After centrifugation at 20 000 \times g for 30 min to remove insoluble material, the supernatants were collected and the quantity of the whole protein was determined at OD 405 nm.

Western Blotting

Aliquots of the cell lysates and 1 μ g of recombinant PrP were separated on 15% SDS-PAGE gels and subsequently transferred onto a nitrocellulose membrane by semi-dry blotting. For the immunoblotting experiments, the membranes were incubated with mAb 3F4 (1:5 000) overnight at 4 °C in TBS-T (10 mmol/L Tris-HCl, pH 7.8, 100 mmol/L NaCl, 0.05% Tween 20) containing 5 g/100 mL nonfat milk and subsequently incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5 000) for 1 h at 37 °C. PrP specific signals were visualized using the ECL method (Amersham Life Sciences, UK).

Deglycosylation Assay

After mixing with an equal volume of glycoprotein denaturing buffer (New England Biolabs, Hitchin UK), the various cell lysates were heated at 100 °C for 10 min. Subsequently, 50 mmol/L sodium phosphate, pH 7.5, containing 1% NP-40 and 2 μ L of N-glycosidase F (1 800 000 U/mg, New England Biolabs, USA) were added to the samples and the mixtures incubated at 37 °C for 2 h. The PrP signals in each preparation were detected by western blotting as described above.

Immunofluorescence Assay

Cells transfected with various PrP plasmids for 48 h were fixed for 15 min with ice-cold methanol and treated for 5 min with 0.2% TritonX-100. Cells were blocked with 10% FBS in PBS and incubated with mAb 3F4 (1:500) overnight at 4 °C and subsequently mixed with FITC-labeled goat anti-mouse IgG (1:500; Santa Cruz, USA) for 1 h at

room temperature. Cells were visualized under an Olympus fluorescence microscope.

Animal Immunizations

Female Balb/c mice (4-6 weeks old) were assigned to groups ($n=7$) and injected with the different vaccines. A prime-boost strategy was used with the various DNA vaccines (pcDNA3.1-PrP, pcDNA3.1-Ubiq-PrP, pcDNA3.1-PrP-LII, or pcDNA3.1-PrP-ER) and then boosted with recombinant PrP23-231 protein. DNA vaccines (100 μ g) were administered by intramuscular injection into the sural muscle on Days 0, 14, and 28; each leg receiving 50 μ g of DNA vaccine. The protein vaccines (100 μ g) were administered by hypodermic injection into the abdomen 42 and 56 days after the first injection. One control group was injected three times with vector pcDNA3.1 and twice with the protein vaccine, and the other control group (vector control) was injected with vector pcDNA3.1 and twice with PBS replace the prion protein. Prior to immunization, all DNA vaccines were diluted with PBS to a final concentration of 1 μ g/ μ L. Two weeks after the final immunization, the sera and spleens of mice were collected.

ELISA

Serum anti-PrP antibodies were measured by direct ELISA. PrP23-231 was diluted to a final concentration of 200 ng/mL and coated onto 96-well ELISA plates. After blocking with 200 μ L 1% BSA in PBST (phosphate buffered saline, pH 7.6, containing 0.05% Tween-20), 100 μ L of mouse sera (serially diluted in blocking buffer) were added and incubated for 2 h at 37 °C. The plates were then washed three times with PBST and 100 μ L of HRP-conjugated secondary antibody (1:15 000) were added into each well for 2 h at 37 °C. Bound antibody was detected using 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Sigma). The absorbance was measured at 450 nm after terminating the reaction with 2 mol/L H₂SO₄.

ELISPOT Tests

Mouse spleen cells were prepared as previously described^[11] and the cell concentration adjusted to 10⁷ cells/mL after counting the number of live cells by incubation with 0.4% Trypan Blue (Sigma, USA). The frequency of IFN- γ -producing cells within the primed T lymphocyte population was determined using an ELISPOT assay (Mabtech, Sweden). Nitrocellulose-bottomed 96-well plates were coated

similar to that of native PrP in human brain, suggesting that tagging with ubiquitin or the lysosomal signal peptide did not affect PrP glycosylation. A large amount of non-glycosylated PrP was observed in cells expressing PrP-ER, possibly indicating that accumulation of PrP in the ER disturbs the PrP maturation process. After treatment with PNase F, only one PrP band was detected in the PrP preparations (Figure 2A). Taken together, these results indicate that the PrP DNA vaccines can induce the expression of PrP protein. The expression level of the PrP proteins was the similar after transfection for 36 h and 48 h (Figure 2B). To ascertain the PrP-expressing ability of the constructed PrP DNA vaccines in other cell lines, the four PrP expressing plasmids were introduced into CHO and COS-7 cells. PrP-specific signals from both cells treated with the PrP DNA vaccines were identified, whereas no PrP band was observed in cells transfected pcDNA3.1 alone (data not shown).

