

Induction of Immature Dendritic Cell Apoptosis by Foot and Mouth Disease Virus Is an Integrin Receptor Mediated Event Before Viral Infection

Huali Jin, Chong Xiao, Gan Zhao, Xiaogang Du, Yang Yu, Youmin Kang, and Bin Wang*

State Key Laboratory for Agro-Biotechnology, College of Biological Science, China Agricultural University, Beijing 100094, China

Abstract Foot and mouth disease virus (FMDV) has been demonstrated to infect dendritic cells (DC) and reduced its ability to stimulate host immune responses. This study aimed to determine whether non-replicating FMDV could induce apoptosis of the host immune cells. In this study, we have demonstrated that bone marrow derived dendritic cells (BMDCs) were induced to undergo apoptosis in a dose-dependent manner, which was determined by the annexin-V staining, DNA fragmentation, and TUNEL staining methods, after they were treated with the chemically inactivated FMDV *in vitro*. The initiation of apoptosis was apparently via an interaction of the integrin receptor on BMDCs and the RGD motif within the VP1 capsid protein of FMDV. The initiation activated a cascade of apoptotic pathway including reduced expression of Bcl-2, activation of caspases, and release of cytochrome *c* from mitochondria. Pretreatment with BMDCs with LPS prevented the inactivated FMDV induced apoptosis, suggesting immature BMDCs are susceptible to such apoptosis. Taken together, the data demonstrate that the inactivated FMDV induces the apoptosis in BMDCs via the integrin receptor and subsequently triggers the apoptosis signal, suggesting that such induction of apoptosis is likely to impair immune responses against FMDV infection. *J. Cell. Biochem.* 102: 980–991, 2007. © 2007 Wiley-Liss, Inc.

Key words: viral signaling; inactivated FMDV; RGD motif; immature dendritic cell; integrin receptor; apoptosis

Foot and mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, caused by the infection of the foot and mouth disease virus (FMDV) [Bachrach, 1977]. Acute infection of FMDV causes young animals to die, abortions of pregnant ones, and extensive lesions in mouth and feet. During acute phase the FMDV infection, a marked transient lymphopenia occurs and affects all of the lymph cell

subsets, CD4⁺, and CD8⁺, during 2–3 days after the infection [Bautista et al., 2003; Diaz-San Segundo et al., 2006], suggesting an interaction of the FMDV with the host immune cells. Such remarkable changes in immune system have been also seen in other viral pathogens including classical swine fever virus (CSFV) [Susa et al., 1992; Summerfield et al., 2001], human immunodeficiency virus (HIV) [Moses et al., 1998], influenza virus [Lewis et al., 1986], etc. One reason for the lymphopenia during the acute infection is due to virus-induced apoptosis. Lymphocytes, including the T cells, B cells and antigen presenting cell (APC), are usually targets of viral infection to evade host immune responses.

Virus induced dendritic cells (DC) apoptosis is an important mechanism to avoid the host immune surveillances and its eradication. For instance, induction of DC apoptosis by HIV, CSFV, and Influenza virus leads to the retardation of immune system [Hinshaw et al., 1994; Shi et al., 1996; Summerfield et al., 1998; Gougeon, 2003; Stasakova et al., 2005], which

Abbreviations used: FMDV, foot and mouth disease virus; BMDC, bone marrow derived dendritic cells; PI, propidium iodide; TUNEL, transferase uridyl nick end labeling; FITC, fluorescein-5-isothiocyanate; LPS, lipopolysaccharide; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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*Correspondence to: Bin Wang, State Key Laboratory for Agro-Biotechnology, College of Biological Science, China Agricultural University, Beijing 100094, China
E-mail: bwang3@cau.edu.cn

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creates a window period for viral replications and propagations that leads to an immune suppression. The viral induced apoptosis can occur before and after the virus entry. Induction of apoptosis before viral entry has been reported for primary cells or cell lines exposed to type 3 reovirus [Tyler et al., 1995], avian leukosis virus [Brojatsch et al., 1996], bovine herpesvirus 1 [Hanon et al., 1998], vaccinia virus [Ramsey-Ewing and Moss, 1998], sindbis virus [Jan and Griffin, 1999], and murine coronavirus [Liu et al., 2003]. Ostrowski et al. [2005] recently demonstrated that FMDV can infect murine DCs in vitro and subsequently suppress thymus-dependent immune responses in vivo. However, whether this interaction of FMDV and DC induces the DC to undergo an apoptosis in turns to lead the observed immune suppression has not been investigated.

In this study, we examined whether FMDV could induce the apoptosis of murine DC before its infection. The results showed that the inactivated FMDV virion (the chemically inactivated FMDV) induced immature bone marrow derived dendritic cells (BMDCs) to undergo the apoptosis via the interaction between the integrin receptor and RGD motif sequence and triggered the mitochondria associated apoptotic signal pathway. Therefore, this study may reveal one of the important strategies to impair the immune surveillance before the FMDV invades into host cells.

MATERIALS AND METHODS

Animals and Cells

Adult female C57BL/6 mice (6–8 weeks old) were from Beijing Vital Laboratory Animal Technology Company, Ltd (Beijing, China) and received pathogen-free water and food for maintenance. T cells were isolated from the spleen of mice using the T cell isolation kit (R&D Systems, Inc.). B cells were positively selected with anti-CD19 Dynabeads (Jingmei Biotech Co., Beijing, China).

