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Hybrid baculovirus-mediated prolonged hemagglutinin expression and secretion *in vivo* enhances the vaccine efficacy

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ABSTRACT

Viral vectors expressing the major antigen hemagglutinin (HA) are a promising vaccine platform against avian influenza virus (AIV) which poses serious threat to the poultry industry and human health. However, vector vaccines typically express HA transiently within the cells. Conversely, nanoparticles encapsulating and releasing antigens slowly *in vivo* potentiate humoral and cellular immune responses, hence underscoring the benefits of long-term antigen release for potent vaccine efficacy. Baculovirus (BV) is a non-pathogenic insect virus capable of transducing mammalian and avian cells and holds promise as a vaccine vector. Here we constructed a hybrid BV (Bac-FCsHW) harboring an Frt sites-flanking gene cassette expressing a fusion HA protein composed of the HA ectodomain and a GCN4 pII peptide to facilitate trimerization. Bac-FCsHW transduction of cells enabled expression and secretion of HA trimers. Co-transduction of mammalian cells with Bac-FCsHW and Bac-FLPo expressing FLPo recombinase led to FLPo recognition of Frt sites, excision and formation of episomal DNA minicircles that encompassed the HA expression cassette. Such FLPo/Frt-mediated minicircle formation remarkably prolonged and enhanced the expression of secreted HA to at least 28 days. Intramuscular co-injection of Bac-FLPo and Bac-FCsHW into BALB/c mice significantly potentiated the humoral and cellular immune responses when compared with the control groups expressing native intracellular HA, either for a short-term or long-term. These data demonstrated that BV-mediated prolonged expression and release of HA substantiated the vaccine efficacy and implicated the potentials of the FLPo/Frt-based hybrid BV vector as a new AIV vaccine platform.

1. Introduction

Outbreaks of highly pathogenic avian influenza virus (AIV) H5N1 have led to the death or depopulation of numerous poultry birds, and poses a serious threat to the poultry industry worldwide [1]. AIV transmission to humans also occurs through contact with infected birds, and has caused 844 confirmed human cases of H5N1 virus infection and 449 deaths as of December 2015 [2], thus underlying the need to develop vaccines to ward off the threat of emerging AIV pandemic. Current licensed influenza vaccines (*e.g.* inactivated virus) mainly consist of the major antigen hemagglutinin (HA), which is a homotrimer with each uncleaved monomer (HA0) comprising 2 domains (HA1 and HA2) [3]. HA1 forms the immunodominant head that contains most antigenic sites while HA2 forms the stem and the membrane anchor [4]. These vaccines predominantly induce antibodies neutralizing the hypervariable head-domain of HA, hence eliciting low protective immunity against heterologous virus infection [5]. Since cellular immunity is critical for providing protection against heterologous viruses [6], vector vaccines based on adenovirus [7], vaccinia virus [8], modified vaccinia Ankara [9] and poxvirus [1] have captured extensive attention because they can deliver *HA* gene into animals and express HA intracellularly for *de novo* antigen presentation, thereby eliciting immune responses against influenza viruses of different clades.

Aside from these virus vectors, baculovirus (BV) is a non-pathogenic insect virus but can also enter (*i.e.* transduce) various mammalian and avian cells without replication or cytotoxicity [10,11], hence BV has emerged as a promising vaccine vector [12–16]. Furthermore, BV can be engineered to carry the fusion gene

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composed of the HA ectodomain as well as the signal peptide and cytoplasmic tail domain (CTD) of gp64 (a protein anchored on the BV envelope). After infection of insect cells, the chimeric HA is expressed under the control of baculoviral *p10* promoter and incorporated into the BV envelope, hence being displayed on the BV surface [17]. A dual baculoviral vector is further engineered to harbor an additional cassette with the *HA* gene under the control of mammalian promoter, such that the HA-pseudotyped BV can express HA endogenously after transducing mammalian cells [18]. Intramuscular (i.m.) administration of the BV vector that simultaneously expresses and displays HA into BALB/c mice provokes HA-specific immune responses [18,19].

Although vector vaccines provoke strong cellular immune responses, the antigen expression is transient and the expressed HA is retained in the cells. In recent years, various nanoparticles have been developed to encapsulate and release antigens *in vivo* over a long period of time, which potentiates humoral and cellular immune responses [20–24]. The slow and persistent antigen release provides a longer time window for recruiting immune cells and promotes the interaction between the antigen and antigen presenting cells [21], thus underscoring the benefits of long-term antigen release for potent vaccine efficacy.

Similar to other vaccine vectors, BV confers short-term expression because BV genome (~134 kb) does not replicate inside mammalian cells and is rapidly degraded [10]. To extend the transgene expression, we developed an FLP/Frt-based hybrid BV system that prolongs the transgene expression in a broad range of mammalian cells [25–27]. More recently, we developed an advanced system that exploits the codon-optimized FLP (FLPo) [28]. The new FLPo/Frt-based BV system consists of two BV: Bac-FLPo expressing FLPo and the substrate BV harboring the Frt-flanking transgene cassette. After co-transduction of cells, the expressed FLPo recognizes the Frt sites and excises the Frt-flanking cassette off BV genome, hence catalyzing recombination and formation of episomal DNA minicircles encompassing the transgene cassette. The minicircle can persist in the cells for a longer term while the BV genome is rapidly degraded [28]. Such FLPo/Frt-based hybrid vector has been used to engineer stem cells for expression of various secreted growth factors to stimulate tissue regeneration [29–32].

