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Long-term humoral immunity induced by CVC1302-adjuvanted serotype O foot-and-mouth disease inactivated vaccine correlates with promoted T follicular helper cells and thus germinal center responses in mice

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ABSTRACT

Long-lasting humoral immunity is one of the necessary criteria for a successful vaccine. In our previous study, it was demonstrated that the immunopotentiator CVC1302 could improve the humoral immunity induced by the foot and mouth disease virus (FMDV) killed vaccine (KV) with only one dose. Significantly higher FMDV-specific antibody titers and more persistent antibody responses were observed in pigs receiving CVC1302-adjuvanted KV (KV-CVC1302) than in those inoculated with KV alone. In this study, we show that CVC1302 enhances murine IgG responses to FMDV by promoting a potent T follicular helper cell (T_{FH}) response, which directly controls the magnitude of the germinal center (GC) B cell response. These results indicate a need for studies to assess the capacity of CVC1302 to enhance the efficacy of FMDV KV immunization in pigs, and provide new insights into the development of FMDV vaccines.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, which causes substantial economic losses in the swine industry worldwide [1]. FMD was listed at the head of the class A diseases of the animal health code by the International Organization of Animal Health (OIE).

Foot-and-mouth disease virus (FMDV), the causative agent of FMD, belongs to the genus *Aphthovirus* of the family *Picornaviridae*, containing seven serotypes (A, O, C, Asia I, and Southern African Territories (SAT) 1, 2, and 3) [2]. In China, serotype O of FMDV is currently prevalent, and its control still relies mainly on vaccination with inactivated vaccines [3]. However, one dose of the available commercial FMDV inactivated vaccines cannot induce efficient humoral immunity against FMDV infection, which not only causes economic loss, but also inhibits the progress of FMD prevention and control.

In our previous study, an immunopotentiator CVC1302, containing muramyl dipeptide (MDP), Monophosphoryl lipid A (MPL), and β-glucan, was determined to successfully improve the efficacy of serotype O FMDV killed vaccine.

Long-term humoral immunity was observed by the detection of liquid-phase blocking antibody titers in pigs after receiving just one dose of KV-CVC1302. One of the criteria for successful vaccines is the generation of long-lived antibody responses to provide protection against subsequent infection, which in turn relies on a robust germinal center (GC) response [4]. The size and quality of the GC response are directed by T follicular helper (T_{FH}) cells, which provide growth and differentiation signals to GC B cells and mediate the positive selection of high-affinity B cell clones in the GC, thereby determining which B cells exit the GC as long-lived plasma cells (LLPCs) and memory B cells (MBCs) [5]. Studies in mice indicate that LLPCs, localized primarily in specialized niches in the bone marrow (BM), can survive up to the lifetime of the mice, even in the absence of regeneration by B cells [6].

MF59, an adjuvant used in human vaccines, is known to induce persistent high-affinity functional Ab titers. Recent studies have indicated that MF59 mediates its B cell adjuvanticity by promoting T_{FH} cells and thus GC responses [7–9]. Hence, in this study, we aimed to decipher the molecular and cellular mechanisms whereby CVC1302 induces a long-term humoral immune response

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and whether CVC1302 directly, or indirectly, mediates GC responses to induce long-term antibody immunity. This knowledge may improve the rational design of FMDV vaccine candidates.

2. Materials and methods

2.1. Mice

Six-week-old BALB/c female mice were purchased from Yang Zhou University. The study and protocol were approved by the Science and Technology Agency of Jiangsu Province. The approval ID is NKYVET 2015-0066, granted by the Jiangsu Academy of Agricultural Sciences Experimental Animal ethics committee. All animal studies were performed in strict accordance with the guidelines of Jiangsu Province Animal Regulations (Government Decree No. 45).

2.2. Vaccine, adjuvants, and immunizations

Commercially available serotype O FMDV inactivated vaccine was purchased from CAVI (Lanzhou, China, Lot. No: P160719J).

CVC1302 was prepared as an adjuvant for a water-in-oil emulsion formulation. Briefly, MDP, MPL, and β-glucan were simultaneously dissolved in sterile water as the aqueous phase, then mixed thoroughly with the oil phase. The recipe for the adjuvants was as described in the Chinese patent license with the registered number 201310042983.0 (Supplemental Fig. 1). One volume of adjuvant was fully mixed with nine volumes of FMDV killed vaccine before injection. The FMDV vaccine mixed with CVC1302 was named KV-CVC1302.

BALB/c mice (seven mice per group per experiment) were immunized i.m. once with 50 μL KV-CVC1302, KV, or PBS as control, in each hind leg quadriceps (100 μL/mouse).

2.3. Quantification of Ag-specific Abs in serum

Serum samples of individual mice were collected at 14, 28, 56, 90, 120, and 150 days post-immunization (dpi) and evaluated for serotype O FMDV-specific liquid-phase blocking antibody titers. The levels of IgG1 and IgG2a isotype antibodies at 14, 28, and 56 dpi were determined by indirect ELISA.

