Autophagy-targeted vaccine of LC3–LpqH DNA and its protective immunity in a murine model of tuberculosis

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A R T I C L E   I N F O

Article history:
Received 21 June 2013
Received in revised form 7 February 2014
Accepted 25 February 2014
Available online 12 March 2014

Keywords:
Autophagy
Mycobacterium tuberculosis
LC3
LpqH
Ag85B
Protective immunity

A B S T R A C T

The development of more effective antituberculosis vaccines would contribute to the control of the global problem of infection with Mycobacterium tuberculosis (MTB). Recently, the highlighted importance of autophagy in the host immune response against MTB has attracted the attention of researchers. However, the vaccines targeted at autophagy remain to be developed. In this study, we report on an autophagy-targeted vaccine of 19 kDa MTB lipoprotein (LpqH) DNA that harbors another gene coding microtubule-associated protein light chain-3 (LC3), which transports LpqH to autophagosomes and displays enhanced protective efficacy against MTB. After the transfection of pCMV-LpqH DNA, a significant increase LC3 II was detected in RAW264.7 cells, which was similar to that observed with treatment with rapamycin, a reagent used to induce autophagy. To target autophagy, the gene coding LC3, as a marked protein of autophagosome, was linked to the LpqH gene to express an LC3–LpqH fused protein. Interestingly, LC3–LpqH fused protein was determined to be transported to an autophagosome, which was demonstrated by the co-localization of GFP-LC3 with LC3–LpqH at autophagosomes. Notably, the mice immunized with LC3–LpqH/Ag85B displayed decreased mycobacterial loads in the lungs and spleen when challenged with virulent MTB by intravenous infection, which was consistent with increased IgG2a in serum and IFN-γ and IL-2 produced by splenocyte. In conclusion, our study demonstrates that an LC3–LpqH DNA vaccine could have autophagy as its target, which contributes to the enhancement of the Th1 immune response and vaccine protective efficacy.

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1. Introductions

Mycobacterium tuberculosis (MTB) infection remains a major cause of morbidity and mortality throughout the world. There were 8.8 million new cases of tuberculosis (TB) in 2010 resulting in 1.1 million deaths [1]. The current vaccine, Mycobacterium bovis bacillus Calmette-Guérin (BCG), has variable protective efficacy, ranging from 0% to 85% in different studies [2]. In the past few decades, based on the immunodominant nature of MTB antigens and their protective immunity in the host, various types of new vaccines have been developed.

LpqH, a 19-kDa lipoprotein, is a cell wall-associated lipoprotein present in M. tuberculosis. It was originally identified as a major antigen of M. tuberculosis on the basis of its recognition by murine antibodies raised against crude bacterial extracts [3]. Furthermore, LpqH exhibits rather unique properties. It not only stimulates a strong adaptive immune responses in MTB-infected humans and animals [4] but also induces the secretion of large amounts of IL-12 from macrophage cultures [5]. In addition, on account of its lipoproteinaceous nature, LpqH is one of a number of molecules that mediate the innate response to MTB and the major histocompatibility complex class I (MHC) antigen presentation pathway [6,7]. Lipoprotein has been recently described to be involved in autophagy activation in human monocytes via TLR1/2/CD14 receptors and the AMP-activated protein kinase signal pathway [8].
Autophagy, particularly macroautophagy, is a conserved catabolic process in eukaryotic cells. Recently, it has been recognized that autophagy plays an important role in innate and adaptive immunity defense against intracellular pathogens, such as M. tuberculosis. Using this mechanism, increased levels of antigens will be transported into autophagosomes or autophagic vacuoles to be degraded for peptide presentation, which promotes a protective immune response [9,10]. The induction of autophagy by pharmacological or immunological means can eliminate M. tuberculosis in macrophages, providing for the possibility of enhanced protective efficacy of vaccines designed to activate autophagy [11]. Interestingly, the efficacy of the Ag85B DNA vaccine is increased in murine models when autophagy is induced by using a DNA plasmid to disrupt the mTOR, the mammalian target of rapamycin [12].

