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A single dose of the novel chimeric subunit vaccine E2-CD154 confers early full protection against classical swine fever virus

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ABSTRACT

Classical swine fever is an economically important, highly contagious disease of swine worldwide. Subunit vaccines are a suitable alternative for the control of classical swine fever. However, such vaccines have as the main drawback the relatively long period of time required to induce a protective response, which hampers their use under outbreak conditions. In this work, a lentivirus-based gene delivery system is used to obtain a stable recombinant HEK 293 cell line for the expression of E2-CSFV antigen fused to porcine CD154 as immunostimulant molecule. The E2-CD154 chimeric protein was secreted into the medium by HEK293 cells in a concentration around 50 mg/L in suspension culture conditions using spinner bottles. The E2-CD154 immunized animals were able to overcome the challenge with a high virulent CSF virus strain performed 7 days after a unique dose of the vaccine without clinical manifestations of the disease. Specific anti-CSFV neutralizing antibodies and IFN-γ were induced 8 days after challenge equivalent to 14 days post-vaccination. The present work constitutes the first report of a subunit vaccine able to confer complete protection by the end of the first week after a single vaccination. These results suggest that the E2-CD154 antigen could be potentially used under outbreak conditions to stop CSFV spread and for eradication programs in CSF enzootic areas.

1. Introduction

Classical swine fever (CSF) is a highly contagious and frequently fatal disease, which is responsible for significant losses in the swine industry worldwide. It is endemic in Asia, some areas of Central and South America and in many Eastern European countries with sporadic occurrence in Western Europe [1–3]. The etiological agent is an enveloped RNA virus that belongs to the *Pestivirus* genus of the *Flaviviridae* family [4].

The most efficient vaccines currently available against CSFV are live attenuated vaccines. Among of them, the Chinese vaccine strain (C-strain) has been introduced into many other countries because of its high efficacy and safety [5]. Immune responses elicited by these vaccines do not allow differentiating infected from vaccinated animals (DIVA). To overcome this drawback, subunit recombinant marker vaccines based on viral proteins have been considered as a promising alternative against CSFV [6].

The glycoprotein E2 is a homodimer exposed on the outer surface of the virus and mediates its entry into the target cells and it is considered the best target for the development of a marker vaccine. Several vaccines based on E2 have been generated using different expression systems [7–13]. E2 subunit vaccines induce clinical protection 14 days after vaccination, with total prevention of horizontal transmission but incomplete prevention of vertical transmission. In double-dose vaccination with E2, the neutralizing antibodies can be detected 7 days after booster, indicating that the efficacy of the E2 subunit vaccines is lower than that of modified live vaccines [2,3]. Until now, only live attenuated vaccines have been able to achieve full protection at day 5 after a single inoculation [2,3,5].

The CD154 molecule (CD40 ligand) is a glycoprotein that belongs to the tumor necrosis factor superfamily. The interaction of CD154 with its receptor CD40 is essential in promoting humoral and cellular immune responses [15]. CD40-CD154 ligation

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provides signals for activation and maturation of dendritic cells [14–16], B cell activation, clonal expansion, germinal center formation, isotype switching and generation of B cell memory [17]. Several studies have shown that CD154 can act as a molecular adjuvant enhancing the immune response [18–24].

This work describes a new potential CSF subunit marker vaccine based on a recombinant E2 protein fused to the extracellular domains of porcine CD154 and emulsified in Montanide ISA50 V2 oil adjuvant. The rationality is boosting the cellular and humoral immune response against E2 to confer an early protection against CSFV similar to the one provided by live attenuated vaccines.

2. Materials and methods

2.1. Lentiviral vector expression system

The coding sequence of the *Sus scrofa* CD154 extracellular domain (210 aa) was obtained by chemical synthesis (GeneArt™, Germany) (GeneBank Access number: AB040443). Four repeated units of Gly-Gly-Gly-Gly-Ser were included as a spacer at the 5′. This DNA fragment was inserted in the *EcoR* I-*Sal* I restriction sites after the 6 histidine tail attached to the carboxy terminal end of the previously cloned E2 sequence [10,11]. Afterward, a PCR-amplified DNA fragment containing the CMV enhanced promoter and E2-CD154 gene was cloned into the *EcoR*V site in the pLW vector (pLW-E2CD154). For lentiviral particles production, the plasmids from ViraPower™Lentiviral Expression System (Invitrogen) and pLW-E2CD154 vector, were transfected into 293FT cells, using the polyethylenimine-based transfection method [25].