Dendritic Cell Culture

DCs were cultured from mouse bone marrow, as previously described [Lutz et al., 1999], with slightly modification. Briefly, bone marrow cell suspensions, prepared from femurs and tibiae of mice, were cultured at 5×10^6 cells/ml with RPMI-1640 (Gibco, Eggenstein, Germany) supplemented with 10% FBS, 20 ng/ml murine

recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF), in a 10 cm diameter dish. On days 3 and 6, a half of the medium was replaced with a fresh one with 20 ng/ml GM-CSF. At day 8, the CD11c⁺ DCs were purified by magnetic beads conjugated with anti-mouse CD11c antibodies (e-Biosciences, Inc.).

Inactivated FMDV and Synthetic Peptides

The chemically inactivated FMDV was prepared by BEI method in a BSL-3 laboratory at JinYu Group Corporation (Inner Mongolia, China) as described previously [Patil et al., 2002], and followed by the centrifugation on 30–50% of sucrose gradient [Collen et al., 1984]. The concentration of the inactivated FMDV was analyzed by the Bradford micro-assay (Bio-Rad) [Bradford, 1976].

Peptides were synthesized by BL Chemical, Inc. (Shanghai, China) and dissolved in 5% dimethyl sulfoxide in phosphate-buffered saline (PBS) at the concentration of 1 mg/ml and stored at -20°C .

Inactivated Virus Treatment

BMDCs isolated from the culture were seeded at 10^6 /ml of fresh medium without the GM-CSF in 48-well plates (Costar, Corning, NY) after three time washes by PBS. The cells were treated with the inactivated FMDV at different concentrations for 0.5–24 h at 37°C in serum-free media. At the end of treatment, cells were washed with two times of PBS and replaced with a fresh medium.

Viability Assay

Cells were mixed with 0.45% of trypan blue (Sigma, St. Louis, MO) for 10 min and the viable cells were determined under a light microscope with a hemocytometer.

Annexin-5 Binding Assay

The annexin V-fluorescein-5-isothiocyanate (FITC) binding and propidium iodide (PI) staining assay were used to assess apoptosis of cells as described previously [Koopman et al., 1994] with 1×10^6 cells stained with the annexin V-FITC (eBioscience, CA) and PI (1 $\mu\text{g}/\mu\text{l}$, Molecular Probes, Portland). Stained cells were analyzed via a FACSClibur and the CellQuest software (BD Biosciences, CA). Annexin-V positive cells were determined as apoptosis cells.

Inhibition of Apoptosis

Inhibition by neutralizing antibodies.

The inactivated FMDV were pretreated with 50 μ l the bovine neutralizing antibodies (the bovine FMDV specific antibodies have a neutralizing titer at 1:300 and were obtained from the JinYu Group) or negative control bovine anti-sera for 30 min at 37°C, and then used to incubate with the BMDCs for further 24 h.

Ligand inhibition. Fibronectin (fbn), a natural ligand (Sigma–Aldrich) for the integrin receptor, at 10 μ g/ml was co-incubated with BMDCs and the different amounts of the inactivated FMDV for 24 h at 37°C.

Inhibition by peptide. BMDCs were co-incubated with the indicated concentrations of VP1 peptide containing the RGD sequence or VP2 without the RGD sequence as a control and the 40 μ g inactivated FMDV for 24 h at 37°C.

Inhibition by maturation reagents. BMDCs were co-incubated with various amounts of lipopolysaccharide (LPS) or GM-CSF and the 40 μ g inactivated FMDV for 24 h at 37°C or the BMDCs were preincubated with a fixed concentration of LPS at various times at 37°C, and then reacted with 40 μ g of the inactivated FMDV for additional 24 h.

Inhibition of endocytosis. BMDCs were co-incubated with the various concentrations of rapamycin or FK506 and 40 μ g of the inactivated FMDV for 24 h at 37°C.

The apoptosis was determined from all the above experiments by the annexin-5 binding assay.

DNA Fragmentation Analysis

DNA fragmentation was analyzed as described previously [Zhang et al., 1998] with 1×10^6 of BMDCs.

FITC-Labeled-Dextran Assay

BMDCs (1×10^6) were washed and resuspended in 2 ml RPMI-1640 medium, and then incubated with the FITC-dextran to 50 μ g/ml at 37°C for 15 min. After washed with ice-cold PBS for three times, samples were read by a FAC-SClibur and analyzed with Cell Questpro software (BD Bioscience).

TUNEL Assay

The transferase uridyl nick end labelling (TUNEL) assay was performed with the use of the TUNEL detection reagent according to

manufacturer's instruction (Promega). All samples were analyzed by the FACSClibur and Cell Questpro software (BD Bioscience).

FACS Assay

For cell surface proteins or intracellular staining of BMDCs, single cell suspensions were prepared from the spleens and Fc receptors were blocked with an excess amount of anti-Fc antibodies (BD PharMingen). Cells were washed with ice-cold PBS. For the intracellular staining, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin (Sigma–Aldrich). For staining of the surface proteins, appropriate concentrations of phycoerythrin-labeled antibodies (eBioscience, CA) were added to permeabilized cells for 30 min on ice followed by washing twice with cold PBS. Samples were processed in FACSCalibur and data were analyzed with Cell Questpro software (BD).

Cell Extract Preparation

Cell were lysed in a lysis-buffer (20 mM HEPES, pH 7.6, 75 mM NaCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 0.1% Triton X-100, 0.1 mM Na₃VO₄, 50 mM NaF, 0.5 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 100 μ g/ml bezenesulfonyl fluoride). The cell lysate was centrifuged at 10,000 rpm for 10 min and the precipitates were discarded. Then the protein concentrations were determined by the Bradford assay [Bradford, 1976].