LC3 is one of the key components involved in autophagy, the cellular process mediating the degradation and turnover of macromolecules and organelles. Under nutrient-rich conditions, LC3 is usually dispersed throughout the cytoplasm in a dissociated form (LC3-I). In cases where autophagy is induced, such as nutrient depletion or rapamycin (a classical reagent to induce autophagy by inhibiting mTOR) treatment, LC3-I converts to its phosphatidylethanolamine-conjugated LC3-II form and then localizes to both sides of autophagosome [13]. The mechanism underlying LC3s regulation of autophagy is still under investigation, but it is clear that the subsequent recruitment of LC3-II is essential for the initiation of autophagy, elongation and closure of the autophagosomes in concert with other autophagy-associated gene proteins. LC3 is also crucial for autophagosome maturation during which the outer autophagosomal membrane fuses with the lysosome, and the subsequent breakdown of the inner membrane results in the exposure of the segregated cytoplasmic material to lysosomal hydrolases for degradation. Growing evidence indicates that autophagy may be involved in MHC class I and II antigen processing and presentation of certain endogenous synthesized peptides. Therefore, the specific targeting of antigens to autophagy by fusion with the LC3 protein may represent an effective vaccine strategy for enhancing CD4+ T and CD8+ T cell responses.

In this study, we determined that LpqH induced a greater expression of LC3-II and localized to the autophagosome when fused with LC3 via genetic engineering in vitro. Furthermore, this autophagy-targeted vaccine of LC3–LpqH DNA displayed enhanced protective efficacy of Th1-type immunity against MTB in mice.

2. Materials and methods

2.1. Plasmids, cells and animals

The virulent MTB strain H37Rv, the pCMV-Ag85B, recombinant Ag85B protein, pEGFP-LC3, RAW264.7 and GFP-LC3–RAW264.7 cells were prepared as previously described [14,15]. To construct pCMV–LpqH, the LpqH gene was amplified from the genomic DNA of H37Rv by PCR with specific primers (sense–tgatggcggggaagctgatgac; antisense–ggtgatgccggagctcattgtc), and then the PCR product was subsequently cloned into the EcoRI and BglII sites of the pCMV-HA vector. To construct pCMV-LC3–LpqH, the cDNA was prepared using reverse transcriptase PCR from triturates from Balb/c mice, and the LC3 gene was amplified with specific primers (sense–ttatagattgccctcagccccgtc; antisense–gctgagctcagggctagttc), and then the PCR products were subcloned into the pCMV-LpqH plasmid, which was responsible for HA–LC3–LpqH fusion protein expression. These primers were synthesized by Sangon Biotech Company. Female Balb/c mice (6–8 weeks old) were purchased from the animal center of Anhui University of Science and Technology and raised carefully in accordance with the National Institutes of Health Guidelines on Animal Care. All experimental procedures were approved by the Animal Care and Use Committee of Anhui University of Science and Technology (Permit numbers: AUST 2012-0632).

2.2. Cell transfection

Transient transfection was performed using Lipofectamine™ 2000 reagent as previously described [16,17]. Raw264.7 cells or GFP-LC3–RAW264.7 cells were plated in 6-well plates with DMEM medium containing 10% fetal calf serum (FCS) and then cultured in a humidified 37 °C/5% CO2 incubator overnight. For transfection assays, Raw 264.7 cells were transfected with 1 μg/ml, 2 μg/ml, 4 μg/ml pCMV-LC3–LpqH or pCMV vectors, using the β–actin as the internal control. At 24–48 h after transfection, the cells were harvested, washed with PBS and then lysed with lysis buffer on ice for 30 min with vortexing. After centrifugation at 15,000 × g for 15 min, the supernatants were collected and subjected to Western blot analysis. Quantification of autophagy was performed based on the LC3–II band intensity. For positive control of autophagy activation, cells were incubated in complete medium that contained 50 nM rapamycin (Sigma) for 4 h. In vitro transfection of pCMV-LpqH (1 μg/ml, 2 μg/ml, 4 μg/ml and 8 μg/ml) into Raw264.7 and pCMV-hLC3–LpqH (1 μg/ml, 2 μg/ml, 4 μg/ml and 8 μg/ml) into THP-1 cells was described in S1 Text.

