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Full Length Research Paper

Phenotypic and functional modulation of porcine monocyte-derived dendritic cells for foot-and-mouth disease virus

Hai-yan Shen[1#], Jiaying Wang[1#], Li-jun Chen[1], Ming-qiu Zhao[1], Chun-mei Ju[1], Ming Liao[1], Jian-min Zhang[1], Jin-ding Chen[1]* and Hong-zhuan Wu[2]

[1]College of Veterinary Medicine, South China Agricultural University, 483 Wu Shan Road, Tian He District, Guangzhou 510640, China.
[2]Department of Biological Sciences, Alabama State University, 915 S Jackson St. Montgomery, AL, 36101, USA.

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Dendritic cells (DCs) play an important role in inducing primary antigen-specific immune responses to viral antigens. In this study, the peripheral blood monocyte-derived (PBMC) were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. After 6 days of culture, immature monocyte-derived dendritic cells (Mo-DCs) were generated. The addition of lipopolysaccharide (LPS) during differentiation of Mo-DCs enhanced their ability to stimulate allogeneic mixed lymphocyte reaction (MLR) and alter their ability to produce cytokines. Then, we investigated the interaction between foot-and-mouth disease virus (FMDV) and porcine Mo-DCs *in vitro* and confirmed that the immunological phenotype and function of porcine Mo-DCs were modulated during FMDV infection. A down-regulated expression of MHC II and CD1 were observed at 48 h post FMDV infection. In addition, the infected porcine Mo-DCs exhibited ultrastructural morphological changes, FMDV-infected porcine Mo-DCs failed to stimulate T cell proliferation *in vitro*. Moreover, infection of porcine Mo-DCs *in vitro* induced the secretion of IFN-γ and the suppressive cytokine IL-10 in porcine Mo-DCs. Results indicated that the down-regulation of MHC II and CD1 molecules and the increased secretion of the IFN-γ and IL-10 cytokines might be the mechanisms that FMDV uses to evade the host immune responses.

Key words: Dendritic cells, foot-and-mouth disease virus, MHC II, modulation, cytokines.

INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and are essential initiators and regulators of the immune response and have efficient cytokine producing capacity (Johansson et al., 2003; Unternaehrer et al., 2007). DCs play a crucial role in immune responses to viruses. A combination of unique properties makes them the key APCs involved in antiviral T-cell priming (Banchereau et al., 1998).These properties include the ability to acquire antigen in infected tissues, to migrate to secondary lymphoid organs and to provide the co-receptor signals required for effective helper and cytotoxic T-cell activation. Conversely, certain viruses use a variety of strategies to avoid recognition by the host immune system (Orange et al., 2002; Benedict et al., 2002; Yewdell et al., 2002). The active induction of immune suppression is one mechanism by which viruses escape clearance (Rouse et al., 1986). Several viruses are known to target dendritic cells (DCs) and impair antiviral T cell responses (Knight et al., 1997; Fugier-Vivier et al., 1997; Andrews et al., 2001; Sevilla et al., 2000; Kruse et al., 2000; Ho et al., 2001).

Foot-and-mouth disease virus (FMDV) causes a highly contagious viral disease of cloven-hoofed animals and

*Corresponding author. E-mail: jdchen@scau.edu.cn Tel: +86-020-85288017. Fax: +86-020-85280242.

Abbreviations: DC, Dendritic cells; **PBMC,** peripheral blood monocyte-derived; **GM-CSF,** granulocyte-macrophage colony-stimulating factor; **IL -4,** interleukin-4; **LPS,** lipopolysaccharide; **MLR,** mixed lymphocyte reaction; **FMDV,** foot-and-mouth disease virus; **APC,** antigen-presenting cell; **TEM,** transmission electron microscope.

#These authors contributed equally to this work.

remains a major animal health concern, affecting agricultural economics worldwide (Ferguson et al., 2001). This pathogen is responsible for repeated epidemics around the world that invariably inflict significant economic damage on the agricultural and wider economies of the affected regions (Grubman et al., 2004). It has been reported that during acute infection with FMDV in swine, there is an immunosuppressive stage characterized by T cell unresponsiveness (Diaz-San Segundo et al., 2006; Bautista et al., 2003). Throughout this stage, a severe but transient lymphopenia affecting all T cell subsets correlates with the appearance of viremia. However, when viremia is cleared, both the number of lymphocytes and the altered T cell function, start to recuperate normal levels. These effects on the early host immune response provide the perfect conditions for viral spread through the organism and subsequent shedding to the environment. Also, the interaction of FMDV with DCs has been previously described by others who have reported an absence of detectable FMDV replication in mature DCs *in vitro* (Bautista et al., 2003; Guzylack-Piriou et al., 2006; Harwood et al., 2008). In view of these studies together with the central role played by DCs in the induction of innate and adaptive immune responses mentioned earlier led us to examine the interactions of FMDV and porcine Mo-DCs.