Preparation of Mitochondrial Fractions

Cell were lysed in a lysis-buffer as described above and then centrifuged twice at 750g for 10 min at 4°C, and the supernatant was then centrifuged at 10,000g for 10 min at 4°C, and the resulting pellets was used as the mitochondria fractions. The supernatant was further centrifuged at 100,000g for 2 h at 4°C to remove any mitochondrial contamination. The resulting supernatant was used as the cytosolic fractions [Li et al., 1997].

Western Blot Analysis

Protein extracts at 25 μ g from cells were loaded onto 12% SDS–polyacrylamide gels and transferred electrophoretically to the PVDF membranes (Millipore, MA) and subsequently reacted with a 1:1,000 diluted primary antibodies and 1:500 the secondary antibodies conjugated to horseradish peroxidase, followed with five washes by PBST. The immunoreactive

bands were visualized by using enzyme reaction according to manufacturer's instruction (Amersham, Pharmacia Biotech).

Inhibition of Caspase Activity

Caspase activities were irreversibly inhibited by treatment of cells with the indicated concentrations of each corresponding caspase inhibitor (R&D Systems, MN). The pan-caspase inhibitor Z-VKD-FMK, and the caspase-specific inhibitors used were Z-WEHD-FMK (caspase-1 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD (caspase-8 inhibitor), and Z-LEHD (caspase-9 inhibitor). The BMDCs were preincubated with indicated inhibitors for 30 min at 37°C, and then reacted with 40 µg of the inactivated FMDV for additional 24 h.

Statistics

The data were subjected to Student's *t*-test to assess the significant difference and indicated as **P* < 0.05 and ***P* < 0.01.

RESULTS

Inactivated FMDV Induces Apoptosis of BMDCs

Firstly, we used the annexin-V staining, which measures the translocation of phosphatidylserine to the out of the plasma membrane, to determine whether inactivated FMDV could induce apoptosis of DC, T, and B cells. After 24 h of treatment with the inactivated FMDV, BMDCs, but not T cells or B cells, exhibited a significant increased uptake of the annexin-V staining in a dose and time dependent manner (Fig. 1A,B).

Secondly, chromosomal DNAs were isolated from the BMDCs before and after 24 h treatment and used to analyze their fragmentations. As depicted in Figure 1C, DNAs from the treated BMDCs were fragmented in a time depended manner, suggesting the apoptotic process occurred in BMDCs.

Thirdly, to confirm annexin-V and PI staining results, the TUNEL method, which detects the

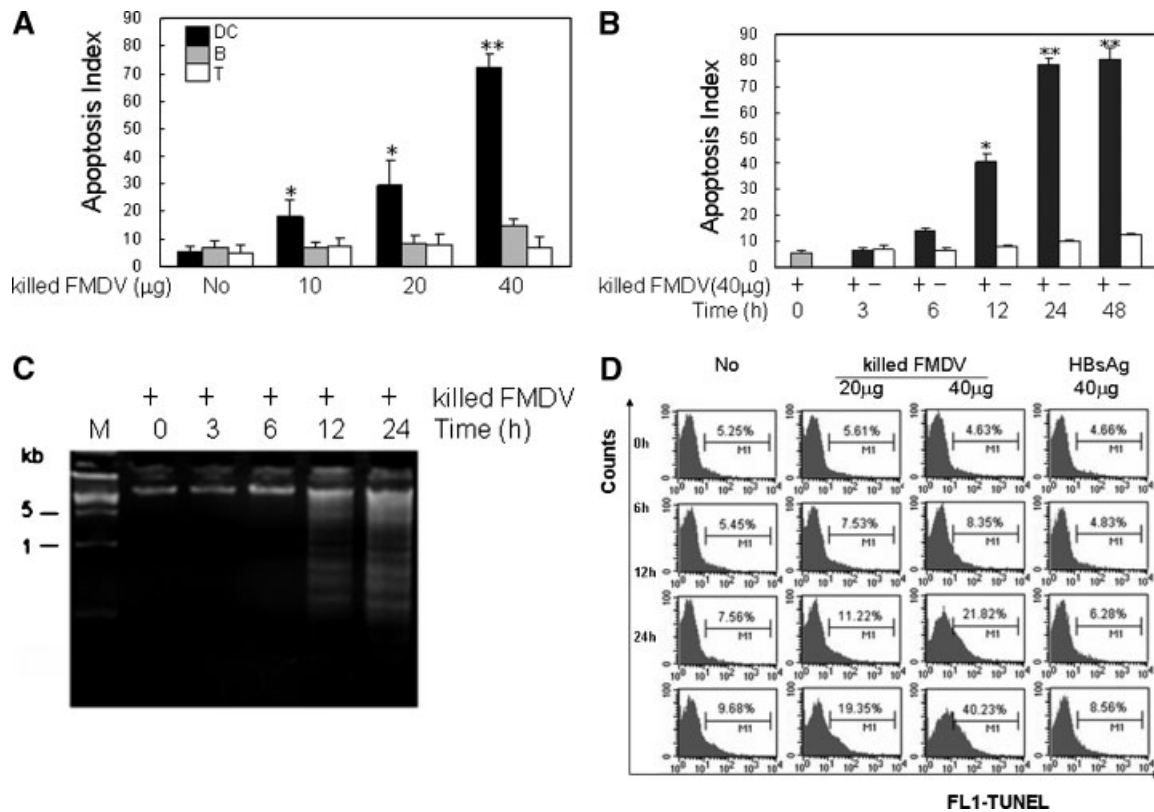


Fig. 1. The inactivated FMDV induced the apoptosis of BMDC. The inactivated FMDV induced a dose-dependent (A) and time-dependent (B) apoptosis to the BMDCs, but not to T cells or B cells. BMDCs, T cells and B cells isolated from spleen of C57 mice were treated with different concentrations of the inactivated FMDV for 24 h, and the degree of apoptosis was determined by annexin-V-FITC and PI staining. C: BMDCs were treated with

