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Enhanced immune responses against Japanese encephalitis virus using recombinant adenoviruses coexpressing Japanese encephalitis virus envelope and porcine interleukin-6 proteins in mice

Hanyang Liu[a,1], Rui Wu[a,1], Kai Liu[a], Lei Yuan[a], Xiaobo Huang[a,b], Yiping Wen[a,c], Xiaoping Ma[a], Qigui Yan[a], Qin Zhao[a,b], Xintian Wen[a,b,*], Sanjie Cao[a,b,*]

[a] Research Center of Swine Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China
[b] Laboratory of Zoonoses, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China
[c] Sichuan Science-observation Experiment of Veterinary Drugs and Veterinary Biological Technology, Ministry of Agriculture, Chengdu 611130, China

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ABSTRACT

Japanese encephalitis is a reproductive disorder caused by Japanese encephalitis virus (JEV) in swine. Previous studies have demonstrated that recombinant adenovirus serotype 5 (Ad5) may be a potential vaccine candidate because it can express JEV envelope epitopes and induce immune responses against JEV. Still, it will be necessary to develop an adjuvant that can enhance both humoral and cellular immune responses to the recombinant antigen delivered by non-replicating Ad5. In this study, we investigated the systemic immune responses of BALB/c mice immunized with recombinant adenovirus expressing JEV envelope epitopes in combination with porcine interleukin-6 (rAdE-IL-6).The rAdE-IL-6 immunized group had the highest titers of anti-JEV antibody as detected by an enzyme-linked immunosorbent assay (ELISA), as well as the highest levels of neutralizing antibody (1:75) as detected by a serum neutralization test. Similarly, higher concentrations of interferon-gamma (834.7 pg/ml) and interleukin-6 (IL-6) (229.7 pg/ml) were detected in the rAdE-IL-6 group using an ELISA assay. These data indicate that immunized BALB/c induce a strong cellular response against rAdE-IL-6. Furthermore, after challenge with the virulent JEV SCYA201201 strain, the rAdE-IL-6 group generated an immune protective response 70% greater than that of the control group, indicating that rAdE-IL-6 induced a protective immune response against JEV challenge in mice. The results from this study demonstrated that IL-6 is a strong adjuvant that can enhance both humoral and cellular immune responses in mice. Furthermore, a recombinant adenovirus coexpressing JEV envelope epitopes and porcine IL-6 protein may be an effective vaccine in animals.

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1. Introduction

Japanese encephalitis virus (JEV), a zoonotic pathogen transmitted by mosquitoes, is a major cause of central nervous system infections in humans and stillbirths in swine (Huang et al., 2014). JEV belongs to the genus *Flavivirus* of the family Flaviviridae. JEV E protein is the major virulence factor and is responsible for a number of important viral processes including attachment, fusion, penetration, cell tropism, virulence, and attenuation (Misra and Kalita, 2010).

To date, several approaches have been investigated to develop a successful JEV vaccine, including use of an inactivated virus (Bharati and Vrati, 2006), live-attenuated virus (Yu, 2010), subunit particles (Hua et al., 2014), and naked DNA (Kulkarni et al., 2012). In general, several studies have suggested that there is concern associated with the immunogenicity and safety of a mouse brain-derived inactivated vaccine, which were also limited by the requirement for multiple vaccinations and the cost of the vaccine (Andersen and Ronne, 1991; Takahashi et al., 2000). Moreover, safety has been the dominant concern associated with using replication-competent vaccines, because attenuated viruses have the potential to revert to virulent form and hard to be licenced (Diagana et al., 2007; Yang

* Corresponding authors at: Research Center of Swine Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China.
E-mail addresses: leohanyang001@gmail.com (H. Liu), wurui1977@163.com (R. Wu), rsghb110@126.com (X. Huang), mxp886@sina.com.cn (X. Ma), yanqigui@126.com (Q. Yan), xintian3211@126.com (X. Wen), csanjie@sicau.edu.cn (S. Cao).
[1] Both the authors contributed equally to this study.

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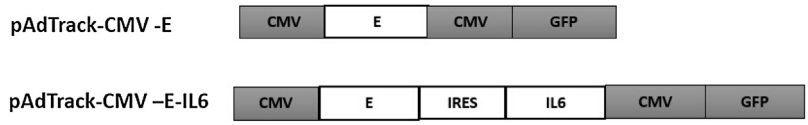

Fig. 1. Construction of recombinant transfer vectors. Two shuttle vectors (pAdTrack-CMV-E and pAdTrack-CMV-E-IL6) were constructed. First, the E gene DNA fragment was cloned into pAdTrack-CMV vector through restriction site of Bgl II and Sal I (pAdTrack-CMV-E). Second, IRES (600 bp) and IL-6 (586 bps) were fused by overlapping PCR and cloned to pAdTrack-CMV-E vector through restriction site of Sal I and Xhol I.

et al., 2014). With regard to protein subunit vaccines, a lack of efficacy has been a significant limitation (Laddy et al., 2008).

Recently, adenoviruses have shown incredible promise as vectors for recombinant vaccine development (Kim et al., 2014; Li et al., 2008; Tutykhina et al., 2013). Besides being safe, these viruses have the capacity to effectively induce both humoral and cellular immune responses. For example, adenovirus serotype 5 (Ad5) vectored vaccines have been studied extensively and may induce potent and protective immune responses against several pathogens in a variety of animal models (Herbert et al., 2014; Khanam et al., 2006; Singh et al., 2014). Due to encouraging preclinical results, the prospects of a large-scale clinical trial using this type of vaccine vehicle are becoming more likely.