FMDV-specific antibody titers of immunized mice were analyzed using liquid-phase blocking ELISA (LPBE) detection kits (LVRI, Lanzhou, China) according to the manufacturer's specifications. Briefly, 50 μL of twofold serial dilutions of serum samples and 50 μL of FMDV antigen (1:20 dilution) were added to a U-bottomed 96-well plate and incubated for 1.5 h at 37 °C. The mixtures then were transferred into a 96-well ELISA plate pre-coated with rabbit anti-FMDV polyclonal antibody and incubated for 1 h at 37 °C. After five times washes, plates were incubated with 50 μL of guinea pig antiserum against FMDV O serotype for 30 min at 37 °C. Then 50 μL of rabbit anti-guinea pig IgG/HRP was added to the 96-well plate after a washing step (total of five times with PBST) and incubated for 30 min at 37 °C. The plate was washed and 50 μL of the substrate/chromophore mixture (H_2O_2/OPD) was added to each well, and the plate was incubated for 15 min at 37 °C in the dark. Finally, 50 μL of stop solution was added to each well, and absorbance at 492 nm was measured within 15 min of stopping the reaction [10].

The levels of FMDV-specific IgG1 and IgG2a antibodies were measured by indirect ELISA. Briefly, 50 μL of FMDV antigen (1:20 dilution) were added to a 96-well ELISA plate pre-coated with rabbit anti-FMDV polyclonal antibody and incubated for 1 h at 37 °C. The plate was washed five times and 50 μL of the serum samples (1:512 dilution) was added and incubated for 1 h at 37 °C. Then, 50 μL of rat anti-mouse IgG1 or IgG2a/HRP (Arigo, China) was added and incubated for 30 min at 37 °C. TMB (50 μL) was added to each well after washing, and the plate was incubated in the dark at 37 °C for 15 min. The reaction was stopped by addition 50 μL of 2 M H_2SO_4, and the absorbance at 450 nm was measured within 15 min of stopping the reaction.

2.4. In vitro re-stimulation of Ag-specific T cell responses

To access the frequency of FMDV-specific, IFN-γ-secreting $CD3^+CD4^+$ T cells induced by vaccination, samples of approximately 1×10^6 cells/well were prepared from pools of inguinal lymph nodes (LNs) excised from three mice/group, and stimulated with inactivated FMDV (10 μg/mL), or medium, overnight at 37 °C, in the presence of 5% CO_2, before the addition of a protein transport inhibitor cocktail (eBioscience, USA). Follwing an additional incubation for 5 h at 37 °C, in the presence of 5% CO_2, cells were incubated with Fc blocking reagent (Miltenyi Biotec, USA) in PBS plus 1% FBS (HyClone, Thermo Scientific, USA) for 10 min at 4 °C, stained with anti-CD3 FITC and anti-CD4 PE-Vio770 (Miltenyi Biotec, USA) and then fixed and permeabilized with intracellular fixation and permeabilization buffer (eBioscience, USA). For determination of the frequency of Ag-specific IFN-γ-producing $CD3^+CD4^+$ T cells by intracellular staining, cells were stained with anti-IFN-γ PE (Miltenyi Biotec, USA).

Cells were analyzed with a BD Accuri C6 (BD Biosciences, USA). Data analyses were performed using FlowJo version 7.6.1 software.

The expression levels of IFN-γ induced by injection of PBS, KV, or KV-CVC1302 were measured by real-time RT-PCR and commercial ELISA kits.

For detection of the expression levels of IFN-γ mRNA, samples of 4×10^6 cells/well were prepared from pools of inguinal LNs excised from three mice/group, and stimulated with FMDV (10 μg/mL), or medium, at 37 °C, in the presence of 5% CO_2. After 18 h incubation, lymphocytes were harvested and the total RNA was extracted by Trizol (Invitrogen, USA), and reverse transcribed in a 20 μL reaction mixture. The cDNA product (2 μL) was amplified in a 20 μL reaction mixture containing Bright Green 2 × qPCR Master Mix (abm, Canada) and 0.2 μM of each of the forward and reverse gene-specific primers. Each cDNA amplification was performed in triplicate. PCR amplifications were performed using a Roche Light Cycler® 480. The thermal cycling conditions were 10 min at 94 °C, 40 cycles of 15 s at 94 °C and 1 min at 60 °C. Gene expression was measured by relative quantity as described previously [11]. The primers of all detected genes are listed in Supplemental Table 1.

For analysis of IFN-γ release, 4×10^6 cells/well were prepared from pools of inguinal LNs excised from three mice/group, and stimulated with FMDV (10 μg/mL) or medium at 37 °C, in the presence of 5% CO_2. After 72 h incubation, the culture supernatant was harvested and the presence of IFN-γ was tested with commercial mouse IFN-γ immunoassay ELISA kits (Angle Gene Technologies, Nanjing, China) according to manufacturer's instructions. The concentrations of IFN-γ in the samples were determined based on the standard curves.

2.5. Flow cytometric analysis

For the characterization of GC B cell responses, the inguinal LNs were harvested and pooled per mouse after i.m. immunization. LN single-cell suspensions were prepared by homogenization and then incubated with Fc blocking reagent in PBS plus 1% FBS (HyClone, Thermo Scientific, USA) for 10 min at 4 °C. Approximately 1×10^6 LN cells were stained for 30 min at 4 °C with the following mAbs: anti-B220 APC (Miltenyi Biotec, USA); anti-GL-7 PE; and anti-CD38 PE-Cyanine7 (eBioscience, USA).

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