40 µg of the inactivated FMDV for the indicated time points, and their total DNAs were extracted and 2 µg of each sample were loaded on the 1.5% agarose gel for the DNA fragmentation analysis, lane M is a DNA marker. D: The induction of apoptosis to BMDCs after being treated with two different concentrations of the inactivated FMDV at various times was further confirmed by TUNEL method.

nucleic DNA damage during induction of apoptosis [Koopman et al., 1994], was utilized. High concentrations of the inactivated FMDV were used to treat BMDCs for 0–48 h. After 24 h, the number of TUNEL-positive DCs was reached to a peak level. In contrast, BMDCs treated with HBsAg or a blank control at the same concentrations and in the same time periods were showed little apoptosis (Fig. 1D).

From the three independent analyses, the data suggest that the inactivated FMDV induced BMDCs to undergo the apoptosis in the dose- and time-dependent manners.

Inactivated FMDV Induces Apoptosis of BMDCs by Interacting With the Integrin Receptor on the Cell Surface

It has been well demonstrated that the G-H loop of capsid protein VP1 of FMDV initiates viral infection by binding to integrin receptors on the host cells via a highly conserved RGD triplet motif [Mason et al., 1994]. To determine if the apoptosis of BMDCs induced by the inactivated FMDV is also initiated via this

ligand-receptor communication, bovine anti-sera that neutralizes FMDV infection was used to pretreat the inactivated FMDV before incubating with the BMDCs. As shown in Figure 2A, such antibodies could specifically block the apoptosis of BMDCs while the control bovine sera did not. To further confirm this interaction has taken place, fbn, a natural ligand for integrin receptor, was used at the time BMDCs was exposed to the inactivated FMDV. This ligand treatment shown that the apoptosis of BMDCs induced by the inactivated FMDV was also blocked (Fig. 2B).

To narrow the binding region on the VP1 of FMDV, we employed a VP1 peptide containing the RGD integrin receptor binding sequence (aa133–147) and a peptide from the VP2 region (aa165–179) as a control at various concentrations to test if a signal from the peptide binding could induce the similar apoptotic event. As showed in Figure 2C, BMDCs treated with both peptides did not induce the apoptosis. However, when the VP1 peptide, but not the VP2 peptide, was added to BMDCs culture with the inactivated

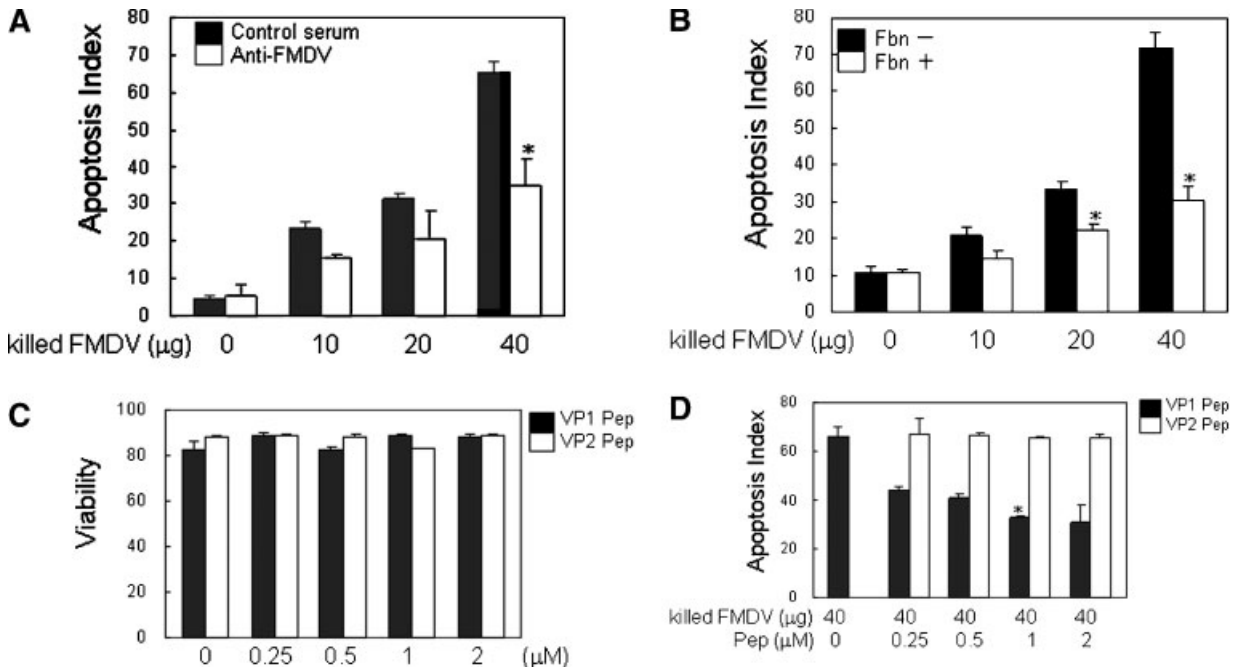


Fig. 2. Apoptosis of BMDCs induced by the inactivated FMDV was limited by interaction between RGD motif of the virus and integrin receptor on cells. **A:** Bovine neutralizing antibodies against FMDV infection blocked the apoptosis of BMDCs induced by the inactivated FMDV. **B:** Fibronectin, the natural ligand for the integrin receptor, blocked the apoptosis of BMDCs induced by the inactivated FMDV. **C:** Treatment of a peptide containing the RGD or without the RGD motif did not induce the

apoptosis of BMDCs. The cells' viability was tested by trypan blue staining after they did not show a positive for the annexin-V staining. **D:** Treatment with the RGD containing peptide blocked the inactivated FMDV induced apoptosis of BMDCs. The cells were pretreated with the peptide with or without the RGD motif for 30 min before incubation of the inactivated FMDV. The apoptosis index was determined by the annexin-V staining.

