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Foot-and-mouth disease virus infection of dendritic cells triggers phosphorylation of ERK1/2 inducing class I presentation and apoptosis

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cloven-hoofed animals. This pathology is caused by foot-and-mouth disease virus (FMDV). Over time, the development of vaccines to prevent the spread of this illness became essential. Vaccines currently used contain the inactivated form of the virus. However, vaccination generates an immune response different to that induced by the infection. We investigated whether these differences are related to intracellular mechanisms on dendritic cells (DCs). As a result, we demonstrated that the internalization of infective virus triggered the phosphorylation of ERK1/2, which was involved in the activation of caspase-9, the intrinsic pathway of apoptosis and the delivery of viral peptides on MHC class I molecules. While, inactivated virus (iFMDV) did not affect this pathway or any function mediated by its activation. As described, infectious virus in DCs was also associated to autophagy LC3 protein and was associated to lysosomal protein Lamp-2; contrary to observe for the iFMDV. Strikingly, the processing of viral antigens to accommodate in class I molecules does not appear to involve the proteasome. Finally, this increased presentation promotes a specific cytotoxic response against infectious virus.

1. Introduction

Foot-and-mouth disease virus (FMDV) is a non-enveloped RNA virus belonging to the *Picornaviridae* family, responsible of high contagious and acute viral disease affecting cloven-hoofed animals. Although the primary interaction of virus is with the epithelial cells of mucosa [1], other cells as dendritic cells (DCs) can participated of this interaction, both in natural host and mice [2,3]. DCs-virus crosstalk culminates with the modification in the DCs "maturity" state, determining finally the adaptive immune response [3–5]. An interesting fact is the immune differences observed between the infectious and the inactivated particles. While FMDV induces a rapid onset of neutralizing thymus-independent antibodies which quickly cleared the virus from circulation [2], the vaccination, which used inactivated virus, induced a T-dependent response that required a longer timing to activate [6,7]. Therefore, FMDV-specific MHC class I-restricted CD8+ lymphocytes were reported after infection and vaccination [8]. While, the CD4+ response does not appear to play a central role in the resolution of acute infection by FMDV [9].

Several works have been assessed to evaluate which mechanisms are central in the interaction of FMDV and immune system. Thus, Kaur et al. shows that Akt activation is relevant in the cascade of interferons (IFNs) [10], which culminates with the induction of cellular biological effects of type I IFNs. The early production of IFN-α after FMDV infection was demonstrated both in mice and a natural hosts, however the intracellular signaling pathways involved are not known [11,12]. Strikingly, an association of PI3K/Akt with divergent functions after viral infections was extensively showed [13].

Abbreviations: FMDV, foot-and-mouth disease virus; iFMDV, inactivated FMDV; DCs, dendritic cells; MHC, class I major histocompatibility complex class I; MAPK, mitogen-activated protein kinase.

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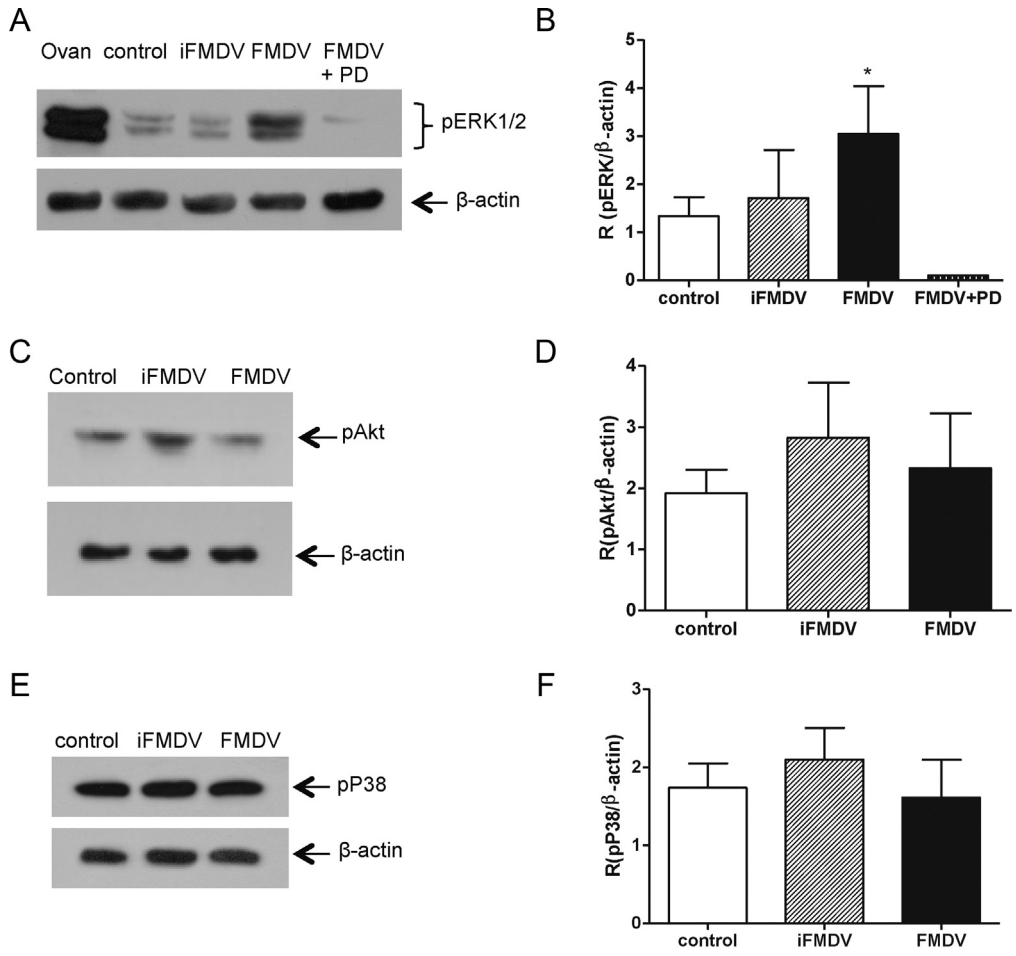