Vaccination with PrP DNA Vaccines Followed by Protein Boosting Induces PrP-specific Antibody Responses

The PrP protein vaccine used was a recombinant

human PrP segment (PrP23-231) without the signal peptide and the GPI anchor sequence. The PrP protein of anticipated weight showed about 95% purity by SDS-PAGE and western blotting (Figure 3). Serum from each mouse were collected and serially diluted from 1:200 and measured using an established ELISA after the fifth immunization. A clear PrP-specific antibody response (1:1600) was elicited with the PrP DNA vaccines followed protein boosting (Figure 4A). However, the protein-alone control groups (except the PrP-ER DNA vector group) showed a weaker PrP-specific antibody response,

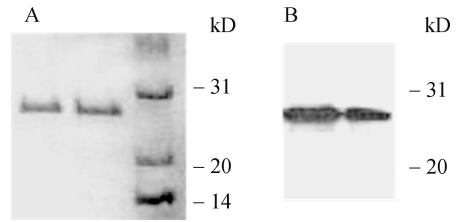


Figure 3. Purification and identification of recombinant human PrP (PrP23-231) from E.coli. (A) 12% SDS-PAGE gels. (B) western blotting with mAb 3F4. The two left lanes contain preparations of purified PrP23-231.

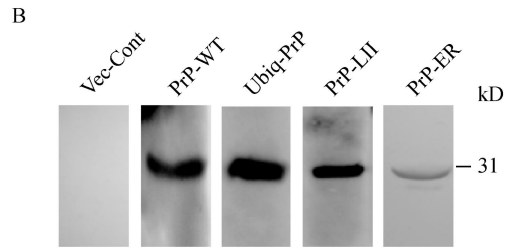
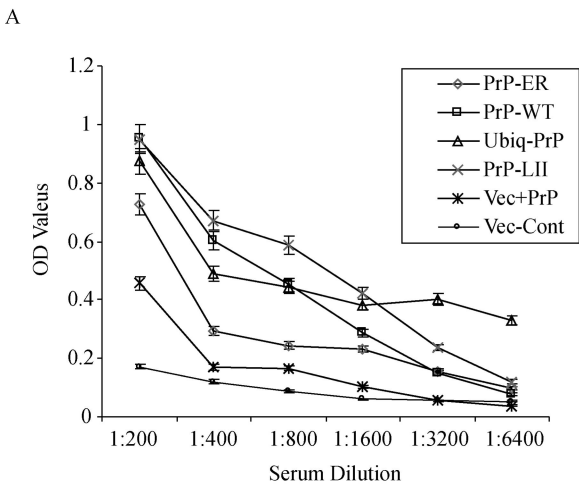


Figure 4. Measurement of PrP-specific antibodies after vaccination. (A) sera PrP antibodies were detected by ELISA. (B) sera PrP antibodies identified by Western Blotting.

with significantly lower levels (<1:1600) than those seen after immunization with DNA prime - protein boost ($P < 0.05$; Figure 4A). Using the rule of P/N value ≥ 2.1 , the titers of PrP antibody in the groups of PrP-WT, Ubiqu-PrP, PrP-LII, and PrP-ER were 1:3 200, >1:6 400, >1:6 400, and 1:3 200, respectively, while that of Vector+PrP was 1:200 (Figure 4B). To examine the specificity of the anti-serum from

immunized mice, aliquots of serum from each mouse were pooled the same groups. A specific signal in the sera of all the vaccination groups was detected by western blotting with recombinant human PrP23-231, but not in the control (pcDNA3.1) (Figure 4C). This suggests that immunization with the PrP DNA plus protein vaccines elicited PrP-specific antibody responses in these mice.

