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Recombinant baculovirus vaccine containing multiple M2e and adjuvant LTB induces T cell dependent, cross-clade protection against H5N1 influenza virus in mice

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ABSTRACT

H5N1, highly pathogenic avian influenza poses, a threat to animal and human health. Rapid changes in H5N1 viruses require periodic reformulation of the conventional strain-matched vaccines, thus emphasizing the need for a broadly protective influenza vaccine. Here, we constructed BV-Dual-3M2e-LTB, a recombinant baculovirus based on baculovirus display and BacMam technology. BV-Dual-3M2e-LTB harbors a gene cassette expressing three tandem copies of the highly conserved extracellular domain of influenza M2 protein (M2e) and the mucosal adjuvant, LTB. We showed that BV-Dual-3M2e-LTB displayed the target protein (M2e/LTB) on the baculoviral surface and expressed it in transduced mammalian cells. BV-Dual-3M2e-LTB, when delivered nasally in mice, was highly immunogenic and induced superior levels of anti-M2e IgA than the non-adjuvanted baculovirus (BV-Dual-3M2e). Importantly, after challenge with different H5N1 clades (clade 0, 2.3.2.1, 2.3.4 and 4), mice inoculated with BV-Dual-3M2e-LTB displayed improved survival and decreased lung virus shedding compared with mice inoculated with BV-Dual-3M2e. The enhanced protection from BV-Dual-3M2e-LTB is mediated by T cell immunity and is primarily based on $CD8^+$ T cells, while mucosal antibodies alone were insufficient for protection from lethal H5N1 challenge. These results suggest that BV-Dual-3M2e-LTB has potential to protect against a broad range of H5N1 strains thereby providing a novel direction for developing broadly protective vaccines based on cellular immunity.

1. Introduction

Over the past two decades, H5N1 highly pathogenic avian influenza (HPAI) has become a significant threat to the health of commercial poultry, wildlife and humans [1]. Moreover, H5N1 virus constantly undergoes reassortment with other influenza virus subtypes, resulting in the occasional emergence of highly pathogenic H5 variants with pandemic risk [2–6]. Vaccination has been an important strategy for the prophylaxis of H5N1 influenza infection in Chinese domestic poultry since 2004 [7]. However, the current H5N1 influenza vaccines generate protective antibodies against the highly variable viral glycoproteins hemagglutinin (HA) and neuraminidase (NA), and the vaccine strain must be regularly updated to match the antigenicity of the circulating viruses [7,8]. From 2004 to 2015, the Chinese government successively introduced a series of H5N1 influenza vaccines derived from different H5 HA phylogenetic clades, including Re-1 (clade 0), Re-4 (clade 7), Re-5 (clade 2.3.4), Re-6 (clade 2.3.2.1), Re-7 (clade 7.2) and Re-8 (clade 2.3.4.4) [9]. Despite the progress achieved in controlling H5N1 HPAI, the development of conventional inactivated vaccines cannot keep pace with genetic evolution in the virus, which impedes the ability of the current vaccination strategy to provide sufficient protective effects. Therefore, development of a novel influenza vaccine providing broad cross-protection against the currently circulating H5N1 viruses, and which does not need to be frequently updated, is a high priority.

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The extracellular domain of influenza matrix protein 2 (M2e) remains highly conserved among avian, swine and human influenza A viruses [10,11]. Moreover, that an anti-M2e monoclonal antibody is capable of reducing viral replication in vitro and in vivo implies that M2e may serve as a promising vaccine target [12,13]. Numerous studies have shown that vaccination with M2e-based vaccine constructs can induce M2e-specific immune response as well as broad-spectrum protection against divergent influenza virus [14]. Apart from the considerable progress made thus far in preclinical studies, several M2e-based vaccine candidates are now undergoing clinical trials in humans; such vaccines include the M2e-HBc VLP vaccine (ACAM-FLU-A^{TM}) [15] and the recombinant subunit vaccine STF2.4 × M2e (VAX102) [16]. That these vaccines have reached this stage indicates their potential safety, immunogenicity, and protective efficacy. More recently, the TCN-032 human monoclonal antibody, which is directed against the N-terminus of M2e, was evaluated in a phase II study with enrolled volunteers, where it showed therapeutic benefits during infection with influenza [17]. Taken together, use of the M2e antigen is now considered a highly desirable approach by which a universal influenza vaccine could be developed.

Naive M2e, a 24-aa small polypeptide, is considered to be poorly immunogenic when delivered alone. Previous studies have reported that increased immune responses can be obtained by fusing M2e to an appropriate carrier or in combination with vaccine adjuvants or delivery systems [14]. *Escherichia coli* heat-labile enterotoxin (LT) is one of the most powerful adjuvants known, attributed to its ability to elicit robust mucosal and systemic immune responses through mucosal and other routes of administration [18]. To remove the enterotoxicity of LT, the nontoxic B subunit (LTB) has been used as a substitute with the type of strong adjuvant properties that can significantly improve the immunogenicity when combined with certain other antigens [19–21]. Furthermore, LTB can be chemically or genetically fused with micro-molecular antigens, like epitope-based peptide, to form a bigger more immunogenic fusion construct serving as a powerful carrier molecule [22].