Fig. 1. FMDV activates ERK1/2 MAPK pathway in DCs. 4×10^6 of DCs in 500 μl of RPMI 1% FCS were prewarmed for 30 min at 37 °C. Cells were incubated in the presence of medium (Control), iFMDV or FMDV (MOI, 10) for 30 min at 37 °C. In some cases before to treat DCs with FMDV, cells were incubated with PD98059 to inhibit ERK1/2 phosphorylation. The samples were analyzed by western blot (WB) as described in Section 2. Pervanadate (OVAN) treated DCs were used as positive phosphorylation controls. We show the representatives WB to ERK1/2, Akt and p38 (A, C and E, respectively). B, D and F, histograms of relativization obtained by quantitative densitometric analysis of ERK1/2, Akt and p38, respectively, normalized with β-actin densitometric units. The bars represent the mean ± SE. Asterisk represents statistical significance ($^*P < 0.05$, $N = 7$).

It is known that apoptosis proceeds through two activation mechanisms; intrinsic and extrinsic pathways, both pathways converge in the activation of caspase-3, while caspase-8 and 10 are central in cellular death mediated by the extrinsic pathway. The intrinsic pathway is activated by pro-apoptotic factors such as cytochrome C and caspase-9 [14], but also it was related to activation of JNC and p38 mitogen-activated protein kinase (MAPK) [15]. It was demonstrated that the interaction of the capsid protein VP1 of FMDV causes the deactivation of Akt pathway inducing apoptosis of BHK-21 cells through the activation of several pro-apoptotic proteins [16]. However, in the presence of both infectious and inactivated whole viruses, these authors showed the opposite effect. Interestingly, the use of a soluble recombinant DNA-derived VP1 induced the cleavage of pro-caspase 3, 7 and 9 after BHK-21 treatment.

Moreover, the formation of autophagosomes during cellular entry of FMDV was associated to Atg5 and LC3 proteins [17,18]. Intriguingly, it was demonstrated in macrophages from murine bone marrow that this process is dependent of the extracellular-signal-regulated kinase (ERK1/ERK2) phosphorylation. Notably, Wang et al. [19] showed that the infection of a human line with enterovirus 71, a single positive-stranded-RNA virus, induces the phosphorylation of MEK1/ERKs kinases.

Here, we decided to deepen in the intracellular mechanisms responsible of functional effects of FMDV. We have shown that FMDV interaction with DCs triggers ERK1/2 MAPK phosphorylation inducing the activation of caspase-9; intrinsic pathway of apoptosis and this effect is most noticeable in the spleen plasmacytoid DCs subset. Finally, it was found that the viral particles were rapidly associated to lysosomal vacuoles allowing their presentation on class I molecules and the activation of a specific cytolytic response.

2. Materials and methods

2.1. Mice

All experiments were carried out using 2-month-old virgin male BALB/c mice purchased from National University of La Plata (Buenos Aires, Argentina). They were housed four per cage and kept at 20 ± 2 °C under an automatic 12 h light–dark schedule. Animal care was in accordance with institutional guidelines.