This study investigated whether FMDV can efficiently infect porcine Mo-DCs and the consequential morphological and functional changes of DCs by examining expressions of immune stimulatory markers in DCs and cytokines induced in porcine Mo-DCs after FMDV infection. Elucidating the cross-talk between DCs and FMDV would provide insights into developing improved vaccines and diagnostic assays specific for FMDV.

MATERIALS AND METHODS

Porcine and virus strain

Healthy dual-hybrid boars referred to the Development Service Center of Quality Animal Products of Guangdong province in China were used in this study. The FMDV O/HK/01(GenBank accession no: EU400597) was propagated on BHK-21 cells (Zhao et al., 2008). The titer of the virus stock was 1.592×10^7 PFU/ml as determined by the plaque test based on the cytopathic effect (CPE) in BHK-21 cells.

Preparation of porcine Mo-DCs

The preparation and culture of porcine Mo-DCs were performed according to a method described previously (Johansson et al., 2003). Monocytes were obtained by plastic adherence of PBMC using 6-well microtiter plates (Greiner company, Germany), subsequently, the recombinant swine GM-CSF (25 ng/ml) and recombinant swine IL-4 (10 ng/ml) (Biosource company, U.S.A.) were added on the first day of the culture. Half of the medium was replaced every third day. On day 6, cells were collected, washed twice and used as immature porcine Mo-DCs. For induction of maturation, immature porcine Mo-DCs were cultured in RPMI - 1640 medium containing 1 µg/ml LPS (Sigma, U.S.A.) for 48 h (Carrasco et al., 2001; Paillot et al., 2001). Cultured cells were photographed every day with a digital camera on an inverted microscope. Simultaneously, the surface morphology of the cells was observed by FEI-XL30 scanning electron microscope (PHILIPS, Netherlands).

Ultrastructural observation by electron microscopy

The cultured FMDV-infected and mock-infected porcine Mo-DCs were removed from the wells. Cells were fixed in 2.5% glutaraldehyde at 4°C for 2 h, washed in PBS, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in acetone and embedded in epon. Ultrathin sections were stained with lead citrate and uranyl acetate and analyzed with a FEI-Tecnai 12 (PHILIPS, Netherlands) transmission electron microscope (TEM).

Flow cytometry analyses of cell surface molecule expression

The cultured FMDV-infected porcine Mo-DCs samples were transferred to a 1.5 ml-centrifuge tube, washed twice with PBS, they were stained at 4°C for 30 min with the following monoclonal antibodies: Mouse anti-Pig CD1-R-PE (Southern Biotec, Birmingham, USA), mouse anti-porcine SLA-II-DR-FITC and mouse anti-pig CD172a-FITC (Serotec, the United Kingdom) or isotype-matched antibodies. Analyses were performed by flow cytometer and CellQuest software (BD Company, Vantage, USA). The un-infected porcine Mo-DCs samples were used as controls.

Phagocytosis assays

Phagocytic function of FMDV-infected and un-infected porcine Mo-DCs were evaluated using 0.1% neutral red solution. The cells were exposed to 0.1% neutral red solution for various lengths of time in RPMI-1640 medium at 37°C. After incubation, cells were washed three times with PBS, then 1% SDS was added to lyse the cells. Samples were measured in triplicate at the 570 nm wavelength by the Multiskan EX spectrophotometer (Bio-TEK Company, USA).

T lymphocyte proliferation assay

The cultured FMDV-infected and un-infected porcine Mo-DCs were incubated with mitomycin-C (MMC) for 30 min and then, the cells were harvested and washed twice with the medium. Lymphocytes were isolated from the porcine peripheral blood by Ficoll-Paque density gradient centrifugation and the monocytes were removed by plastic adherence. Lymphocytes were co-cultured with the MMC-incubated Mo-DCs at ratios of 1:10, 1:100 and 1:1000 at 37°C for 4 days in 96-well plates. Cultures were incubated at 37°C with 5% CO_2 for 4 days. The medium was discarded; MTT (Thiazolyl blue, Amresco) was added at 20 µl/well and incubated for 4 h at 37°C with 5% CO_2. The medium was discarded and 100 µl of DMSO was added to each well. Plates were oscillated slightly for 10 min at room temperature. Proliferation of lymphocytes was measured at 492 nm wavelength by the Multiskan EX spectrophotometer (Bio-TEK , USA).