2.3. SDS-PAGE and Western blot

Samples were boiled for 5 min in the presence of 4 × SDS-PAGE-loading buffer (250 mM Tris–HCl pH 6.8, 40% glycerol, 8% SDS, 0.57 M β-mercaptoethanol, 0.12% bromophenol blue). Equal amounts of protein were run on 12% SDS-PAGE gels and transferred onto a PVDF membrane. The membrane was blocked for 3 hr at room temperature in 5% milk in PBS/Tween 20 (0.1%) and then probed with anti-LC3 (Protein Technology Group) or anti-HA (Protein Technology Group) for bonding LC3–LpqH–HA fusion protein overnight at 4 °C. After washing with PBST, the membrane was probed with appropriate HRP-conjugated goat anti-rabbit IgG (Protein Technology Group) for 1 h at 37 °C. Finally, primary antibodies were visualized using the enhanced chemiluminescence (ECL) purchased from Genebase Company.

2.4. Fluorescence microscopy and image acquisition

Raw 264.7 cells with stable expression of GFP-LC3 were subjected to immunofluorescence assay, after pCMV-LC3 or pCMV-LC3–LpqH transfection for 12 h. The cells were fixed for 10 min in 1% paraformaldehyde. After wash with PBS, the cells were permeabilized with 0.1% Triton X-100 for 3 min. Following wash, anti-HA antibodies (Protein Technology Group) were added to stain overnight at 4 °C. After three washes, secondary antibodies conjugated to rhodamine (Protein Technology Group) were added and the incubation continued at 37 °C for 60 min. Images were taken and processed by a fluorescence microscope system using the Olympus IX73 microscope.

2.5. Challenge with M. tuberculosis

Female Balb/c mice 6–8 week of age were used for vaccination and further infection. At first, they were randomly divided into four groups: pCMV-Ag85B, pCMV-LpqH/pCMV-Ag85B (both constructs administered together), pCMV-LC3–LpqH/pCMV-Ag85B (both constructs administered together) and pCMV vector. Then, the mice were immunized intramuscularly three times at 3-week intervals. Three months after the final DNA immunization, different groups
of mice were infected intravenously through the tail-vein with 1 × 10⁶ CFU of the MTB H37Rv strain. The mice spleens and lungs were dissected out, homogenized and plated for bacterial colonies at 1, 2, 4, and 8 weeks after infection. One portion of the spleen and lung tissue was homogenized and subjected to serial dilutions. An aliquot was plated on solid Middlebrook 7H10 agar plates and incubated at 37°C for 4 weeks. The colonies were counted and the results were expressed as log₁₀ CFU.

2.6. Detection of anti-Ag85B antibodies

Two weeks after the last immunization, the titers of anti-Ag85B IgG1 and IgG2a antibodies in the sera from immunized mice were detected by ELISA using recombinant Ag85B purified by our laboratory as previously described [15]. Briefly, 5 μg/mL recombinant Ag85B protein in carbonate–bicarbonate buffer were incubated in 96-well plates overnight at 4°C. Next, 5% bovine serum albumin in PBS was added for 2 h at 37 °C. After removal of the supernatant, individual serum sample were added in duplicate and the incubation was continued for 1 h at 37°C. After three washes, HRP-conjugated goat anti-mouse IgG1 and IgG2a (1:500, Sigma, St. Louis, MO, USA) were added and the incubation was continued for 1 h at 37°C. After washing three times, tetramethyl benzidine substrate was added for a color reaction, and the reaction was stopped by adding 1 M H₂SO₄. The optical density (OD) was measured at 450 nm.

2.7. Cytokine release assay

Two weeks after the last immunization, the mice were sacrificed, and their splenic lymphocytes were prepared by a routine method. Splenocytes from individual mice were stimulated in duplicate with 10 μg/mL rAg85B in 24-well plates. After 72 h, culture supernatants were harvested, and the presence of cytokines, including IFN-γ, IL-2, IL-4 and IL-10, were detected using commercial mouse cytokine immunoassay ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions, as previously described [18].

2.8. Statistics

For the statistical analysis of the bacterium titles, a non-parametric Mann–Whitney test was used. All other statistical analyses were performed using an unpaired two-tailed Student t-test. The p value of significant differences is reported, and p < 0.05 or p < 0.01 was considered significant. The plotted data represent the mean plus standard deviation (SD), unless otherwise stated.