FMDV, the FMDV induced apoptosis was significantly decreased (Fig. 2D), suggesting a competition on the RGD sequence from the VP1 peptide that lead to block the apoptotic signaling, and also suggesting that virus may be internalized into BMDC before triggering the apoptosis.

To exclude the possibility of apoptosis induced by the inactivated FMDV was via Fas–FasL interactions, we tested that the expression of FasL on the BMDC surface after treated with the inactivated FMDV at 24 h. The expression of FasL was not changed when compared with the untreated or an irrelevant protein treated control (data not shown). Moreover, no changes in FasL expression were observed on BMDCs treated at different doses of the inactivated FMDV (data not shown), suggesting no involvement of the Fas–FasL.

Inhibition of Endocytosis of BMDC Blocks Its Apoptosis

BMDC has an ability to endocytose substances as its one of the important functions, we next to determine if the inactivated FMDV is endocytosed into BMDC when it initiates the apoptosis. Rapamycin, a blocker for the endocytosis [Hackstein et al., 2002], and FK506, a chemical with the similar structure without the blocking function, were used to treat the BMDC before reacting with the inactivated FMDV. We first tested that the uptaken of the FITC-labeled dextran beads was significantly inhibited in a dose-dependent manner by the rapamycin, but not by the FK506 (Fig. 3A). We then demonstrated that the pretreatment of rapamycin indeed significantly suppressed the BMDCs apoptosis induced by the inactivated FMDV (Fig. 3B), suggesting that the induction of apoptosis may require an endocytotic process within the BMDC upon the FMDV binding.

Inhibition of Bcl-2 Expression by the Inactivated FMDV

Nineteen proteins in Bcl-2 family have been identified in mammalian cells, which play essential role in regulating apoptosis [Tsujimoto, 2003; Danial and Korsmeyer, 2004]. As a Western blot data shown in Figure 4A, the expression of Bcl-2 was rapid decreased after the BMDCs exposure to the inactivated FMDV for 3 h; whereas the expressions of Bax and Bcl-XL were not affected. This

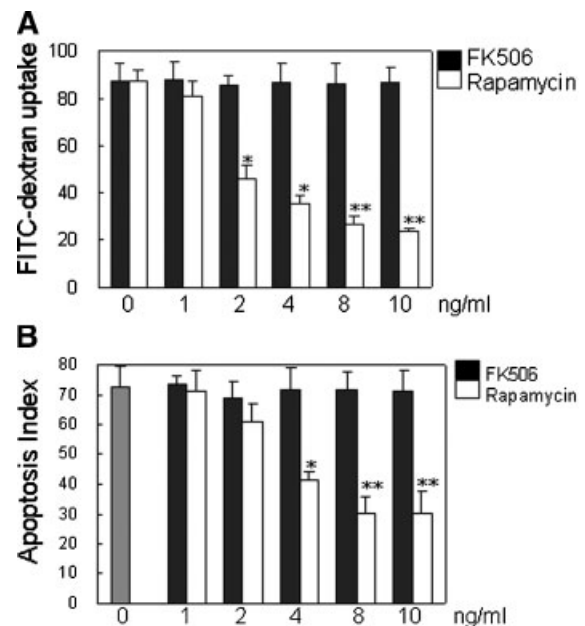


Fig. 3. Inhibition of endocytosis of BMDCs blocks the apoptosis induced by the inactivated FMDV. **A:** The FITC-labeled dextran beads were fed into BMDC culture after the rapamycin treatment at different concentrations. The endocytosed beads were analyzed by FACS to examine the inhibition of rapamycin to the BMDC. The similar structured FK506 was used a negative control. **B:** BMDCs were pretreated with different concentrations of rapamycin or FK506 for blocking the apoptosis of induced by the 40 μ g inactivated FMDV. The apoptosis index was determined by annexin-V staining.

suggests that the induction of apoptosis in BMDCs by FMDV is mainly via reduction of the Bcl-2 expression.

Inactivated FMDV Induces Cytochrome *c* Release in BMDCs

Another key factor in the induction of apoptosis is the release of cytochrome *c* from mitochondria. Subcellular fractionation experiments showed that treatment of BMDCs with the inactivated FMDV induced a significant release of the cytochrome *c* from mitochondria into the cytosol after 4 h treatment. Such increase of the cytochrome *c* in the cytosolic fraction was related directly with a reduction of the mitochondria-containing fraction (Fig. 4B).

Loss of Mitochondrial Membrane Potential After Treatment of the Inactivated FMDV

Loss of the transmembrane potential of mitochondria has been shown to occur prior to the caspase activation and is linked to cytochrome *c* releasing in many apoptotic cells [Kluck et al., 1997]. To determine whether the