Detection of cytokine secretion

Concentrations of IL-10 and IFN-γ cytokines were quantified in cell free-supernatants of FMDV-infected and un-infected porcine Mo-DCs using Opti-EIA kits (Biosource, Carlsbad, USA) according

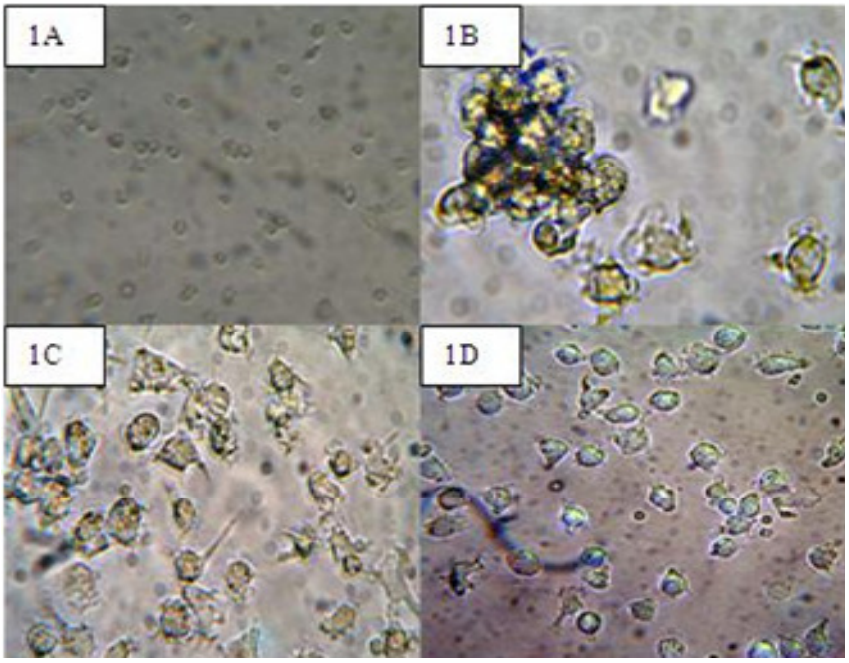

Figure 1. Swine GM-CSF, IL-4 and LPS induced differentiation of monocytes into dendritic-like cells. (A) Peripheral blood mononuclear cells (PBMC) from swine were isolated and cultured in RPMI-1640 medium; (B) cells were cultured for 3 days in the presence of GM-CSF and IL-4; (C) the immature DCs displayed a typical veiled morphology after being cultured for 6 days; (D) the mature DCs were in a suspended or semi-suspended state. A large number of cells presented typical dendritic appearance on the 8th days. (A) Magnification 200×; (B-D) magnification 400×.

to the manufacturer's instructions.

Statistical analyses

All values were presented as mean ± S.D. and statistical analyses were performed using the SPSS 11.0 for windows software. Tukey's test was conducted to determine the differences among treated groups. The means of paired groups were analyzed by a paired Student's t-test. Significance was indicated by a probability of $p < 0.05$.

RESULTS

Generation of DCs and the modulation of its ultrastructural characterizations after FMDV-infection

Porcine monocytes selected from purified PBMCs by plastic adherence (Figure 1a) were cultured in the presence of the combinations of cytokines swine GM-CSF and IL-4. After 3 days of culture in growth medium with cytokines, adherent aggregates were visible (Figure 1b). After 6 days of culture, the cells presented a typical veiled morphology (Figure 1c) compared with the initial culture. A large number of cells presented typical dendritic appearance on day 8 (Figure 1d). Results of scanning electron microscope showed that the cells have a typical veiled appearance (Figure 2). Overall, the adherent PBMC-derived porcine cells showed morphologic features characteristic of DCs, indicating that the porcine Mo-DCs were cultured successfully. Simultaneously, the ultra-structure of FMDV-infected and mock-infected porcine Mo-DCs was observed under the TEM, which showed that porcine Mo-DCs had pronounced protrusions and microvillous projections of their plasma membrane, mitochondrion, endoplasmic reticulum and cellular nucleus (Figure 3a). In contrast, the intracytoplasmic lysosomes of FMDV-infected porcine Mo-DCs increased (Figure 3b) and a few of the FMDV-infected porcine Mo-DCs collapsed (Figure 3c) or the nuclei are pyknosised (Figure 3d).

DCs down-regulate the expression of immunologically important molecules after FMDV infection

The important role which DCs play as professional APCs in antiviral immune a response depending on the fine-tuned and coordinated expression of adhesion, costimulatory and MHC molecules. By using flow cytometry, we investigated whether FMDV affects the level of these immunologically relevant molecules. Our results show that in comparison to un-infected cells the expression of MHC-II and CD1 on FMDV-infected immature DCs was strongly down-regulated 48 h post infection, whereas

