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A heterologous prime-boost Ebola virus vaccine regimen induces durable neutralizing antibody response and prevents Ebola virus-like particle entry in mice

Tan Chen [1], Dapeng Li [1], Yufeng Song [1], Xi Yang, Qingwei Liu, Xia Jin, Dongming Zhou**, Zhong Huang*

Vaccinology Division, CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200031, China

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ABSTRACT

Ebola virus (EBOV) is one of the most virulent pathogens known to humans. Neutralizing antibodies play a major role in the protection against EBOV infections. Thus, an EBOV vaccine capable of inducing a long-lasting neutralizing antibody response is highly desirable. We report here that a heterologous prime-boost vaccine regimen can elicit durable EBOV-neutralizing antibody response in mice. A chimpanzee serotype 7 adenovirus expressing EBOV GP (denoted AdC7-GP) was generated and used for priming. A truncated version of EBOV GP1 protein (denoted GP1t) was produced at high levels in *Drosophila* S2 cells and used for boosting. Mouse immunization studies showed that the AdC7-GP prime/GP1t boost vaccine regimen was more potent in eliciting neutralizing antibodies than either the AdC7-GP or GP1t alone. Neutralizing antibodies induced by the heterologous prime-boost regimen sustained at high titers for at least 18 weeks after immunization. Significantly, in vivo challenge studies revealed that the entry of reporter EBOV-like particles was efficiently blocked in mice receiving the heterologous prime-boost regimen even at 18 weeks after the final dose of immunization. These results suggest that this novel AdC7-GP prime/GP1t boost regimen represents an EBOV vaccine approach capable of establishing long-term protection, and therefore warrants further development.

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Despite the 2013–2016 Ebola virus disease (EVD) outbreak has passed (Heeney, 2015), the development of prophylactic vaccines for Ebola virus (EBOV) remains an urgent international priority (Kanapathipillai et al., 2014).

The envelope glycoprotein (GP) of EBOV mediates viral attachment and receptor binding (Cote et al., 2011; Kondratowicz et al., 2011) and is the main inducer of neutralizing antibodies (Bornholdt et al., 2016; Corti et al., 2016; Lee et al., 2008; Misasi et al., 2016; Wilson et al., 2000; Zhang et al., 2016). Therefore, almost all of the EBOV vaccine candidates under investigation are based on GP (Mire et al., 2016; Ohimain, 2016; Ye and Yang, 2015).

In nonhuman primates (NHPs), GP-specific antibodies either induced by active immunization (Marzi et al., 2013; Stanley et al., 2014; Sullivan et al., 2009) or administered passively (Kudoyarova-Zubavichene et al., 1999; Marzi et al., 2012) can confer protection against EBOV challenge. It has been shown that GP-specific serum antibody titer is correlated with protection against EBOV infection (Wong et al., 2012). In addition, treatment with GP-targeted neutralizing monoclonal antibodies can provide protection in animal models of EBOV infection (Kudoyarova-Zubavichene et al., 1999; Marzi et al., 2012), indicating neutralizing antibodies play an important role in protection against EBOV infection. Besides neutralizing antibodies, cellular immune responses may also contribute to vaccine-induced protection against EBOV infection, as it has been shown that depletion of $CD8^+$ cells in vivo could abrogate protection in NHPs that had received an adenovirus-vectored experimental EBOV vaccine (Sullivan et al., 2011).

Efforts have been made to develop effective EBOV vaccines using a variety of approaches (Mire et al., 2016; Ohimain, 2016; Ye and

* Corresponding author. Institut Pasteur of Shanghai, 320 Yueyang Road, Shanghai 200031, China.
** Corresponding author. Institut Pasteur of Shanghai, 320 Yueyang Road, Shanghai 200031, China.
E-mail addresses: dmzhou@ips.ac.cn (D. Zhou), huangzhong@ips.ac.cn (Z. Huang).
[1] These authors contributed equally to this work.