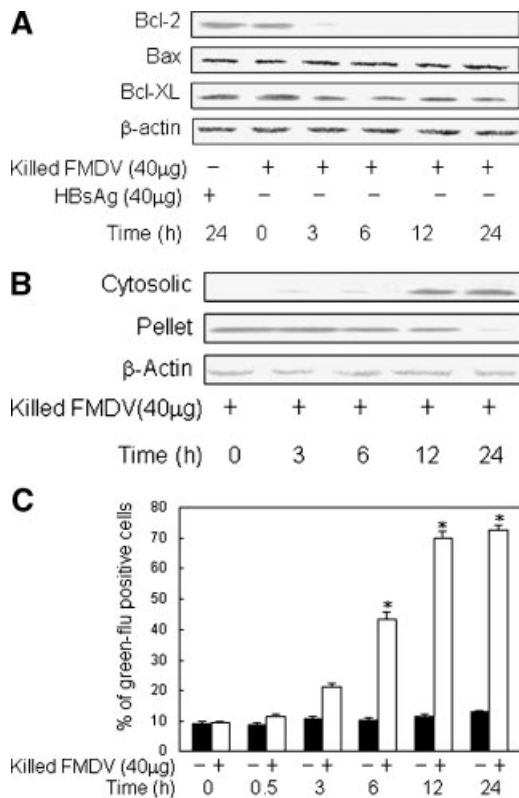


Fig. 4. The induction of apoptosis by the inactivated FMDV to BMDCs is mediated by mitochondria signaling. **A:** Proteins were extracted from the BMDCs after treated with the inactivated FMDV or HBsAg as the negative control at different times, and subjected into SDS-PAGE and immunoblot analysis by anti-BCL2, anti-Bax, and anti-Bcl-XL specific antibodies. Anti-actin antibody was used as an internal protein concentration control. **B:** Proteins extracted from cytosols or mitochondria fractions of the BMDCs after treated with the inactivated FMDV at different times and subjected into SDS-PAGE and immunoblot analysis by anti-cytochrome c antibody. **C:** The inactivated FMDV treatment weaken the mitochondria membrane potential in BMDCs. BMDCs were treated with 40 μg of the inactivated FMDV at different times and an intra-mitochondria dye (MitoCapture kit, BioVision) was used to stain the mitochondria, and intracellular fluorescence was analyzed by FACS. Green fluorescence showed the loss of transmembrane potential of mitochondria in BMDCs.

inactivated FMDV treatment also induces the loss of transmembrane potential of mitochondria in BMDCs, an intra-mitochondria dye (MitoCapture kit, BioVision) was used to stain the cells and intracellular fluorescence was analyzed by FACS. As shown in Figure 4C, more BMDCs were exhibited green fluorescence after 6 h treatment with the inactivated FMDV, indicating a loss of transmembrane potential of mitochondria. In contrast, few BMDCs at indicated times were showed green fluorescent

staining, indicating a normal transmembrane potential for the mitochondria.

Inhibition of Caspases Blocks BMDC Apoptosis

In most cases, the initiation and execution phases of the apoptotic process involve activation of a family of aspartate-specific cysteine proteases called caspases. To test whether the activities of caspases were critical for the apoptosis of BMDCs induced by the inactivated FMDV, chemical inhibitors specific for pan or specific caspases were added to BMDC cultures before exposed to the inactivated FMDV. As shown in Figure 5A, addition of a broad-spectrum caspase inhibitor (z-VAD.fmk) significantly blocked the apoptosis. Similarly, the inhibitors of caspase-3, 8, and 9 have been shown to partially block the apoptosis Figure 5B. Inhibitions of caspase 8 is suggesting that apoptosis in BMDCs induced by the inactivated FMDV is partially mediated by caspase 8. The inhibitions of caspases 3 and 9 are suggesting that the downstream of the apoptosis signaling cascade are activated and the apoptosis process is irreversible. In contrast, the caspase-1 inhibitor was totally unable

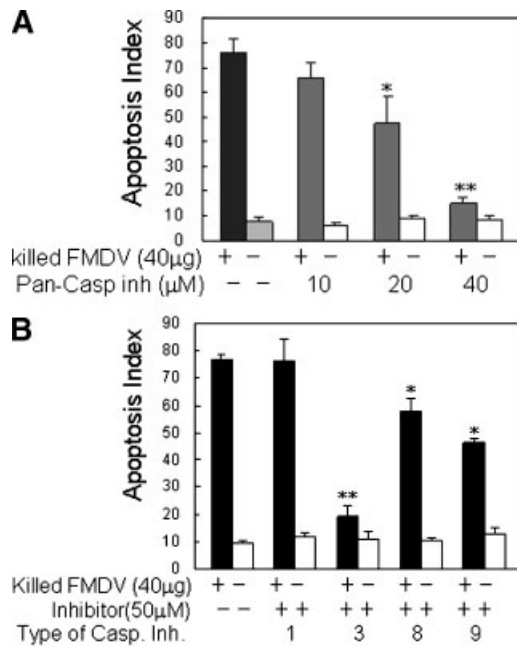


Fig. 5. The inactivated FMDV induced apoptosis of BMDCs is mediated by the caspase cascade. BMDCs were treated with different concentrations of the pan-caspase inhibitor (A), or 50 μM of the specific inhibitors of the caspase family (B) in the presence or absence of 40 μg of the inactivated FMDV for 24 h. The percentage of apoptosis of BMDCs was determined by the annexin-V staining.