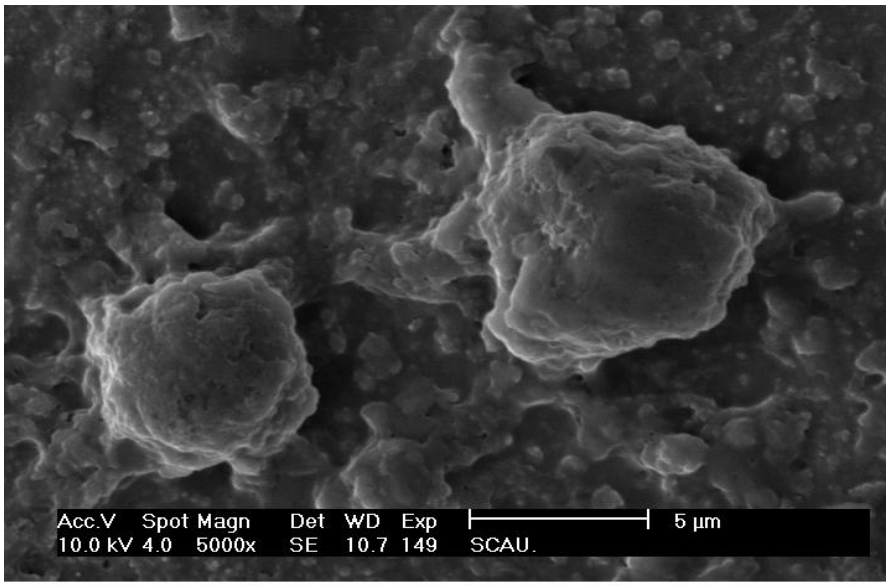

Figure 2. Morphological features of porcine Mo-DCs. The surface of porcine Mo-DCs is rough and even erupt a lot of pleat and irregular ecptoma around the cell body that almost covered the cell body. Magnification 5000×.

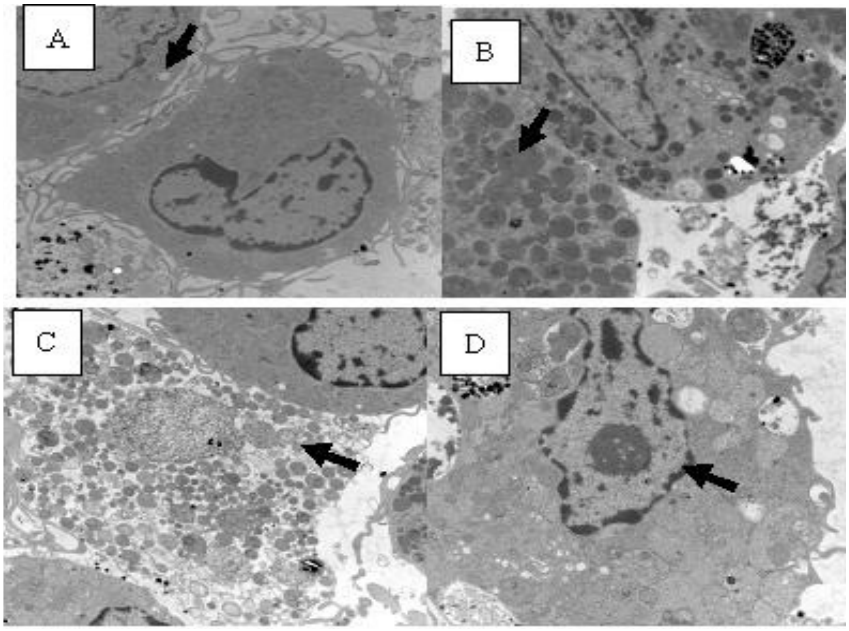

Figure 3. (A) Ultra structure of DCs grown from normal, porcine blood mononuclear cells with GM-CSF plus IL-4 and LPS. Low power view (Magnification 890×) shows three profiles of DCs. Thin cytoplasmic processes and affluent organelles have been seen; (B) most DC intracytoplasmic lysosomes were increased; (C) a few DCs collapsed; (D) the nuclei of very few DCs pyknosised. (B-D) Magnification 1200×.

CD172a was slightly modulated in infected cells at this time point. FMDV infection of already mature Mo-DCs also significantly alter the pattern of surface molecules MHC-II and CD1 (Figure 4).

Taken together, these experiments demonstrated the down-regulation of MHC and CD1 molecules on the cell surface of immature and mature DCs that have been infected with FMDV *in vitro*. Such a modulated DC phenotype indicates that infection with FMDV constitutes a block maturation signal for immature DCs.

