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Efficacy of an autophagy-targeted DNA vaccine against avian leukosis virus subgroup J

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ABSTRACT

Infection with the avian leukosis virus subgroup J (ALV-J) can lead to neoplastic disease in chickens, inflicting significant economic losses to the poultry industry. Recent reports have identified inhibitory effects of ALV-J on autophagy, a process involving in innate and adaptive immunity. Inspired by this connection between autophagy and immunity, we developed a novel DNA vaccine against ALV-J which includes co-administration of rapamycin to stimulate autophagy. To measure the efficacy of the developed prototype vaccine, five experimental groups of seven-day-old chickens was immunized three times at three-week intervals respectively with vector, pVAX1-gp85, pVAX1-gp85-LC3, pVAX1-gp85 + rapamycin and pVAX1-gp85-LC3 + rapamycin through electroporation. We then tested their antibody titers, cytokine levels and cellular immune responses. The immunoprotective efficacy of the prototype vaccines against the challenge of the ALV-J GD1109 strain was also examined. The results showed that the combination of pVAX1-gp85-LC3 and rapamycin was able to induce the highest antibody titers, and enhance interleukin(IL)-2, IL-10 and interferon (IFN)-γ expression, and the chickens immunized with the combination of pVAX1-gp85-LC3 and rapamycin showed the highest percentage of CD3+ CD8+ T lymphocytes. Based on our results, we suggest that stimulating autophagy can improve the efficacy of DNA vaccines and that our DNA vaccine shows the potential of being a candidate vaccine against ALV-J. This study provides a novel strategy for developing vaccines against ALV-J.

1. Introduction

Avian leukosis virus subgroup J (ALV-J), first isolated from a commercial layer flock by Payne et al. [1], is a new avian leukosis virus. The genome of ALV-J encodes the gap, pol and env genes. The envelop protein, gp85, covering the virus, is a type of glycoprotein encoded by env with an epitope related to virus neutralization which determines the antigenicity of ALV-J [2]. It also has an epitope that interplays with the receptors and is critical to identifying the phenotype of the virus [3]. In addition, the protein serves as an assistant to help ALV-J infect susceptible cells and induce the production of specific antibodies among infected chickens [4]. Recently, ALV-J has caused increasing mortality, tumor occurrence, and emaciation among chickens and other livestock in the global poultry industry and the high costs of eradicating such virus has been plaguing poultry farmers worldwide [5–9]. It is fortunately found that using CpG ODN as an adjuvant or encapsulating recombinant gp85 protein with liposomes can help protect chickens from ALV-J [10,11]. However, despite the recent development of a chimeric multi-epitope DNA vaccine against ALV-J for use in chickens [12,13], there has been no effective commercial vaccine against ALV-J to date.

Macroautophagy (referred hereafter as autophagy) is a conserved cellular pathway of regulating and maintaining the cytoplasmic environment in eukaryotic cells through degrading long-lived protein and damaged organelles [14]. There is an important relation between autophagy and immunity [15,16]. Not only can autophagy directly eliminate pathogens through capturing, isolat-

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ing and digesting autophagosomes, it also influences innate and adaptive immunity [17,18], including antigen processing and presentation. Autophagy happens when cytoplasmic constituents are delivered to lysosomes to be hydrolyzed and thereby can facilitate the process of endogenous antigen presentation by major histocompatibility complex (MHC) class II molecules [19]. Autophagy might also help the loading of antigens onto MHC class I molecules when the proteasomal degradation system, which is a main component of MHC class I antigen presentation, is blocked [20].

Microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3) is a protein involved in autophagy within animal cells. It is mainly positioned on the surface of preautophagosomes and autophagosomes, and is involved in the process of autophagy [21]. LC3 is divided into 3 subtyples, LC3A, LC3B, and LC3C among which LC3B is regarded as a specific marker protein of autophagosomes, and to some extent as a specific diagnostic indicator of autophagy [22]. Studies also show that MHC class II presentation to CD4+ T cell clone could be significantly enhanced if we target the Influenza Matrix Protein 1 (MP1) to autophagosomes via fusion to the autophagosome-associated protein Atg8/LC3 [19].

The important role of autophagy in antigen presentation presents an opportunity to exploit this process for the development of novel vaccines. A previous study has shown that the efficacy of the BCG vaccine can be enhanced through stimulating autophagy, and increasing antigen presentation in mouse dendritic cells [23]. In recent years, several research studies of DNA vaccines based on autophagy have been reported. Meerak et al. developed a DNA vaccine that incorporates an autophagy-inducing plasmid, which enhances host immune responses to a DNA vaccine against the *Mycobacterium tuberculosis* (MTB) antigen delivered by chitosan particles in mice [24]. Hu et al. reported that the autophagy-targeted vaccine, LC3-LpqH, displays enhanced protective efficacy of Th1-type immunity against MTB in mice [25]. In addition, Ravindran et al. demonstrated the key role of virus-induced GCN2 activation in dendritic cells in initiating autophagy and enhancing antigen presentation to both CD4+ and CD8+ T cells [26].