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Yang, 2015). A few EBOV candidate vaccines based on recombinant vesicular stomatitis virus (rVSV) or adenoviral vectors have advanced into clinical trials (Martins et al., 2016; Osterholm et al., 2016). Thus far, results from early phase clinical trials are encouraging, demonstrating that viral vectored EBOV vaccines, such as rVSV-ZEBOV (Agnandji et al., 2016; Huttner et al., 2015) or chimpanzee adenovirus 3 expressing EBOV GP (termed ChAd3-EBO-Z) (Milligan et al., 2016; Tapia et al., 2016), are able to induce EBOV GP-specific binding and neutralizing antibodies. However, the immunogenicity of viral vectored EBOV vaccines may be compromised due to pre-existing and/or vaccination-induced anti-vector immunity, and, as a consequence, a heterologous booster may be required to elicit long-term immune response (Stanley et al., 2014; Ewer et al., 2016; Milligan et al., 2016). In this study, we designed and evaluated a novel heterologous prime-boost EBOV vaccine regimen, in which a chimpanzee serotype 7 adenovirus expressing the full-length EBOV GP (designated AdC7-GP) was used for priming and a truncated GP1 recombinant protein (designated GP1t) for boosting.

For construction of AdC7-GP, the codon-optimized full-length GP gene (Li et al., 2016a) derived from a recently identified EBOV isolate (GenBank accession no.: KJ660347) was cloned into the E1-deleted region of the molecular clone of AdC7 (Chen et al., 2010), resulting in pAdC7-GP. Using the same strategy, green fluorescent protein (GFP) gene was cloned into the AdC7 vector to generate the construct pAdC7-GFP. Based on a method described previously (Zhou et al., 2010), recombinant adenoviruses were rescued by transfection with *Pac*-I linearized plasmid (pAdC7-GP or pAdC7-GFP) into HEK293 cells. Virus was rescued, expanded, and purified by cesium chloride density-gradient centrifugation and desalination. The resulting adenoviruses (denoted AdC7-GP and AdC7-GFP) were characterized by Western blotting and immunostaining assays using anti-GP mAbs 13C6 and 6D8 as described previously (Li et al., 2016a). While GFP signal was observed in the cells infected with AdC7-GFP but not AdC7-GP (Fig. 1A), robust and MOI-dependent GP expressions were only detected in AdC7-GP infected cells, but not in the mock or AdC7-GFP infected samples (Fig. 1B–C). These results indicate the successful generation of recombinant adenoviruses expressing GFP or EBOV GP.

To produce soluble GP recombinant protein, we constructed a plasmid (designated pMT-GP1t) for the expression in *Drosophila* S2 cells (Invitrogen) of a truncated form of GP1 (designated GP1t), which lacks the N-terminal signal peptide and the C-terminal mucin domain. A DNA fragment encoding the amino acids 34–296 of EBOV GP was amplified from the codon-optimized GP gene (Li et al., 2016a), and cloned into the backbone vector pMT/BiP/V5-HisA (Invitrogen), yielding the plasmid pMT-GP1t. Transfection of S2 cells with pMT-GP1t, selection of transgenic cell lines expressing GP1t, and large scale expression and purification of recombinant GP1t protein from supernatant of pMT-GP1t transgenic cell cultures, were performed according to a previously described protocol (Li et al., 2016b). High yields (up to ~100 mg protein per liter of supernatant) of purified GP1t were consistently obtained. SDS-PAGE and Western blot analyses showed that the GP1t migrated as a band of ~44 kDa (Fig. 2A), which is larger than the predicted mass (35 kDa) based on its amino acid sequence. To examine whether the larger mass was due to glycosylation, we treated purified GP1t with glycosidase PNGase F or Endo H. As shown in Fig. 2B, both PNGase F and Endo H fully cleaved GP1t, generating predicted 35 kDa bands, suggesting N-glycans associated in GP1t are mostly high-mannose or/and some hybrid oligosaccharides.

