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Effects of inactivated porcine epidemic diarrhea virus on porcine monocyte-derived dendritic cells and intestinal dendritic cells

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ABSTRACT

Porcine epidemic diarrhea (PED) is a serious infection in neonatal piglets. As the causative agent of PED, porcine epidemic diarrhea virus (PEDV) results in acute diarrhea and dehydration with high mortality rates in swine. Dendritic cells (DCs) are highly effective antigen-presenting cells to uptake and present viral antigens to T cells, which then initiate a distinct immune response. In this study, our results show that the expression of Mo-DCs surface markers such as $SWC3a^+CD1a^+$, $SWC3a^+CD80/86^+$ and $SWC3a^+SLA\text{-}II\text{-}DR^+$ is increased after incubation with UV-PEDV for 24 h. Mo-DCs incubated with UV-PEDV produce higher levels of IL-12 and INF-γ compared to mock-infected Mo-DCs. Interactions between Mo-DCs and UV-PEDV significantly stimulate T-cell proliferation *in vitro*. Consistent with these results, there is an enhancement in the ability of porcine intestinal DCs to activate T-cell proliferation *in vivo*. We conclude that UV-PEDV may be a useful and safe vaccine to trigger adaptive immunity.

1. Introduction

Porcine epidemic diarrhea (PED) is a serious infection in neonatal piglets where morbidity and mortality can be 80 to 100%. Transmission of PED is fecal-oral, no vector or reservoir has been implicated in its spread. It was first observed in feeder pigs and fattening swine in England in 1971. The causative agent, porcine epidemic diarrhea virus (PEDV), is an enveloped, single-stranded, positive sense RNA virus belonging to the Order *Nidovirales*, the family *Coronaviridae*, subfamily *Coronavirinae* and the genus *Alphacoronavirus* (Chasey and Cartwright, 1978; Pensaert and de Bouck, 1978), had spread epidemics in multiple swine-producing countries in Europe during the 1970s and 1990s (Nagy et al., 1996; Van Reeth and Pensaert, 1994). PEDV was first detected in U.S. swine in May 2013 (Stevenson et al., 2013). The United States Department of Agriculture's (USDA) National Veterinary Services Laboratories (NVSL) confirmed the first PED diagnosis in the United States on May 17, 2013 in Iowa. Then PEDV has wiped out an estimated 10% of the US pig population, helped push pork prices to record highs, and raised questions about US oversight of the livestock industry. In Asia, PED first occurred in Japan in 1982 (Takahashi et al., 1983) and outbreaks have been rare in China. Since October 2010, a severe PED epizootic has been affecting pigs of all ages that is characterized by high mortality rates among suckling piglets in many provinces of China, resulting in tremendous economic losses (Chen et al., 2012; Li et al., 2012; Pan et al., 2012; Park et al., 2011; Zheng et al., 2013). Design and development of safer and more efficacious vaccines against PEDV is a high-priority target in the world.

Dendritic cells (DCs) widely distributed beneath the intestinal epithelium and act as a portal for virus invasion (Banchereau et al., 2000; Khaiboullina et al., 2013). DCs are the most potent antigen-presenting cells and the key initiators of adaptive immune responses to play a central role in regulating the balance between tolerance and immunity in the intestinal mucosa. As the key initiators, they can present antigen to trigger T cell responses against an array of invading pathogens (Crowley et al., 1990; Steinman, 1991) including PEDV. DCs maturation can be initiated by many stimuli such as innate lymphocytes, bacteria and lipopolysaccharide (LPS) (Roake et al., 1995). There is an increase in the surface expression of MHC II and costimulatory molecules (such as CD80 and CD86) in fully mature DCs but a decrease in the capacity to sample antigens (Cella et al., 1997). The induction of DC maturation is beneficial for the generation of T lymphocyte responses, which is essential for the development of piglet vaccines.

An efficient protection against PEDV is vaccination. Current commercial PEDV vaccines are live attenuated PEDV vaccines (Kweon et al., 1999). But when the small intestine mucosal immunity is insufficient, or the live attenuated PEDV mutates into virulent strain, the virus proliferates in the small intestine and can be easily spread in the piglets to cause PED. Inactivated whole-virus vaccines can solve the secure problem and be sufficiently effective as well.