Vaccination with PrP DNA Followed by Protein Boosting Induces PrP-specific T-cell Mediated Responses

To evaluate the possible T-cell mediated immune responses, spleen cells from each mouse were separately prepared in the 2nd weeks after the final immunization. The harvested spleen cells were stimulated with the recombinant proteins PrP23-231, PrP23-90, or PrP91-231, and the γ -IFN-secreting cells counted (Figure 5A). The average number of the positive cells in the PrP-WT, Ubiqu-PrP, PrP-LII, PrP-ER, Vec+PrP and vector control groups (Vec-Cont) stimulated with PrP23-231 were 87, 261, 274, 483, 121, and 16 SFUs/ 10^6 cells, respectively. The average

number of the positive cells induced by Ubiqu-PrP, PrP-LII, PrP-ER was significantly higher than that induced by the Vec+PrP group ($P < 0.05$) after stimulation with PrP23-231. However, there were 537, 482, 336, 720, 274, and 43 SFUs/ 10^6 cells in the preparations stimulated with PrP23-90 (Figure 5B). Compared with the protein control groups, the PrP-WT, PrP-LII, PrP-ER DNA groups showed higher numbers of γ -IFN-positive cells after stimulation with PrP23-90 ($P < 0.05$). Stimulation with PrP91-231 failed to induce γ -IFN-positive cells in any of the vaccination groups [average number of positive cells < 50 SFUs/ 10^6 cells (Figure 5B)]. This indicates that the T-cells epitopes on PrP might be located at the N-terminus.

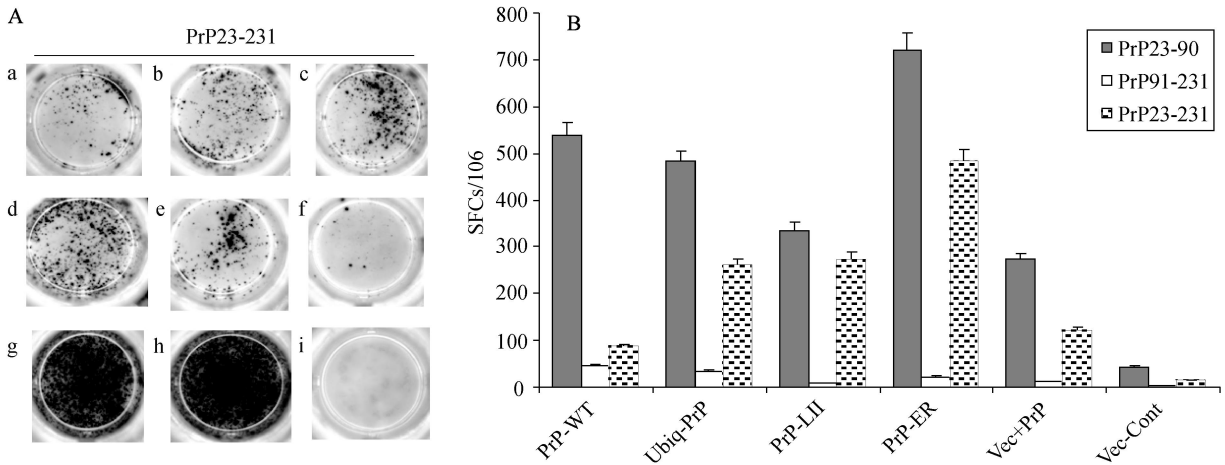


Figure 5. PrP-specific T-cell responses after vaccination. (A) ELISPOT assays with recombinant PrP23-231. Panel a: PrP-WT; panel b: Ubiqu-PrP; panel c: PrP-LII; panel d: PrP-ER; panel e: Vec + PrP; panel f: Vec control; panels g and h: positive controls; panel i: negative control. (B) The average number of IFN- γ -secreting cells (SFUs/ 10^6 cells) in each vaccinated group.

DISCUSSION

There is increasing evidence that points toward an effective role for the immune system in combating Prion diseases^[3,5]. Recent studies report that humoral responses to PrP can be induced not only in PrP knockout (Prnp0/0) mice or xenogeneic systems, but also in wild-type mice, using strong immunization procedures. These reports clearly demonstrate that specific antibodies can delay the onset of disease^[6-7]. In this study, four different DNA vaccines broke PrP immune tolerance and, subsequently, inducing specific humoral and/or cellular responses using a prime-boost immunization regime.

As a conservative host-encoded protein, the immune system appears not to respond to prion

infections in a classical way because no production of anti-PrP Abs^[12]. Indeed, the two doses of prion protein vaccine induced only extremely weak PrP-specific humoral and cellular responses in the experimental mice. Recently, DNA vaccines have been widely used against many infectious diseases, including TSE. Previous studies show that prion DNA vaccines can induce specific immune responses^[4]. Thus, four prion DNA vaccine constructs were prepared and tested for immunogenicity. Of these, immunizations with Ubiqu-PrP and PrP-LII induced relatively high levels of PrP-specific antibodies. A previous study shows that expression of ubiquitin and targeting proteins as fusion proteins enhances antigen uptake and presentation via MHC class-I molecules^[13]. The COOH-terminus of the lysosomal membrane protein facilitates the presentation of MHC class II antigens via lysosomal targeting^[14].