2.2. Lentiviral transduction and cell cloning

Lentiviral transduction was carried out in 24 wells plate HEK 293 cells (ATCC CRL-1573) containing 2×10^4 cells per well in 2 mL of DMEM containing 10% fetal bovine serum (FBS) and 4 mg/mL hexadimethrine bromide (Polybrene; Sigma). Tittered virus was added at different infection multiplicity (MOI; 0, 5, 50 and 100) with four replicates per condition and the cells were incubated at 37 °C, in 8% CO_2. All experiments included a background sample. After overnight incubation, transduction media was replaced with 2 mL of fresh media containing blasticidin at 0, 2.5, 5 and 10 μg/mL. Blasticidin-resistant cell colonies were picked and transferred to 12-well plates about 10–14 days later, when the entire control cells died. The cell pool generated at MOI 50 and resistant to 2.5 μg/mL of blasticidin was cloned by limiting dilution in 96 well/plate. Two to three weeks later, 12 clones were picked randomly and expanded.

To perform Southern blot analysis, genomic DNA (gDNA) was isolated using Wizard Genomic DNA Purification kit (Promega) and digested *Eco*R I, and then it was transferred to Hybond N+ membrane (Amersham) and hybridized with E2 DNA probe.

2.3. Adapting adherent HEK 293-E2-CD154 cells to suspension culture

After the HEK 293-E2-CD154 cells reached 90% of confluence in DMEM supplemented with 10% of FBS, the medium was gradually substituted by CPCHO (CIM, Cuba) varying the ratio CPCHO/DMEM as follows: 25/75, 50/50, 75/25 and 100/0. Medium change was performed every 48 h. Detached cells were recovered by centrifugation at 400*g* for 5 min in each medium change. After adaptation to suspension culture in Erlenmeyer, cells were grown in spinners of 120 mL without medium change during 9 days. One mL of medium was removed every day to measured E2-CD154 protein concentration in the culture supernatant by ELISA. This experiment was performed in duplicate.

2.4. Purification of E2-CD154 protein

The harvested medium from the spinner culture of HEK 293-E2-CD154 stable clone cells was clarified by centrifugation and further filtrated through a 0.2 μm filter. Then, 1/10 vol of a concentrated buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 1 mM imidazole pH 7.4 was added to the medium and applied onto a Ni-NTA Agarose column (Sepharosa Fast Flow, Qiagen). Purification was performed according manufacturer's instructions (more details in supplementary information files).

2.5. Swine immunization and challenge trial

Purified E2-CD154 was formulated in Montanide ISA50™ V2 (SEPPIC) using a 60/40 proportion of aqueous/oil phase. "Water in oil" emulsion was produced in an Ultra-Turrax T25 basic homogenizer (IKA Works Inc.).

Nine weeks old Crossbred Duroc/Yorkshire swines (25–30 kg) (CENPALAB, Cuba), belonging to a non-vaccinated and CSF-free herd were used. Animals were fed with 2 kg/per animal/per day of commercial feed (ALYco CENPALAB, Cuba) and water *ad libitum*.

Trials were carried out under appropriate high containment conditions following the animal welfare regulations and standards according to EU Directive 2010/63/EU and good clinical practices (VICH GL9, 2000). Swine were allocated at random in three experimental groups which were immunized once by intramuscular injection with: 50 and 100 μg of purified E2-CD154 antigen formulations (group 1 and 2, respectively, N = 5), and Placebo (10 mM NaH_2PO_4 pH 7.4 emulsified in Montanide ISA50 V2) (group 3, N = 3). Blood and serum samples were taken at days 0 and 7 post vaccination (dpv) and 7, 14, 21 and 28 post challenge (dpc). Challenge was conducted at 7 dpv by intranasal inoculation of 2×10^3 LD_{50} of CSFV high virulent Margarita strain, genotype 1.4 [26].

Clinical symptoms were scored for 28 days after challenge according Mittelholzer et al., 2000 [27] with minor modifications (Table S1).

2.6. Neutralizing antibodies detection

Serum samples were screened for the ability to neutralize the cell culture adapted Margarita CSFV strain (National Center for Animal and Plant Health, Mayabeque Cuba) using NPLA [28] as described in the Manual of World Organization for Animal Health (OIE, 2014). The assay was revealed with the anti E2 Mab CBSSE2.3 (CIGB-SS, Cuba) conjugated to horseradish peroxidase followed by DAB substrate. The presence of viral replication was determined by visual inspection at the optical microscope. The last serum dilution without any signal of virus replication was considered as the neutralizing titer.

2.7. Viral isolation and detection

Heparinized blood samples collected at days 8, 14, 21 and 28 dpc from vaccinated animals were used for viral isolation. For placebo group, samples were taken at day 7 pc and at the moment of sacrificed. In addition, at the time of sacrificed, tonsils, spleens and ileum were also collected.

The organs (approximately 1 cm^3) were macerated in 1 mL of DMEM (Sigma, St Louis, USA) supplemented with 5% fetal calf serum, penicillin (100 IU) and streptomycin (100 μg). The homogenates were resuspended in 4 mL of DMEM and allowed to settle for 1 h at RT. Afterward, samples were centrifuged at 1200 rpm for 15 min and the supernatant transferred and preserved in cryovials (Sigma-Aldrich, USA) at −70 °C. Viral isolation was performed in PK15 cells trough three serial passages in 24 wells microplates

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