In the present study, we found that inactivated PEDV induced DCs maturation and significantly influenced the stimulation of the T cell response *in vitro* and *in vivo*.

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2. Materials and methods

2.1. Animals

The number of 30 three-week-old Yorkshire, Landrace, and Large White cross-bred pigs were fed and maintained at experimental animal breeding center in JAAS. All animal experiments were carried out in accordance with the regulations and guidelines for laboratory animals at Nanjing Agriculture University (Nanjing, China).

2.2. Preparation of inactivated virus and Escherichia coli K88

The PEDV (CV777) (Egberink et al., 1988) was propagated on Vero cells in DMEM (GIBCO, USA) with 5% fetal bovine serum (FBS; GIBCO, USA). The CV777 strain was obtained from Jiangsu Academy of Agricultural Sciences. Virus was collected by three freeze-thaw cycles when 85% of the Vero cells showed cytopathic effects (CPE), then purified the collection by sucrose gradient centrifugation (Krempl and Herrler, 2001). The tissue culture infective dose 50 (TCID50) of the purified CV777 strain was calculated using the Reed and Muench method. UV inactivated PEDV (UV PEDV) are produced by exposing the virus to ultraviolet rays for 6 h at an optimal cross linking value (0.120 J/cm^2). *E. coli* K88 (enterotoxigenic *E. coli* K88, Invitrogen, USA) was grown overnight in Luria broth (LB) to a density of 1×10^8 cfu mL^{-1}. Bacteria were heat killed in water bath at 95 °C for 30 min, diluted in FCS-free cell culture medium, and incubated to confirm the absence of viable cells.

2.3. Generation of Mo-DCs

Peripheral blood was obtained from five healthy female pigs (three week old, derived from combination of the Yorkshire, Landrace, and Large White breeds, fed and maintained at the experimental animal breeding center at JAAS). Peripheral blood mononuclear cells (PBMC) were isolated immediately by density centrifugation using Histopaque (1.077 g L^{-1}) (Sigma, MO, USA) after venous extraction and incubated in plates at 10^6 cells/mL in 1640 RPMI medium (GIBCO, USA) with 10% fetal calf serum (MULTICELL, Australia). After 18 h, PBMC were separated from monocytes that had adhered to the plate bottom by washing with phosphate-buffered saline (0.01 M PBS, pH 7.2). Next, 20 ng mL^{-1} porcine granulocyte-macrophage colony-stimulating factor (GM-CSF, Invitrogen, CA, USA) and 20 ng mL^{-1} interleukin 4 (IL-4, BioSource, CA, USA) were added to allow the effective differentiation of monocytes to immature Mo-DCs over a 6 day period. Half of the medium was replaced by fresh GM-CSF and IL-4 supplemented medium every three days. For further maturation, immature Mo-DCs were stimulated by addition of 2 μg mL^{-1} lipopolysaccharide (LPS, Sigma, Japan) for 24 h.

2.4. Flow cytometry

Both immature and mature Mo-DCs were harvested by centrifugal separation and gentle pipetting. Harvested cells were washed three times with phosphate-buffered saline (0.01 M PBS, pH 7.2). Cells (5×10^5 cells mL^{-1}) were stained with the four monoclonal antibodies: FITC-conjugated mouse anti-swine monoclonal antibody to swine histocompatibility leukocyte Ag II-DR (SLA-II-DR) (LifeSpan BioSciences, USA), FITC conjugated mouse anti-human monoclonal antibody to the co-stimulatory molecules cluster of differentiation 80/86 (CD80/86) (Abcam, Hongkong), FITC conjugated mouse anti-porcine monoclonal antibody to cluster of differentiation 1a (CD1a) (Abcam, Hongkong), and PE conjugated mouse anti-porcine monoclonal antibody to Swine Workshop Cluster 3a (SWC3a) (Abcam, Hongkong). After three washes with 0.01 M PBS (pH 7.2), the cells were suspended in 200 μL 0.01 M PBS (pH 7.2) and analyzed on a FACS Calibur (BD Bioscience, Cowley, UK).