3. Results

3.1. LpqH induces an increase in the amount of LC3-II

Recent reports indicate that autophagy induction can eliminate intracellular MTB through enhanced interaction between mycobacterial phagosomes and autophagosomes [19]. To verify the induction of autophagy mediated by LpqH, we first analyzed the levels of endogenous LC3 in RAW264.7 cells treated with LpqH or rapamycin by Western blot assay. This analysis indicated that upon rapamycin or LpqH treatment, LC3-I and LC3-II were expressed in RAW264.7 cells as compared to internal control β-actin (Fig. 1A). Furthermore, as shown in Fig. 1B, LC3-II levels upon LpqH treatment were significant higher than pCMV vector treatment. Consistently, rapamycin treatment also induced a strong increase in LC3-II levels. Therefore, LpqH treatment can cause an increase in LC3-II levels, that is, there is a certain relationship between LpqH and autophagy induction. In addition, dose-dependent increase of LC3-II was found in dose range of 1–4 μg/mL pCMV-LpqH as shown in Fig. S1.

3.2. Detection of expression of pCMV-LC3–LpqH plasmid in vitro

Based on the above results, we constructed a pCMV-LC3–LpqH DNA plasmid and explored whether pCMV-LC3–LpqH was expressed in vitro. RAW264.7 cells were transiently transfected with the recombinants. The protein expression levels were detected by Western blotting with the obvious bands probed by anti-HA antibodies. In addition, we analyzed the relationship between protein levels and the dose of the recombinants (Fig. 2). We found that the LC3–LpqH-HA fusion protein was expressed effectively in transfected murine macrophages in a DNA plasmid dose-dependent manner. What is more, the expression of pCMV-hLC3–LpqH and its influence on LC3-II were characterized in human macrophages. As shown in Fig. S2, hLC3-LpqH presented a dose-dependent increase in range of 1–4 μg/mL pCMV-hLC3–LpqH, and also a dose-dependent increase of LC3-II was subsequently found.
3.3. LpqH colocalizes with the autophagy marker LC3

To further test the autophagy induction of LpqH, we investigated GFP-LC3 colocalization with LC3–LpqH. GFP-LC3–Raw 264.7 cells were prepared as previously described [14] and were transfected with pCMV-LpqH or pCMV-LC3–LpqH. As shown in Fig. 3, the autophagosomes were marked as green puncta by GFP-LC3, which partially overlapped with LC3–LpqH in the pCMV-LC3–LpqH-transfected cells as yellow puncta, whereas, this autophagosomal marker did not colocalize with LpqH in pCMV-LpqH-treated cells but instead diffused in red. Therefore, these data suggest that the LpqH localized to autophagosomes was targeted by LC3.

3.4. Ag85B-specific antibody and cytokines response to vaccines

To analyze the immunogenicity of different vaccines, mice were immunized with DNA plasmid according to the immunization schedule (Fig. 4A). IgG1 and IgG2a indicate Th2- and Th1-type immune responses in mice, respectively. As shown in Fig. 4B, no specific anti-Ag85B antibodies were detected in the empty vector-immunized mice. When the titers of the specific IgG isoferes were investigated, the group immunized with pCMV-LC3–LpqH/pCMV-Ag85B displayed the highest IgG2a titer, whereas significant increases in IgG1 were found in the groups immunized with pCMV-LpqH/pCMV-Ag85B and pCMV-Ag85B. The changes in the IgG isoferes between pCMV-LpqH/pCMV-Ag85B and pCMV-LC3–LpqH/pCMV-Ag85B groups suggested that the immune response was skewed from Th2- to Th1-type dominance.

In addition, we characterized the functional phenotype of the antigen-specific T cell response in mice. Splenocytes from immunized mice were tested for the secretion of the cytokines IFN-γ/IL-2 (Th1-like) and IL-4/IL-10 (Th2-like) upon re-stimulation with recombinant Ag85B. As shown in Fig. 4C–F, the mice that were immunized with pCMV-LC3–LpqH and pCMV-Ag85B together responded to antigen stimulation by producing evidently higher levels of IFN-γ/IL-2 than the other groups, whereas no differences were found in the levels of IL-4/IL-10. The high levels of IFN-γ and IL-2 demonstrate that vaccination with pCMV-LC3–LpqH/pCMV-Ag85B induces potent Th1 responses in mice, confirming the previously mentioned humoral response results.