Given the promise of persistent antigen release for vaccine potency, we aimed to explore the FLPo/Frt-based hybrid BV as a vaccine vector against AIV. We constructed FLPo/Frt-based BV vectors capable of simultaneously displaying and persistently expressing HA. Furthermore, we engineered HA by truncating the transmembrane and CTD domains and fusing with a peptide enabling the trimerization of HA, hoping that the BV-transduced cells were able to persistently express and secret soluble HA trimers. Whether the hybrid BV vectors were able to express HA (secreted and non-secreted form) for a prolonged period of time was evaluated *in vitro*. Whether the prolonged expression of secreted HA induced more potent humoral and cellular immune responses was also evaluated.

2. Materials and methods

2.1. Cell culture and medium

Insect cell Sf-9 was cultured in TNM-FH medium (Sigma) containing 10% fetal bovine serum (FBS, Invitrogen) at 27 °C. HeLa cells and mouse muscle myoblast cell line C2C12 were cultured in T-75 flasks using high glucose Dulbecco's Modified Eagle Medium (DMEM-HG, Sigma) containing 10% FBS and 1% PSA (Penicillin/Streptomycin/Amphotericin B, TOKU-E) at 5% CO_2 and 37 °C.

2.2. Construction and preparation of recombinant BV

Recombinant BV Bac-CE expressing enhanced green fluorescent protein (EGFP) [33] and Bac-FLPo expressing FLPo [28] were constructed previously. The hybrid substrate BV vectors were constructed using pFastBac DUAL® (Invitrogen) as the starting vector, which has 2 multiple cloning sites (MCS I and II) under the baculoviral *p10* and polyhedrin promoter, respectively (Fig. S1). We first PCR-amplified the DNA fragments encoding the WPRE-β-globin poly A signal from pBac-HAW (Fig. S1 and [19]) using the primer pair P1/P2 (Table S1) containing an additional sequence coding for Frt site, and subcloned the DNA fragment into pFastBac DUAL® by *Pst*I/*Avr*II to replace the original SV40 polyA sequence to yield pBac-WPRE-Frt. Meanwhile, we PCR-amplified the *p10* promoter from pBac-HAW using the primer pair P3/P4 (Table S1) containing an additional sequence coding for Frt site and subcloned this DNA fragment into pBac-WPRE-Frt using *Xho*I/*Bst1107*I to replace the original *p10* promoter to yield pBac-FW (Fig. S1). The CAG promoter was digested from pCAGGS plasmid using *Sal*I/*Xba*I and subcloned into the *Xho*I/*Xba*I sites of pBacPAK8 (Clontech). The polyhedrin promoter on pBac-WPRE-Frt was removed using *BamH*I/*Bst1107*I and the CAG promoter was digested using *Bgl*II/*Stu*I and cloned into pBac-FW. The resultant plasmid was designated as pBac-FCW (Fig. S1).

The HA sequence used in this study was derived from H5N2 AIV 1209 (A/chicken/Taiwan/1209/03 strain). The fusion gene comprising gp64 SP ($gp64_{SP}$), HA ectodomain (HA_{ecto}), HA transmembrane domain (HA_{TM}) and gp64 cytoplasmic domain ($gp64_{CTD}$) was directly digested from pBac-HA64 (Fig. S2 and [17]) using *Kpn*I/*Xho*I and subcloned into pBac-FCW to yield pBac-FCW-HA64. Meanwhile, the full-length HA gene encoding the HA SP (HA_{SP}), ectodomain (HAecto), transmembrane domain (HA_{TM}) and CTD (HA_{CTD}) was PCR-amplified from pBac-HAW using primers P5/P6 (Table S1) with *Sal*I/*Not*I site, cloned into TA Cloning vector and subcloned into pBac-FCW-HA64, under the control of CAG promoter. The resultant plasmid was designated as pBac-FCHW.

To construct a BV capable of secreting trimeric HA, the DNA sequences encoding HA_{SP} and HA_{ecto} were PCR-amplified from pBac-HAW using primers P5/P7 (Table S1) while the DNA encoding the GCN4 pII sequence (boxed sequence in Table S1) was chemically synthesized (MDBio, Inc.) and amplified using primers P8/P9 (Table S1). These 2 PCR products were mixed and subjected to 2-stage PCR using primers P5/P9 (Table S1) to yield a fusion gene comprising HA_{SP}, HA_{ecto} and GCN4 pII. The fusion gene was cloned into pBac-FCW-HA64 (Fig. S3) using *Sal*I/*Not*I to yield pBac-FCsHW.

pBac-FCHW and pBac-FCsHW were used to generate the hybrid BV Bac-FCHW and Bac-FCsHW following the instructions of Bac-to-Bac® system (Invitrogen). Single virus clones giving rise to high HA expression levels were picked as described [34]. The viruses were amplified by infecting Sf-9 cells and the virus supernatants were harvested at 3–5 days post-infection, and concentrated by ultracentrifugation (100,000 × g for 1.5 h) using 25% sucrose as the cushion [35]. After centrifugation, the virus was resuspended in phosphate-buffered saline (PBS, pH 7.4) and virus titer was determined by end-point dilution method [36].

2.3. Transduction of mammalian cells

BV transduction of HeLa or C2C12 cells was performed as described [36]. Briefly, cells were seeded to 6-well plates (3×10^5 cells/well), cultured for 12 h and washed twice with PBS (pH 7.4). Depending on the multiplicity of infection (MOI) we used (see Results), a certain volume of virus supernatant was mixed with $NaHCO_3$-free DMEM-HG [37] at a volumetric ratio of 1:4 (total volume was 0.5 ml) and added to the cells, followed by gentle shaking on a rocking plate at room temperature (RT). After 6 h, the

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