2.5. Determination of phenotypic markers by flow cytometric analysis after stimulation of UV-PEDV

To examine phenotypic markers associated with Mo-DCs, cells treated with the four antibodies described above were analyzed using flow cytometry. In our preliminary experiment, we chose three concentrations ($TCID_{50}$ = 1, 10 and 100) of CV777 strain interactions with dendritic cells, the best effect of CV777 infected Mo-DCs is the concentration of $TCID_{50}$ = 10. Thus, the immature and mature Mo-DCs were inoculated with UV-PEDV at a concentration of 10 $TCID_{50}$ cell^{-1} or LPS (2 μg mL^{-1}) for 24 h at 37 °C. Washed three times with cold PBS, and then fixed with paraformaldehyde (4%). Background fluorescence was excluded using isotype controls. Ten thousand cells were collected and analyzed.

2.6. Detection of cytokines in Mo-DCs by ELISA

To detect the secretion of cytokines, supernatants were obtained from immature or mature Mo-DCs (5×10^5 in 6-well plates) that had been treated with UV-PEDV at a concentration of 10 $TCID_{50}$ cell^{-1} or LPS (2 μg mL^{-1}) for 24 h. Levels of secreted IL-12, IFN-γ and IL-10 (R&D systems, USA) were determined using commercial ELISA kits according to the manufacturer's recommendations, and calculated based on the curve generated using the cytokine standards.

2.7. Evaluation of mixed leukocyte reaction (MLR) in Mo-DCs

MLR was evaluated using an automated ELISA reader (Bio-Tech Instruments, USA) and the CCK-8 cell counting kit (Beyotime, China). Cells in 96-well plates were treated with 10 μL CCK-8 solution, and incubated for 2 h at 37 °C. Absorbance for each well was measured at 450 nm using the ELISA reader. The Stimulation Index (SI) was calculated using the formula: $SI = (OD_{\text{sample well}} - OD_{\text{blank well}}) / (OD_{\text{negative well}} - OD_{\text{blank well}})$. Non-stimulated cells were used as negative controls.

For MLR experiments, autologous lymphocytes were purified on a nylon wool column (depletion in B lymphocytes and APC) and used as responder cells (Liang et al., 2013). Varying numbers of responder cells were added to 96-well round-bottomed cell culture plates, T-lymphocytes were co-cultured with UV-PEDV-incubated-DCs (1×10^5 cell/well) at a ratio of 1:1; 1:10 (DCs: lymphocytes). Co-cultures were incubated at 37 °C in 5% CO_2 for 72 h. Control cultures contained responder or stimulator cells only. All experiments were performed three or more times. After 72 h of culture, cell proliferation was determined by CCK-8 assay.

2.8. Animal experimental design

In the first animal experiment, a total of 10 conventional three-week-old piglets were divided into two groups and were maintained in isolation facilities to prevent virus transmission. All piglets were starved 24 h before surgery to reduce the amount of chymus in the intestine to the minimum possible. The treatments of the groups are shown in Table 1. In group I (n1 = 5), the piglets were anesthetized with sodium pentobarbital and a midline incision was made just anterior to the navel (Nielsen and Sautter, 1968). Four treatments were carried out using the twelve jejunum segments containing the Peyer's patches (PPs) per piglet. The first treatment was injection with UV-PEDV 3 h before sacrifice (n2 = 3), the second treatment was injection with sterile 0.01 M PBS 3 h before sacrifice (n2 = 3), the third was injection with UV-PEDV 15 min before sacrifice (n2 = 3), and the fourth was injection with sterile 0.01 M PBS 15 min before sacrifice (n2 = 3). During surgery and treatment, piglets lay on a 37 °C warming pad. At the specified times piglets were sacrificed and their twelve intestinal segments were removed. Piglets in group II (n1 = 5) were not subjected to surgery but were inoculated with UV-PEDV by oral administration. 48 h after inoculation, the piglets were sacrificed and the three