With the exception of adjuvant, the use of a live viral vector for antigen delivery represents an attractive strategy for vaccine development. The baculovirus *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV) has long been a vehicle for efficient production of recombinant proteins in insect cells [23]. In recent years, baculovirus has also been exploited as a vaccine delivery vehicle that can not only mediate transgene expression in a variety of vertebrate cells (termed BacMam technology) but can also display foreign proteins on the virion surface (termed baculovirus display technology) [23,24]. Baculovirus vectors also offer the following advantages as vaccines: good biosecurity in vertebrates, low cytotoxicity to mammalian cells, large insert capacities, proper post-translational modifications and strong adjuvant activities [25–27].

Here, we designed and constructed a recombinant influenza vaccine called BV-Dual-3M2e-LTB. The vaccine comprises three tandem copies of a consensus M2e sequence (3M2e) derived from avian-origin influenza A viruses [11] fused to LTB as the carrier-adjuvant. BV-Dual-3M2e-LTB was genetically engineered using baculovirus display technology and BacMam technology. We assessed whether BV-Dual-3M2e-LTB stimulates humoral and cellular immune responses as well as providing cross-clade protection against highly pathogenic H5N1 viruses following intranasal delivery in BALB/c mice. We also investigated the potential immunological mechanisms underlying protection in the mice, as an indication of further vaccine improvements.

2. Materials and methods

2.1. Viruses and cell lines

HPAI H5N1 viruses from clade 0 (A/chicken/Zhuhai/88/02, ZH/88), clade 4 (A/greylag/Guangdong/73/02, GD/73), clade 2.3.4 (A/chicken/Guangdong/348/08, GD/348), and clade 2.3.2.1 (A/duck/Guangdong/383/08, GD/383) were used in this study. Viruses were propagated in 10-day-old embryonated specific pathogen-free chicken eggs. 50% egg infectious dose (EID_{50}) and mice lethal dose (MLD_{50}) were calculated by Reed–Muench method [28]. Each experiment involving H5N1 viruses was performed in a facility at biosafety level 3.

Sf-9 cells were cultured at 27 °C in Sf-900^{TM} II serum-free medium (Invitrogen, USA). PK-15 cells were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, USA).

2.2. Baculoviruses preparation

DNA sequence encoding three tandem copies of a consensus M2e sequence (MSLLTEVETPTRNGWECKCSDSSD) of avian influenza virus and flexible linker (GGGGSGGGGS) were synthesized by Invitrogen, and cloned into a PMD18-T Simple vector (Takara, Japan) to yield the intermediate plasmid, PMD18-T-3M2e (Fig. 1A). Then the LTB coding sequence was inserted into PMD18-T-3M2e downstream of the 3M2e gene. The cloning strategy used for the recombinant baculovirus transfer vectors, pFast-VSV-G-3M2e and pFast-VSV-G-3M2e-LTB, is shown in Fig. 1A. First, the 3M2e or 3M2e-LTB gene was amplified and inserted as an in-frame fusion between the gp64 signal sequence and mature domain in pBACsurf-1 (Novagen, USA). Next, the gp64-3M2e or gp64-3M2e-LTB fusion gene was cloned into pcDNA3.1(+) (Invitrogen, USA) under the control of the human cytomegalovirus immediate early enhancer/promoter ($P_{CMV\text{-}IE}$). Finally, the DNA fragment containing the expression cassette ($P_{CMV\text{-}IE}$-gp64-3M2e or $P_{CMV\text{-}IE}$-gp64-3M2e-LTB) was cloned into the pFastBac-VSV-G baculovirus transfer vector downstream of the polyhedrin promoter (P_{PH}).

Recombinant baculoviruses were generated with the Bac-to-Bac® system (Invitrogen, USA) (see Supplementary data). Purification and titration of the recombinant baculoviruses and the wild-type AcMNPV (AcMNPV-WT) were conducted following the procedure described by us previously [29].

2.3. Immunogold electron microscopy

Purified baculoviruses were adsorbed onto 200-mesh Formvar/carbon-coated nickel grids and then incubated with a mouse mAb against the M2 protein of the influenza A virus (Abcam, USA) or mouse anti-LTB serum (gifts from Dr. H. Fan, College of Veterinary Medicine, South China Agricultural University) for 1 h at room temperature (RT). After two PBS washes, the grids were exposed to anti-mouse IgG conjugated with 10-nm colloidal gold particles (Sigma, USA) for 1 h at RT. The grids were washed with ultra-pure water and air dried. Finally, the samples were negatively stained with 2% phosphotungstic acid and examined under a JEM-2010HR transmission electron microscope (JEOL, Japan).

2.4. Indirect fluorescence assay (IFA)

PK-15 cells were transduced with BV-Dual-3M2e, BV-Dual-3M2e-LTB or AcMNPV-WT at a multiplicity of infection of 100. At 48 h post-inoculation, the cells were fixed with 4% paraformaldehyde for 20 min at RT, rinsed with PBS, and then incubated with