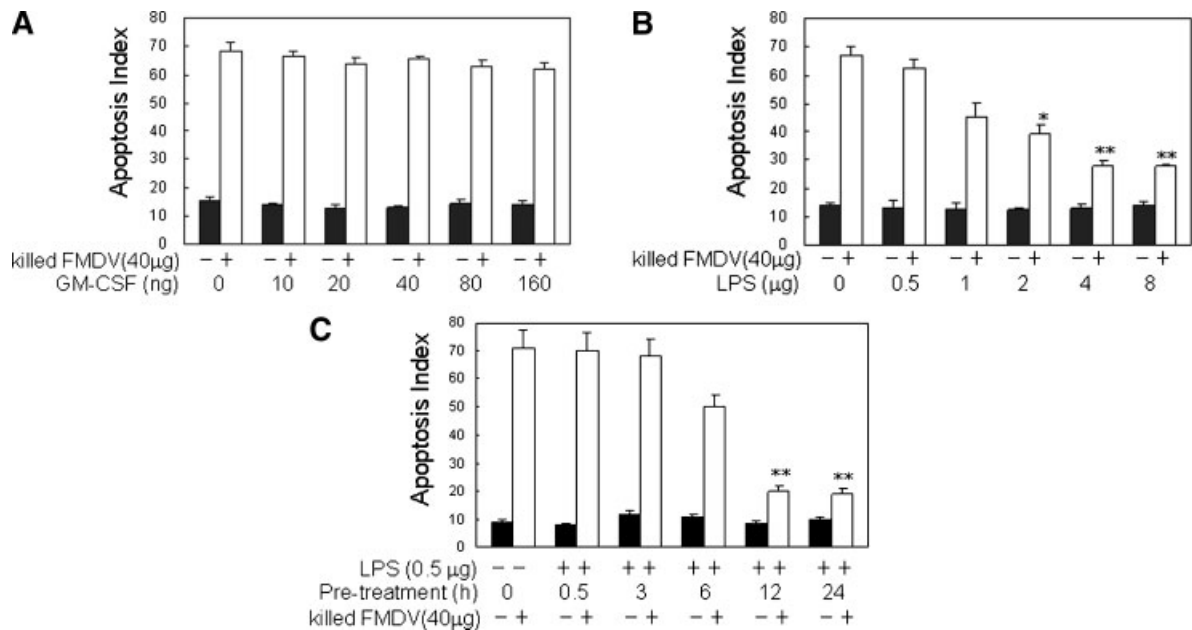


Fig. 6. LPS blocks the apoptosis of BMDCs induced by the inactivated FMDV. BMDCs were cultured in the absence or presence of the inactivated FMDV in medium containing indicated concentrations of GM-CSF (A) or LPS (B). After the culture for 24 h, the percentages of apoptosis were determined by the annexin-5 staining. C: BMDCs were pretreated with 0.5 µg of LPS at various times and then incubated with the inactivated FMDV for additional 24 h. The percentages of apoptosis were determined by the annexin-5 staining. Values are presented as the average mean of triplicate cultures \pm SE.

to block the inactivated FMDV induced apoptosis in BMDCs (Fig. 5B), suggesting the caspase-1 pathway is not involved.

Maturation of BMDCs Leads to Resist to the Apoptosis

To determine what status of DC are affected and undergone the apoptosis by the inactivated FMDV, we examined the effects of co-cultured or pretreated with maturation factors, such as GM-CSF or LPS, on the BMDCs corresponding to the inactivated FMDV induced apoptosis. We first added GM-CSF or LPS at different concentrations to BMDC cultures at the time of treatment with the inactivated FMDV for 24 h. As depicted in Figure 6A, the addition of GM-CSF in culture did not block the inactivated FMDV induced apoptosis. In contrast, BMDCs incubated with LPS became resistant to the apoptosis in a dose-dependent manner at 24 h (Fig. 6B).

Having demonstrated the LPS affecting on BMDCs during the co-cultivation with the inactivated FMDV, we next determined if the

pretreated immature BMDCs with LPS at 0.5 µg/ml, a maturation dose for the DC [Lutz et al., 1999], before incubating with the inactivated FMDV can resist the induction of apoptosis. The result showed that the resistance of BMDCs to the apoptosis has taken place at 6 h and increased significantly after 12 h after pretreated with the LPS (Fig. 6C), suggesting that the prematured BMDCs are more susceptible to induction of apoptosis by the inactivated FMDV.

DISCUSSION

In this paper, we have demonstrated that the inactivated virion of FMDV was able to induce immature BMDCs to undergo an apoptosis. This induction was apparently via the interaction of the RGD loop sequence of VP1 on the FMDV and the integrin receptor on the BMDCs, proceeded to an endocytosis and classical caspase activation cascades for apoptosis, which included the activations of caspase 3, 8, and 9 (Fig. 5B), downregulation of the expression for

Bcl-2 (Fig. 4A) and promotion of the cytochrome *c* releases (Fig. 4B).

Some viruses induce host cells to undergo the apoptosis, particularly to DCs or macrophages, as a strategy to enhance the spread of progeny to neighboring cells to avoid host immune surveillance even before infections. This phenomenon has been reported for many viruses including type 3 reovirus [Tyler et al., 1995], avian leukosis virus [Brojatsch et al., 1996], bovine herpesvirus 1 [Hanon et al., 1998], vaccinia virus [Ramsey-Ewing and Moss, 1998], sindbis virus [Jan and Griffin, 1999], and murine coronavirus [Liu et al., 2003]. In this study, we observed that the inactivated FMDV induces the apoptosis of BMDCs by a contacting before its infection, suggesting that FMDV could disarm the sentinel DC before the DC activating host immune response. This early event may help FMDV to infect and propagate in the host during the acute phase.

FMDV uses the integrins receptors as an entry receptor to initiate its infection *in vitro* and in animals. Binding of the FMDV to its integrin receptors is RGD motif dependent and can be inhibited by synthetic peptides containing this motif [Mason et al., 1994; Jackson et al., 1997]. Our results showed that the neutralizing antibody, the fibronectin and the RGD motif containing peptide blocked the apoptosis of BMDC induced by the inactivated FMDV, which indicated that the communication between the RGD motif and integrin receptor initiated the apoptosis cascade for BMDCs. Furthermore, the triggering of apoptosis was likely occurred after the viral internalization into the BMDC. However, the RGD motif sequence itself is not able to induce the apoptosis as shown in Figure 2C, which suggests that the linear sequence of RGD is not a sufficient triggering signal for the apoptosis and a conformational structure for the sequence may be required.