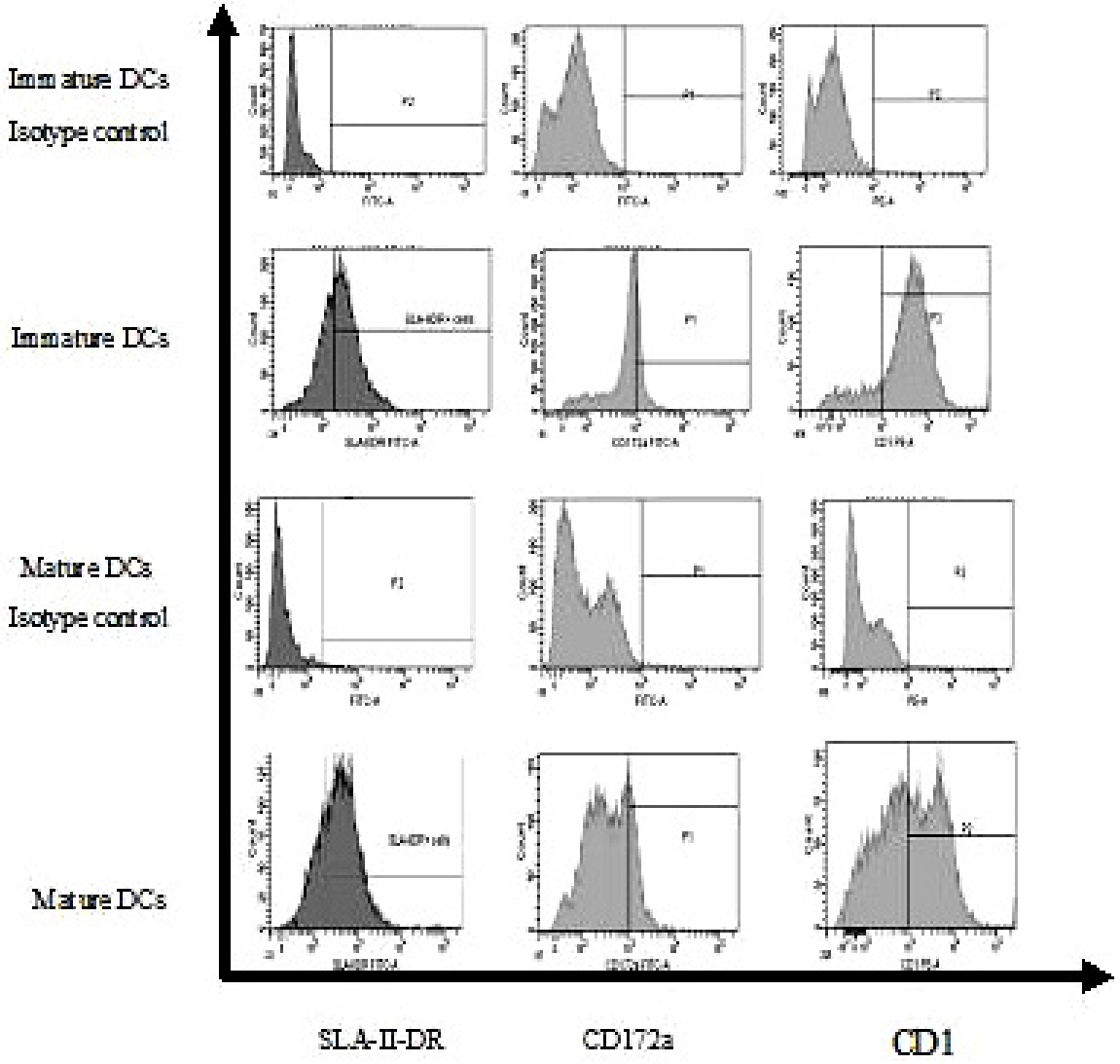

Figure 4. Phenotypic characterization of DC subsets in total cell suspensions of immature DCs, immature FMDV-infected DCs, mature DCs and mature FMDV-infected DCs by two-color immunofluorescence flow cytometric analyses. Immature DCs expressed SLA-II-DR, CD1 and CD172a at 61.50, 83.10 and 24.90%, respectively. Immature FMDV-infected DCs expressed SLA-II-DR, CD1 and CD172a at 27.2, 45.00 and 20.50, respectively, which were decreased compared with mock-infected groups. Mature DCs expressed SLA-II-DR, CD1 and CD172a at 71.70, 50.10 and 19.30%, respectively. Mature FMDV-infected DCs expressed SLA-II-DR, CD1 and CD172a, respectively, at 56.40, 34.00 and 10.50%.

Modulation of phagocytic and T cell proliferatic ability after FMDV infect porcine Mo-DCs

Immature DCs efficiently capture and process antigen (Salter et al., 2001). This DCs function decreases with maturation. Therefore, we want to study the effect of FMDV on the uptake of exogenous antigen. For this type of experiment, immature porcine Mo-DCs (Mo-imDCs) were infected by FMDV for 48 h and the phagocytic function of them were evaluated using 0.1% neutral red solution. As the time of incubation cells with 0.1% neutral red solution prolong (for 40, 80 and 120 min), the amount of phagocytosed neutral red solution were more and more. And that FMDV-infected immature porcine Mo-DCs exhibited higher phagocytic activities than uninfected immature porcine Mo-DCs (Figure 5).

