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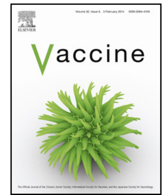

Autophagy-targeted vaccine of LC3–LpqH DNA and its protective immunity in a murine model of tuberculosis

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ABSTRACT

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 (MHCI) 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].

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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 endogenously 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—tatgaattcgggtgaagcgtggactgac; antisense—ggtagatctggaacaggtcacctcgatttc), and then the PCR product was subsequently cloned into the *Eco*RI and *Bgl*II 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—ttaagatctatgccctccgaccggcctttc; antisense—gctggtacctcagaagccgaaggtttcttg), 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 2012-0032).

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% CO_2 incubator overnight. For transactivation 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 h 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

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