3.5. Effect of mycobacterial vaccines on bacterial load

Finally, to determine the protection of mycobacterial vaccines in lungs and spleen against MTB H37Rv infection, we examined the effects of plasmid vaccination on the replication of infected MTB in vivo. Groups of Balb/c mice were injected with pCMV-Ag85B, pCMV-LpqH/pCMV-Ag85B, pCMV-LC3–LpqH/pCMV-Ag85B and pCMV vectors. Then all mice were challenged intravenously with 1 × 10^5 CFU MTB H37Rv. The numbers of MTB loads in the spleens and lungs were determined by in vitro colony formation assays. As shown in Fig. 5, at week 1, in both lungs and spleens, the bacterial loads displayed no significant differences among the four treatments of mice. At week 2, lower loads were found in the lungs of DNA LC3–LpqH/Ag85B-treated mice. The bacterial loads continued to rise up to 4 weeks, reaching their highest level, followed by drops in the number of mycobacteria populating spleens or lungs discovered at week 8. As shown in Fig. 4A, at either week 4 or week 8, DNA LC3–LpqH/Ag85B-treated mice had lower loads compared with DNA-Ag85B treated mice, whereas no evident differences were found between DNA LpqH/Ag85B and DNA Ag85B treated mice in lungs. In addition, the same as in lungs, at either week 4 or week 8, DNA LC3–LpqH/Ag85B-treated mice had lower loads compared with DNA-Ag85B-treated mice, while no evident difference was found between DNA LpqH/Ag85B and DNA Ag85B treated mice in their spleens. The dynamics of the bacterial load indicated that LC3–LpqH/Ag85B can evidently inhibit the replication of MTB and plays a protective role in lungs and spleens against MTB infection.

4. Discussion

Despite great progress in the last couple of decades in the development of novel DNA vaccines against TB, the goal of developing a perfect vaccine has not been realized. Previous results indicate that one of the effective mechanisms of elimination of intracellular pathogens such as M. tuberculosis is the autophagy pathway, which performs an immune defense role [20]. However, one of the major questions is whether mycobacterial antigens can induce autophagy. Here, we analyzed the conversion of LC3-I (cytosolic form) to LC3-II (membrane-bound lipidated form) in RAW264.7 cells treated with LpqH or rapamycin by Western blot assay. We found LpqH induces an increased LC3-II amount, which indicates that the 19-kDa mycobacterial lipoprotein functions to activate the autophagy in RAW264.7 cells, which is consistent with the results reported by Shin et al. (2010) that TLR2/1/C1D14 stimulation by mycobacterial lipoprotein LpqH can robustly activate antibacterial autophagy [8].

Subsequently, to investigate the LpqH-induced autophagy in macrophages further, we explored whether pCMV-LC3–LpqH was expressed in vitro. Our results indicated that the LC3–LpqH fusion protein was expressed in transfected cells effectively and related closely to the dose of the pCMV-LC3–LpqH plasmid, which contributed to the follow-up study of protective immunity. Interestingly, the vaccine efficacy against MTB could be enhanced by inducing autophagy upon rapamycin treatment or intergenic suppression in the murine model, via augmenting
autophagy-mediated antigen presentation [9,12,21]. Given that antigen presentation is enhanced by autophagy, a vaccine with the ability to participate in the autophagy pathway could contribute to better immune protection. In this study, we provide evidence that LpqH localizes to autophagosomes when fused with LC3, directing the packing of cargo and participating in forming the double-layer membrane, which in turn, leads to increased processing and presentation of antigens. This observation prompted us to use a mouse model to evaluate whether this autophagy-targeted vaccine could promote an enhanced efficacy in vivo.

The efficacy of an autophagy-targeted vaccine relies on its protective immunity in humans or murine models of tuberculosis. Previous studies indicate that the detrimental or beneficial activity of LpqH is mediated by its effect on recognition of other antigens present in the vaccine rather than by an immune response to the antigen itself [22,23]. Moreover, several other MTB antigens have been demonstrated to confer obvious protection when delivered as DNA vaccines, such as Ag85B, a major protein secreted by all Mycobacterium species, which can induce a potent protective immune response [15]. In this study, we used a murine model of tuberculosis involving an intravenous route rather than an aerosol route of inoculation because earlier death and inconsistent bacterial growth differences of mice infected via an aerosol route was found (data not shown), which is not suitable for the functional long-term evaluation of vaccines. Notably, we found that mice immunized with pCMV-LC3–LpqH displayed enhanced Th1-type