Another characteristic feature of mature DCs is their capacity for T-cell priming. Therefore, we analyzed the ability of FMDV-infected mature Mo-DCs to stimulate the proliferation of naive allogeneic T cells. Mature DCs were infected with FMDV *in vitro* to evaluate their ability to stimulate T cell proliferation in cocultures with autologous leukomonocytes from peripheral blood. The ability of mature porcine Mo-DCs to stimulate the proliferation in an MLR was measured using MTT assay at 492 nm wavelength. The DCs were used at different stimulator:

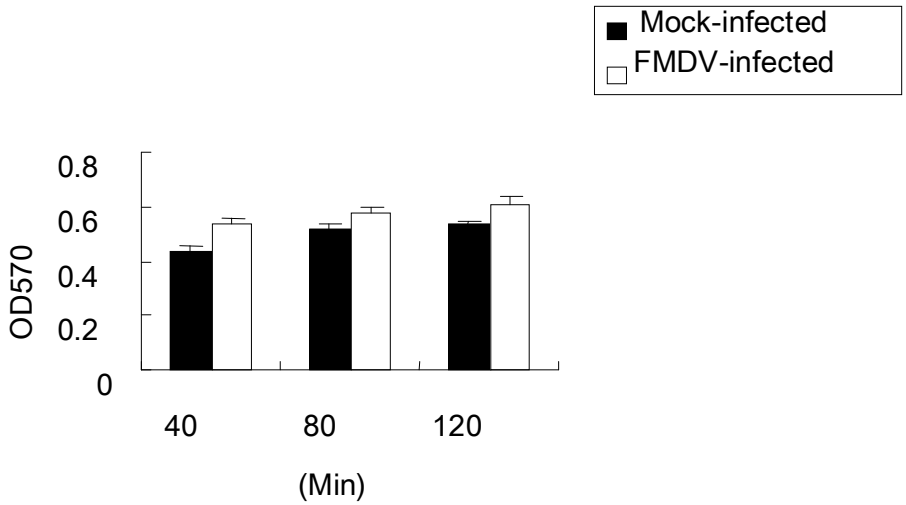

Figure 5. Antigen up taken by immature DCs after infection with FMDV. After 48 h of infection with FMDV, the capacity of FMDV-infected immature DC for antigen uptake phagocytosis was analyzed. The results indicated that phagocytosis of FMDV-infected immature DCs was increased.

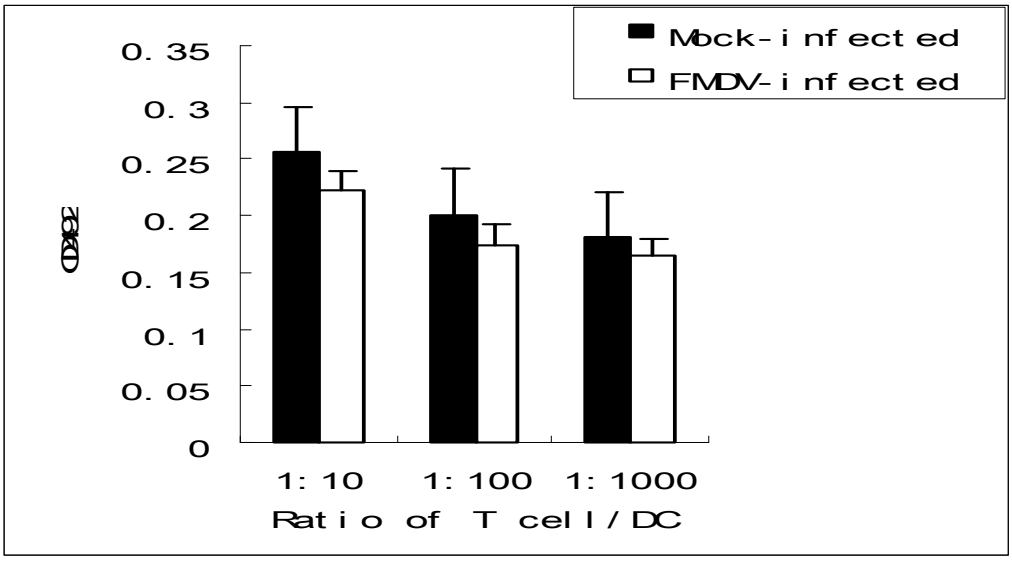

Figure 6. T-cell stimulatory capacity of FMDV-infected DCs. Mock-infected DCs and FMDV-infected DCs were added to allogenic T cells at various ratios (the T/DC ratio is shown on the x axis). After incubation for 4 days, MTT was added to the culture for 4 h. Thereafter, the cells were fixed and MTT incorporation determined by measuring the optical density at wave length of 492 nm

responder cell ratios: 1:1000, 1:100 and 1:10 in the MLR and the stimulatory effect were expressed as OD492 value. The mature porcine Mo-DCs were able to stimulate allogeneic responder cells to proliferation at the stimulator responder ratio 1:1000, 1:100 and 1:10 and the OD492 value obtained with the ratio at 1:10 was higher than the other ratios. Compared with un-infected mature porcine Mo-DCs, the FMDV-infected mature porcine Mo-DCs stimulated proliferation of naive allergenic T cells were decreased, they could not stimulate the proliferation of T cells significantly (Figure 6).