Strong cellular responses were observed in mice immunized with PrP-ER. The ER is considered to play an essential role both in the presentation of both exogenous and endogenous antigens^[15]. These, taken together with the results of the present study, demonstrate that a more active T-cell immune response is highly correlated with the localization of PrP within the ER.

Under our experimental conditions, 10 synthetic peptides spanning aa140-189 failed to stimulate the formation of IFN- γ -producing cells in ELISPOT assays (data not shown). Coincidentally, stimulation with recombinant PrP91-231, which covers the same regions as the above 10 peptides, did not induce enough IFN- γ -producing cells (<50 SFUs/10⁶ cells) in any of the all vaccination groups. In contrast, the NH₂-terminal fragment (PrP23-90) induced a significant number of IFN- γ -producing cells in the spleens of the mice using our prime-boost vaccinating strategy. Our data strongly suggest that the potential PrP T-cell epitopes are located at the NH₂-terminus. The exact molecular basis for T-cell immunity induced by the NH₂-terminal region of PrP remains unknown. One possibility might be its flexible structure, which may more easily provide linear epitopes for T-cell immunity. More precise understanding of the role of the PrP NH₂-terminal region during antigen presentation is required.

REFERENCES

- Venneti, S. Prion diseases. *Clin Lab Med*, 2010; 30, 293-309.
- Prusiner, SB. Prions. *Proc Natl Acad Sci USA*, 1998; 95, 13363-83.
- Aguzzi A, Glatzel M, Montrasio F, et al. Interventional strategies against prion diseases. *Nat. Rev. Neurosci*, 2001; 2, 745-9.
- Fernandez-Borges N, Brun A, Whitton JL, et al. *DNA vaccination can break immunological tolerance to PrP in wild-type mice and attenuates prion disease after intracerebral challenge*. *J Virol*, 2006; 80, 9970-6.
- Féraudet-Tarisse C, Andréoletti O, Morel N, et al. Immunotherapeutic effect of anti-PrP monoclonal antibodies in transmissible spongiform encephalopathy mouse models: pharmacokinetic and pharmacodynamic analysis. *J Gen Virol*, 2010; 91, 1635-45.
- Shimizu Y, Kaku-Ushiki Y, Iwamaru Y, et al. A novel anti-prion protein monoclonal antibody and its single-chain fragment variable derivative with ability to inhibit abnormal prion protein accumulation in cultured cells. *Microbiol Immunol*, 2010; 54, 112-21.
- White AR, Enever P, Tayebi M, et al. Monoclonal antibodies inhibit prion replication and delay the development of prion disease. *Nature*, 2003; 422, 80-3.
- Leitner WW, Hwang LN, deVeer MJ, et al. Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways. *Nat Med*, 2003; 9, 33-9.
- Wang X, Dong CF, Shi Q, et al. Cytosolic prion protein induces apoptosis in human neuronal cell SH-SY5Y via mitochondrial disruption pathway. *BMB Rep*, 2009; 42, 444-9.
- Han J, Zhang J, Yao HL, et al. Study on interaction between microtubule associated protein tau and prion protein. *Science in China, Series C*, 2006; 49, 473-9.
- Cole GA. Interferon-gamma ELISPOT assay for the quantitative measurement of antigen-specific murine CD8⁺ T-cells. *Methods Mol Biol*, 2005; 302, 191-204.
- Rosset MB, Ballerini C, Gregoire S, et al. Breaking immune tolerance to the prion protein using prion protein peptides plus oligodeoxynucleotide-CpG in mice. *J Immunol*, 2004; 172, 5168-74.
- Duncan LM, Nathan JA, Lehner PJ. Stabilization of an E3 ligase-E2-ubiquitin complex increases cell surface MHC class I expression. *J Immunol*, 2010; 184, 6978-85.
- Basha G, Lizée G, Reinicke AT, et al. MHC class I endosomal and lysosomal trafficking coincides with exogenous antigen loading in dendritic cells. *PLoS One*, 2008; 3, e3247.
- Sofra V, Mansour S, Liu M, et al. Antigen-loaded ER microsomes from APC induce potent immune responses against viral infection. *Eur J Immunol*, 2009; 39, 85-95.