![Image of autophagy-mediated presentation and cytokine production](Image)

**Fig. 4.** Immunization, antibody titer and cytokine production. Female Balb/c mice, 6–8 weeks of age, were randomly assigned into four groups for different immunization regimens: pCMV-Ag85B (Ag85B), pCMV-LpqH/pCMV-Ag85B (LpqH+Ag85B), pCMV-LC3–LpqH/pCMV-Ag85B (LC3–LpqH+Ag85B) and pCMV (vector). (A) The mice were immunized intramuscularly three times at 3-week intervals. (B) The level of anti-Ag85B IgG1 and IgG2a in sera was determined by ELISA at 2 weeks after the final boost. Sera from pre-immunized mice were used as a negative control. The IgG2a titers of mice immunized with LC3–LpqH+Ag85B were significantly higher than those immunized with LpqH+Ag85B. Consistently, the LC3–LpqH+Ag85B-immunized mice had lower levels of IgG1 compared with the LpqH+Ag85B-immunized mice. (C–F) For detecting cytokines responses to vaccination, bulk splenocytes from vaccinated mice were stimulated in vitro with Ag85B for 72 h and the supernatants were harvested for cytokine determination using ELISA. The secretion of IFN-γ and IL-2 in the LC3–LpqH+Ag85B group was significantly higher than the LpqH+Ag85B group, whereas no significantly differences in IL-4 and IL-10 were detected between them. All independent experiments were performed in triplicate, and data are presented as the means ± SD (*p < 0.05, **p < 0.01).
immunity, leading to a greater expansion of Ag85B-specific IFN-γ and IL2T cells in the spleen. The Th1-type immune response is considered to be the major host protective response used to control an MTB infection. We recently reported that the efficacy of the Ag85B-DNA vaccine can be enhanced by T-bet as a powerful adjuvant to induce the optimal development of the Th1-immune response [15]. Thus, we finally evaluated the protective immunity response of a LC3–LpqH/Ag85B DNA vaccine in lungs and spleens following infection with 1 × 10^5 CFU of MTB H37Rv strain. We found that at week 2, lower loads were found in the lungs of DNA LC3–LpqH/Ag85B-treated mice. The bacterial loads continued to rise up to 4 weeks, reaching a maximum level, followed by drops in the number of mycobacteria populating spleens or lungs discovered at week 8. In addition, at either week 4 or week 8, DNA LC3–LpqH/Ag85B-treated mice had lower loads compared with DNA-Ag85B-treated mice, whereas no evident difference was found between the DNA LpqH/Ag85B and DNA Ag85B–treated mice in lungs and spleens. During later MTB infection, LC3–LpqH/Ag85B treatment significantly inhibited the replication of MTB infected in the spleens and lungs of mice. These data confirmed that the efficacy of anti-tuberculosis vaccine can be enhanced through targeting of autophagy by the vaccine itself.

Although our results clearly demonstrate a novel and effective vaccine against tuberculosis in vivo, we must emphasize that they were obtained in an intravenously infected model with a large bacterial inoculum. Consequently, these data are not able to predict the efficacy of the vaccine in models with smaller challenge doses or with other routes of infection. Further analyses are needed to link the beneficial effect of the pCMV-LC3–LpqH DNA-vaccine to effects on antigen-presentation-associated molecules, prevention of organ pathological injury, overall survival in this illness, severity of illness, weight fluctuation and other parameters, which would aid in elucidation of the mechanism underlying the enhanced immunoprotection induced by LC3–LpqH.

Taken together, the above results indicate that LpqH exerts anti-MTB activities through autophagy induction, and the protective immunity of LC3–LpqH DNA vaccine is mediated by its effect on recognition of Ag85B rather than the antigen itself. Thus, an autophagy-targeted vaccine can provide a potential route for the development of improved vaccine candidates, as it provides a simple and powerful strategy to enhance vaccine efficacy through engineered access to the autophagic pathway.

**Acknowledgments**

This work was supported by Anhui Provincial Natural Science Foundation (No. 1208085Q162), National Natural Science Foundation Grants of China (No. 81202294, No. 81172778, No. 81302524 and No. 61170172) and Higher Education Key Program of Anhui Provincial Nature and Science Foundation (No. KJ2013A105). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Conflict of interest**: There is no conflict of interest in this study.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.02.069.

**References**