The conformation of GP1t was first evaluated by ELISA using a known conformation-dependent neutralizing monoclonal antibody (mAb) 13C6, which is one of the components of anti-EBOV mAb cocktail, ZMAPP (Qiu et al., 2014). GP1t, but not the control protein BSA, efficiently reacted with mAb 13C6 in a dose-dependent manner (Fig. 2C). Next, we investigated whether GP1t could inhibit EBOV GP-pseudotyped HIV-1 entry into Vero cells. EBOV GP-pseudotyped HIV-1 was generated as described previously (Wool-Lewis and Bates, 1998). Serially diluted GP1t or the control protein BSA was mixed with the pseudotyped virus, and the mixtures were added to Vero cells to allow infection for 6 h. Then, the protein-virus mixtures were removed and replaced with fresh medium. The cells were incubated at 37 °C for 72 h and then assayed for luciferase activity as described previously (Li et al., 2016a). For a given treatment, percent (%) inhibition was calculated as follows: (luciferase activity in infected cells without treatment – luciferase activity in the sample with treatment)/luciferase activity in infected cells without treatment x 100. We found that BSA did not show inhibitory activity at the highest

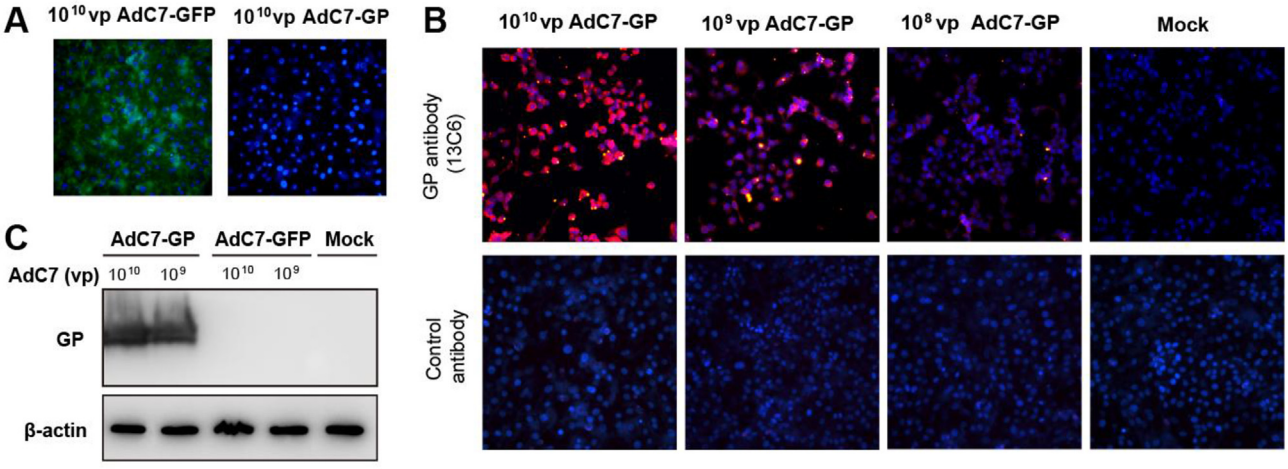

Fig. 1. Characterization of the recombinant adenovirus AdC7-GP. (A) GFP expression in the AdC7-GFP but not AdC7-GP infected cells. RD cells infected with 1×10^{10} viral particles (vps) of AdC7-GFP or AdC7-GP were fixed with 4% paraformaldehyde and stained with Hoechest dye to show nuclei. **(B)** Dose-dependent GP expression in the AdC7-GP infected cells. RD cells infected with the indicated doses of AdC7-GP or mock-infected cells were fixed, blocked and stained sequentially with anti-GP mAb 13C6 and Alexa Fluor 555 conjugated anti-human IgG. The cells were also stained with Hoechest dye (blue) to show nuclei. **(C)** Western blot analysis. Lysates of RD cells infected with AdC7-GP or AdC7-GFP in different doses were subjected to 10% SDS-PAGE, followed by Western blotting with anti-GP mAb 6D8 or anti-β-actin mAb (Sigma-Aldrich). Lysate from mock-infected cells served as the negative control in the assay.

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