In this study, we demonstrate that the immunity of chickens against ALV-J in is enhanced after transfected with a gp85-LC3B vector with the addition of rapamycin using a TERESA DNA delivery device.

2. Materials and methods

2.1. Cell lines, viruses and plasmids

The immortalized chicken embryo fibroblast cell line DF-1 was obtained from ATCC (cell line number: CRL-12203), and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). ALV-J strain GD1109 was isolated from a commercial layer flock [27]. To construct pVAX1-gp85-LC3B and pVAX1-gp85, gp85-LC3B and gp85 genes were amplified and sub-cloned into pVAX1 plasmid. Firstly, cDNA was prepared through processing a sample from DF-1 cells infected by GD1109 at an M.O.I of 1 with the method of reverse transcription PCR. Then, it was used as templates to amplify gp85-LC3B and gp85 genes. To amplify gp85-LC3B fusion gene, we conducted fusion PCR, a process in which gp85 and LC3B genes were amplified individually with specific primers, fusion gp85 and fusion LC3B, respectively (Table S1) before the fusion gene was generated by using the sense primer fusion gp85 and anti-sense primer fusion LC3B (Table S1) to re-amplify the previously amplified gp85 and LC3B. Finally, the gp85-LC3B fusion gene was sub-cloned into the pVAX1 plasmid and DNA sequencing was conducted to validate the sequence of pVAX1-gp85-LC3B. To obtain pVAX1-gp85, gp85 gene was amplified using the primer gp85 (Table S1) and similarly sub-cloned into a pVAX1 plasmid.

2.2. Cell transfections

DF-1 cells were transfected with pVAX1-gp85, pVAX1-gp85-LC3B or a pVAX1 empty vector using jetPRIME Polyplus-transfection (polyPlus, California, America) transfection reagent to confirm *in vitro* expression of the plasmids. For each transfection, 2 μg of plasmid was transfected into DF-1 cells in a 6-well plate. Non-transfected cells were used as blank control (NC group).

2.3. Western blot

DF-1 cells were harvested from 30 mm dishes at nominated times (12, 24, 36 h) post-infection and from 6-well plates 24 h after transfecting plasmid. The cells were washed twice with PBS at 4 °C, added with 200 μL RIPA Lysis B uffer and 2 μL Protease Inhibitor Cocktail, lysed on ice for 20 min, and clarified by centrifugation at 10,000*g* for 20 min at 4 °C. The supernate was boiled for 5 min in the presence of 5 × SDS PAGE-loading buffer. Equal amount of protein was 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 prepared in TBS/Tween 20 (0.1%) and then probed with rabbit anti-human LC3B polyclonal antibody (1:1000; Sigma) or mouse anti-gp85 monoclonal antibody (a kind of gift Professor Zheng Shijun from China Agricultural University) overnight at 4 °C. After being washed with TBST, the membrane was probed with appropriate goat anti-rabbit IgG-HRP (1:10,000; Proteintech Group, Inc) or goat anti-mouse IgG-HRP (1:10,000; Proteintech Group, Inc) for 1 h at 37 °C. Finally, primary antibodies were visualized using the enhanced chemiluminescence (ECL) purchased from Cwbiotech Company. Densitometric analysis of protein expression was carried out using Odyssey Application Software Version 3.0 (LI-COR Biosciences, NE, USA). All western blots were performed in triplicate for each experimental condition.

2.4. Animals

One-day-old specific pathogen free (SPF) chickens (Guangdong Wens Dahuanong Biotechnology Co., Ltd., Guangdong, China) (N = 100) were randomly divided into five groups: gp85-LC3B + Rap, gp85-LC3B, gp85 + Rap, gp85 and vector. Chickens were immunized using a TERESA DNA delivery device three times at three week intervals. Every chicken was transfected with 10 μg plasmid and the two groups injected with rapamycin (Vetec, V900930) were treated with 200 μL of 500 nM.

2.5. Electric pulse stimulations

Electric pulse stimulations were applied as previously described [28]. An electrical field was applied to the area around the injection after the intramuscular (IM) injection of plasmid DNA. Two silver needle electrodes were inserted 3 mm apart into the tibialis anterior (TA) muscles and 6 electric pulses were applied using a TERESA DNA delivery device (Shanghai Teresa Healthcare Sci-Tech Co., Ltd, Shanghai China). The electric pulses were 50 ms in duration and 1 s apart at a voltage of 60 V.

2.6. Antibody and cytokine analyses assays

Two weeks after the last immunization, antibody and cytokine production were measured using ELISA. Blood was collected from all groups of chickens, and serum isolated for further detection. Secreted cytokines and antibodies were measured using a relevant ELISA kit according to instructions of the manufacturers'. Chicken

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