Cytokine secretion by Mo-imDCs in response to FMDV infection, LPS stimulation or a combined treatment

In further experiments, we explored whether FMDV could induce the production of cytokines by Mo-DCs. For this purpose, supernatants from immature Mo-DCs and

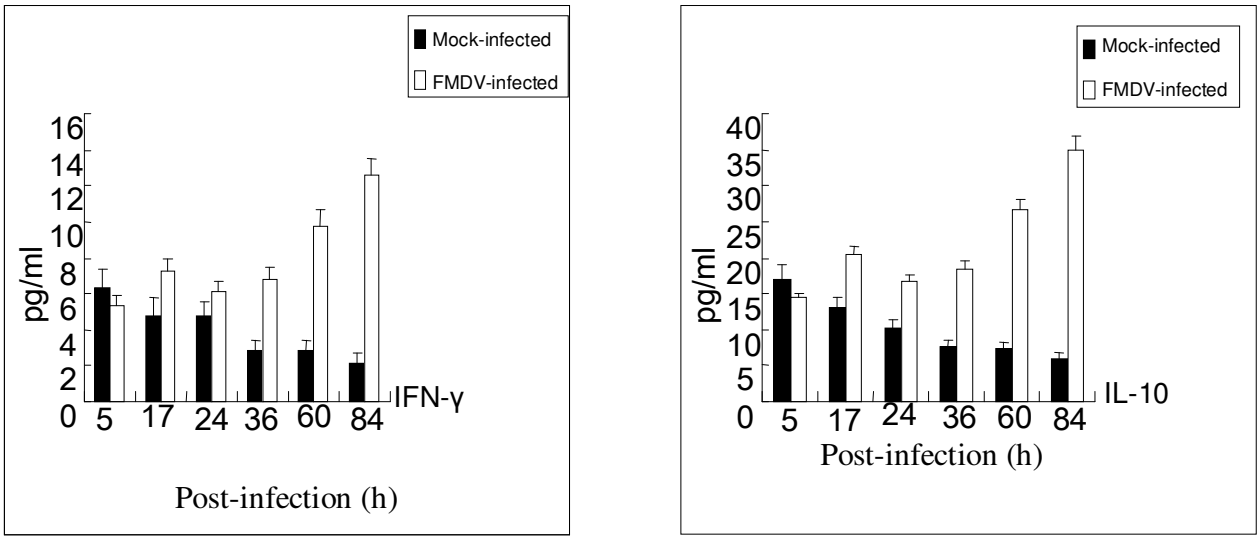

Figure 7. Cytokine production level of Mo-DCs after FMDV infection. Samples from Mo-Dcs were taken at various times post-infection and cytokine concentrations (IFN-γ and IL-10) were assayed by ELISA. Cytokine concentrations in clarified supernatants, expressed as pg/ml.

stimulated with LPS for 5, 17, 24, 36, 60 and 84 h were collected and analyzed for their concentration of IFN-γ and IL-10. Simultaneously, the induction of IFN-γ and IL-10 secretion by immature Mo-DCs following infection with FMDV were analyzed by cytokine specific ELISAs at 5, 17, 24, 36, 60 and 84 h post-infection. Cytokine production levels are shown in pg/ml. Each bar represents the mean ± standard error of triplicate cultures, P values were calculated by the use of the unpaired Student's t-test and significance set at 0.05 levels. The productions of IL-10 and IFN-γ in the immature Mo-DCs only stimulated with LPS were significantly ($p < 0.05$) less and less as the time past. A combination of FMDV infection and LPS stimulation of Mo-imDCs induced significantly ($p < 0.05$) higher productions of IL-10 and IFN-γ compared with the productions by the mock-infected un-stimulated cells (Figure 7).

DISCUSSION

DCs are professional antigen-presenting cells, yet many DC subsets play a major role in innate responses to pathogens. These cells therefore, are critical in regulating the early, non-specific response to viral infection and transitioning to the induction of highly specific immune responses via activation of helper T cells if viral antigens are still present. Based on the central role played by DC in the induction of innate and adaptive immune responses, the importance of studying the interaction of FMDV with DC has already been addressed recently (Bayry et al., 2006; Golde et al., 2008). Plasmacytoid DC (pDC), monocyte-derived DC and bone marrow-derived DC-were shown to be susceptible to FMDV infection (Guzylack-Piriou et al., 2006; Harwood et al., 2008). Following infection, it was possible to detect structural and non-structural viral proteins as well as double-stranded RNA up to 24 h post-infection. In monocyte-derived DC, it was possible to detect small quantities of virus released between 2 and 8 h post-infection, only occurring in the absence of cycloheximide implying a full replicative cycle (Harwood et al., 2008).