The integrin receptors are α/β heterodimeric transmembrane proteins, which have multiple functions, including to mediate a cell adhesion, induce a proliferation, function as mechanotransducers for the kinase activation and cancer metastases [Intengan and Schiffrin, 2000; Goldschmidt et al., 2001; Hynes, 2002]. A recent study has demonstrated that the β 1-integrin receptor could be used to mediate a mechanical stretch-induced apoptosis of smooth muscle cells (SMCs) through the activation of

p38 MAPK [Wernig et al., 2003]. Whether the inactivated FMDV induced apoptosis via the integrin receptor utilizes the same pathway is needed to be investigated.

Caspases have been known to mediate both cell death and inflammation [Thornberry and Lazebnik, 1998; Siegel, 2006]. In this study, the inactivated FMDV-induced apoptosis in BMDCs was blocked by caspase inhibitor. The caspase inhibitors, Z-VKD-FMK (pan-caspase inhibitor) (Fig. 5A), Z-DEVD-FMK (caspase-3-like inhibitor), Z-IETD (caspase-8 inhibitor), and Z-LEHD (caspase-9-like inhibitor) (Fig. 5B), were equally effective in rescuing BMDCs from the inactivated FMDV-induced apoptosis. These results suggest that caspase-3-like, caspase-8-like, and caspase-9-like protease activations are critical for the apoptotic BMDCs in response to microtubule damage. We also showed that the difference on the inhibitions of apoptosis induced by these three caspase-inhibitors, which may be caused by their different roles on the apoptosis induction. In general, caspase-3 functioned as the irreversible inducer of the apoptosis [Cohen, 1997]; caspase-9 is mainly observed to a critical apoptotic stimulus through the mitochondrial dysfunction [Kuida et al., 1998]. However, the caspase-8 is observed to regulate both mitochondrial and the death receptor pathways [Varfolomeev et al., 1998; Chandra et al., 2004]. So that we showed in Figure 5B that the inhibitors for caspase-8 or -9 could only partially block the apoptosis induced by killed FMDV, however the inhibitor for caspase-3 could block the apoptosis twofold over the inhibition obtained from the caspase-8 or caspase-9, suggesting that the killed FMDV may activate the apoptosis of BMDC by caspase-8 or caspase-9 first and subsequently activate the caspase-3 to become the irreversible phase.

It has been well demonstrated to mitochondria is pivotal in controlling cell life or death. Bcl-2 and Bcl-X_L as anti-apoptotic proteins resided in the mitochondria outer membrane have been demonstrated to prevent the activation of caspase 9 and 3 by blocking the release of cytochrome *c*. Our results showed that treatment with the inactivated FMDV downregulated the expression of Bcl-2 (Fig. 4A) and promote cytochrome *c* release (Fig. 4B). Bax as a pro-apoptotic protein in the Bcl-2 family can target to the mitochondria membrane, induce mitochondria damage and initiate the

caspace-dependent cell death [Wolter et al., 1997; Xiao et al., 2005]. However, our result showed that the inactivated FMDV treatment did not affect the Bax expression.

It is well known that immature DC expresses low levels co-stimulatory molecules and inflammation cytokines, but has higher ability to endocytose. In this study, we showed that LPS induced maturation of BMDCs exhibited a resistance to the induction of apoptosis (Fig. 6B,C). This is further supported by the rapamycin treatment experiment since the rapamycin downregulates the endocytosis and promotes the maturation of BMDCs (Fig. 3B). These results suggest that the resistance to the apoptosis induced by the inactivated FMDV may be due to matured BMDCs losing the endocytosis ability during their maturation.

It has been demonstrated that a marked immuno-pathogenesis and lymphopenia has been associated with acute viral infections caused by viruses including the HIV-1 [Moses et al., 1998], measles virus [Okada et al., 2000], CSFV [Susa et al., 1992], and FMDV [Bautista et al., 2003; Diaz-San Segundo et al., 2006]. In the case of FMDV, the induction of transient lymphopenia during acute phase infection affects all subpopulations of lymphocytes, such as CD4⁺ and CD8⁺, during 2–3 days after infection [Bautista et al., 2003]. One reason for the lymphopenia during the acute infection is of a viral induced apoptosis. A depletion of lymphocytes due to apoptosis has also been described in mice infected with a highly virulent influenza A virus (AIV, H5N1) isolated from humans [Tumpey et al., 2000]. Peng et al. [2004] has demonstrated recently that the recombinant VP1 of FMDV could induce the apoptosis of human cancer cells by modulating the Akt signaling pathway. However, no association of lymphocytes apoptosis is investigated. Ostrowski et al. [2005], recently demonstrated that FMDV can infect murine DCs in vitro and subsequently suppress thymus-dependent immune responses in vivo. This interaction of FMDV and DC may induce the DC to undergo an apoptosis. Here, we described for the first time that the FMDV induces a rapid apoptosis in murine bone marrow derived DC (BMDC) before its infection. Since DCs play a crucial role in connection innate and adaptive immune responses, FMDV destroys the DC or affects DC functions, including downregulation of co-

stimulatory molecules, inhibition of cytokine secretion, and maturation and its presentation ability (data not shown), which could lead the host immune system impairment. Although, we have not investigated that the induction of apoptosis in DCs occur after FMDV infection in animals during its acute phase, our results clearly demonstrated that the inactivated FMDV induced immature BMDCs undergone apoptosis rapidly, which may reveal one of the important strategies to knockdown the immune surveillance before the FMDV invades into host cells.

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