There have been several strategies aimed at improving the magnitude of the cellular immune responses induced by recombinant vaccines, such as the use of a molecular adjuvant containing a construct expressing a cytokine or co-stimulatory molecule (Ramanathan et al., 2009; Siva Reddy et al., 2010). Interleukin (IL)-6 is a multifunctional cytokine and was originally identified as a factor that induces immunoglobulin production in activated B cells. More recently, it has also been shown to play an important role in T cell-mediated immune responses (Jones, 2005; La Flamme et al., 2000; Yang et al., 2005). Since the major role of IL-6 is to mediate inflammatory and immune responses (Tanaka et al., 2014), it is an excellent adjuvant candidate for enhancing innate immune responses against viral infections (Morrison and Ryan, 1987).

Taking this into consideration, we evaluated the efficacy of IL-6 as an adjuvant in a recombinant adenovirus expressing JEV E protein to modulate immune responses and protection against a lethal dose of JEV in mice.

2. Materials and methods

2.1. Plasmids, virus strains, and cell lines

In this study we used an Ad-easy vector system (Stratagene, La Jolla, CA, USA), the Japanese encephalitis virus strain SA14-14-2 (Genbank: AF315119), and the inactivated commercial JEV vaccine (Keqian Animal Biological Products Co. Ltd, Wuhanshih, Hubei, China). The JEV SCYA201201 strain (Genbank: KM658163), isolated from pig cerebrospinal fluid in 2012 (Sichuan, China), was maintained in our laboratory.

Human embryonic kidney-293 (HEK-293) and baby hamster kidney (BHK-21) cell lines were maintained in Dulbecco's modified Eagle's medium(DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), penicillin-streptomycin (Life Technologies Inc. Gibco/Brl Div., Grand Island, NY, USA), and 2 mM L-glutamine in a humidified atmosphere at 37 °C, 5% CO2.

2.2. Mice

Female BALB/c mice, purchased from Chengdu Institute of Biological Products (Chengdu, China), were maintained in an animal holding laboratory under controlled conditions (25 ± 1 °C, 40 ± 10% humidity) with free access to a standard mouse diet and water.

2.3. Construction of recombinant adenovirus rAd-E-IL-6

Truncations were made using reverse transcription polymerase chain reaction (RT-PCR) to amplify JEV SA14-14-2 E gene cDNA to desired lengths. Restriction enzyme cleavage sequences for BglII within the forward primer sequences and SalI within the reverse primer sequences allowed for restriction enzyme digest and subsequent cloning of the cDNA into pAdTrack-CMV (pAdTrack-CMV -E; Fig. 1).

RNA was isolated from pig splenocytes stimulated with 5 mg ml^{-1} concanavalin A for 24 h in culture medium and reverse transcribed the RNA into cDNA. The cDNA was used as a template for PCR amplification using IL-6 gene-specific primers.

To achieve a high level of IL-6 expression within the cells, the internal ribosome entry site (IRES) was amplified from pIRES (Clontech Laboratories Inc., Mountain View, CA, USA) and combined with the IL-6 gene using overlapping PCR. The forward primer was designed with a SalI site and the reverse primer had a XhoII site. The resulting IRES/IL-6 fragment was cloned into pAdTrack-CMV-E to generate pAdTrack-CMV −E-IL-6.

Adenovirus recombinants were produced according to the manufacturer's protocol(He et al., 1998). We used rAd with the E1 region replaced with a transgene-free expression cassette (Ad-null) as a control. All recombinant adenoviruses were propagated in HEK-293 cells, purified with a plaque test three times, and named rAdE, rAdE-IL-6, or rAdnull. Viruses were purified using an adenovirus purification miniprep kit (Biomiga, San Diego, CA, USA) and titers were determined with a tissue culture infectious dose 50 (TCID50) assay.

2.4. Western blot analysis

For western blot analysis, recombinant adenoviruses were subjected to a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (EMD Millipore, Billerica, MA, USA). Western blots were performed using standard methods. As the primary antibody, we used anti-JEV E protein mouse monoclonal antibody (Abcam, Cambridge, UK) diluted 1:200 and Horseradish Peroxidase-labeled sheep anti-mouse IgG antibody (Sangon Biotech Co. Ltd., Shanghai, CN) diluted 1:5000 as the secondary antibody. Subsequent bands were visualized using an Immun-Star WesternC detection system (Bio-Rad, Hercules, CA, USA). The same method was used to identify the expression of IL-6 protein, except the primary antibody used was anti-IL-6 -protein rabbit polyclonal antibody (Abcam).

2.5. Immune responses to rAd-E-IL-6 in mice

Female BALB/c mice (6 weeks, 17–22 g) were randomly divided into five groups (10 mice/group) and were immunized intraperitoneally. Three groups of mice were immunized with AdE, AdE-IL-6, or Adnull viruses prepared at 1×10^8 plaque forming units (PFUs) in 100 μL phosphate buffered saline (PBS)-10% glycerol. Another group of mice were immunized with 100 μL of the inactivated commercial JEV vaccine (WH-1 strain) (Chopper Biology Co. Ltd, Wuhan, Hubei, China), which was 1/10th of the recommended

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