In this study, we demonstrated that DCs derived from PBMCs can be modulated by FMDV *in vitro*. FMDV not only changed DC morphology, but also modulated DC immunological phenotype and function. The changes of DC morphology induced by FMDV were markedly. There was not only a great increase of lysosome as judged by electron microscope and cell disruption effects were also observed. The lysosome of FMDV-infected porcine Mo-DCs increased which probably enhance their phagocytose ability for the foreign microorganism. We even observed that very few cells collapsed, which indicate that DCs possibly impacted by FMDV and induce porcine Mo-DCs apoptosis. In this study, the phenotypic alterations, phagocytic and T cell proliferatic modulation of FMDV-infected porcine Mo-DCs were displayed. All these could be the results of the modulation of its ultrastructural characterizations after FMDV-infection.

Harwood et al. (2008) showed that strain differences as well as differences with respect to receptor usage may influence FMDV-DC interaction. For example, cell culture-adapted FMDV uses heparan sulphate rather than integrins as receptor. They have recently shown that cell culture-adapted virus was more efficiently taken up by the DC when compared with integrin-binding virus. In this study, the virus we used was cell culture-adapted. Furthermore, the ultrastructure of FMDV-infected DCs

was affected, suggesting that the cell culture-adapted FMDV was more efficiently taken up by the DCs. The lysosome of FMDV-infected porcine Mo-DCs increased which probably enhanced their phagocytic ability for the foreign microorganism and was in accordance with that of FMDV-infected immature porcine Mo-DCs which exhibited higher phagocytic ability. *In vitro* data suggested that the suppression induced by FMDV infection reduced the ability of DCs to fully stimulate T cells in a mixed-lymphocyte reaction.

Investigating the way of T cell activation after *in vivo* FMDV-infection may provide insights into the signal-regulatory pathway. Such investigation may help understand the innate immune system and immune response modulation due to the ability of DCs to co-express a wide range of antigens on MHC complexes and CD1 molecules (Romani et al., 1994). The group 1 CD1 proteins are thus, likely to be involved in the protective immune response against complex intracellular pathogens (Oseph et al., 2004). In this study, the FMDV-infected immature and mature porcine Mo-DC expressed surface CD1 and MHC-II was down-regulated as a result of FMDV infection. The down-regulated expression of MHC-II could impact the classic signal transduction, which would disorder the immune state of the host. How the CD1 and MHC molecules mediate antigen, recognizes and interact with each other after CD1 expression is down-regulated in the FMDV; infected host is not known. This may be determined with a better understanding of the FMDV pathogenic mechanism.

Immune responses to specific pathogen need to be controlled to an appropriate level to minimize the development of immunopathological injury. The subtle balance between the host defense and pathogen's response is regulated mainly by IL-10 produced during infections. The secretion of IL-10 may prevent or delay the elimination of pathogens, leading to a persistent infection (Mege et al., 2006). IL-10, an interleukin that inhibits a broad spectrum of cellular responses, has been reported to have a role in inducing immunosuppression *in vivo* (Ejrnaes et al., 2006; Brooks et al., 2006). Furthermore, we show that exposure of Mo-DCs to FMDV *in vitro* impairs Mo-DCs function and causes the production of high amounts of IL-10.

Our study showed that FMDV-infected porcine Mo-DCs secreted more IFN-γ and IL-10 over time and the quantity of IL-10 was larger. Data suggested that the host secreted IFN-γ to induce innate immune responses after FMDV infection which should enhance the body's anti-virus capabilities. Simultaneously, the large secretion of IL-10 would suppress the DCs from developing into a mature state, which in turn, may interfere with the antigen presenting role of porcine Mo-DCs. This may be the reasons for persistent FMDV infection, in which infected animals would have become an important source of infection and then, cause the outbreak of FMDV. Rigden et al. (2002) suggested another mechanism that viruses use to evade the host response; viruses may induce the cytokine signaling pathway to create a climate for sustained long-term survival of the virus within the environment of cells. Whether FMDV infection established persistence in this way needs further study.

In summary, we have demonstrated that the exposure of FMDV to porcine Mo-DCs induced the phenotypic and functional modulation. Results suggested that the immune evasion of FMDV is probably due in part to the inhibition of CD1 and MHC II expression and the induction of IL-10 secretion by FMDV-infected porcine Mo-DCs. However, several questions remain regarding the interaction between FMDV and DCs. Answering these questions will provide clues to improving FMDV vaccines and disease control measures, thus benefiting animal health and reducing the economic impact of the disease.

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