2.2. Generation of bone marrow-derived DCs

Bone marrow-derived DCs were obtained as previously described [3]. Briefly, the epiphysis of femurs and tibiae of BALB/c mice were cut and the marrows were flushed out with RPMI 1640 medium. Red blood cells were lysed using 0.083% ammonium chloride. After washing, cells were suspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium (Invitrogen Life

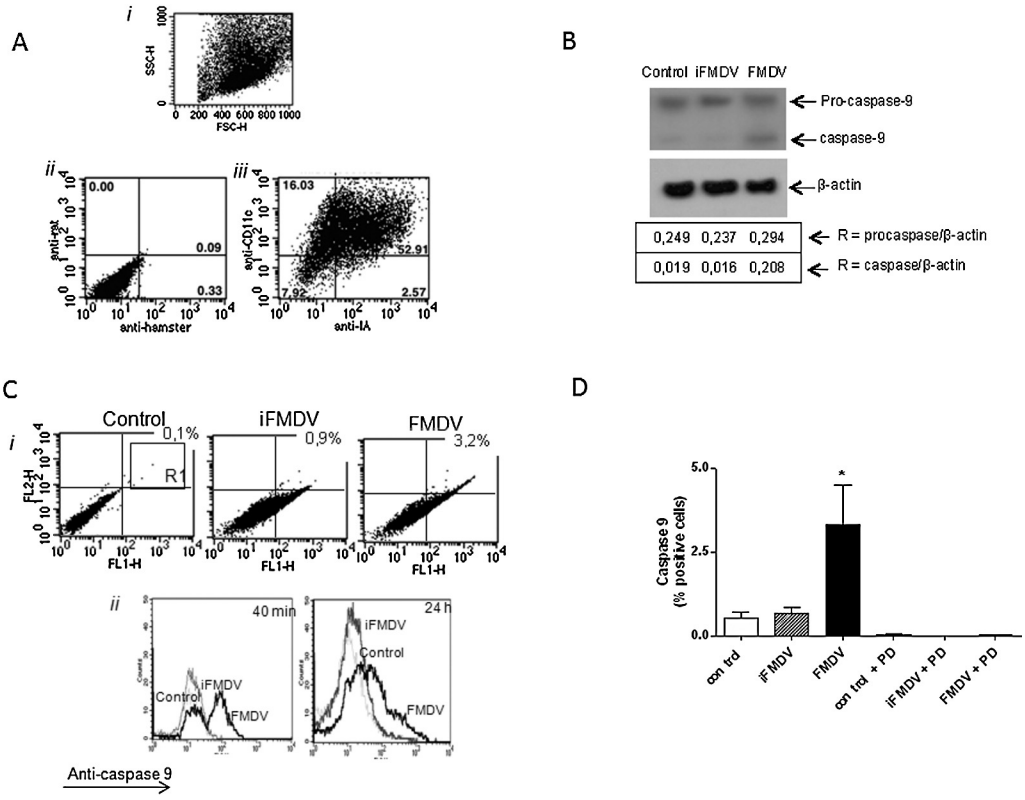

Fig. 2. Analysis of caspase-9 expression on bone marrow DCs. In (A) it is shown the representative dot plots of conventional DCs differentiated from murine bone marrow (A_i). Then DCs were stained with MHC class II molecules and CD11c to determine the purity (A_{iii}) which is compared to isotype control (A_{ii}). (B) As shown the immunoblot analysis of procaspase-9 and the cleaved form, caspase-9, in lysates of DCs exposed to medium, iFMDV or FMDV (10 MOI) for 24 h. β-actin antibody was used as control to quantification of bands. In the table below the figure are shown the relativization of procaspase and caspase-9 proteins densitometric normalized with β-actin as loading control. In (C), we shown a representative experiment of the percentage of caspase-9+ DCs (i) and the histograms of MFI obtained at 40 min or 24 h (R1, ii) from DCs infected with FMDV, and compared with DCs incubated with medium (control) or inactivated FMDV (iFMDV) at the same times. (D) Bars represent the percentage of positive cells expressing caspase-9 after their treatment for 24 h with medium, iFMDV and FMDV or pre-treated with PD before cultured with antigens. After incubation, DCs were analyzed by flow cytometry. Results are expressed as means ± SE (*$P < 0.05$, $n = 10$).

Technologies) supplemented with 10% Fetal Calf Serum (FCS), 5.5×10^{-5} of 2-Mercaptoethanol (Sigma–Aldrich), and 30% conditioned medium from GM-CSF-producing NIH-3T3 cells and cultured for 9 days. Every 2 days, 50% of the medium was aspirated and replaced with fresh medium containing GM-CSF. On day 9 of culture, >85% of the harvested cells expressed MHC class II, CD40, CD80, and CD11c, but no Gr-1 (data not shown).

2.3. Viruses and viral treatment of DCs

The National Service of Animal Health (SENASA, Argentina) and Biogénesis-Bagó (Argentina) provided infectious FMDV (FMDV) and inactivated-FMDV (iFMDV) O1 Campos serotype, respectively, used throughout studies. Infectious FMDV and iFMDV were obtained as previously described [12]. Briefly, FMDV was obtained from vesicles of experimentally infected cattle with 2 passages onto BHK-21 cells. iFMDV was obtained by exposure to binary ethylenimide (BEI). BEI treatment yielded virus that was non-infectious in the FMDV-susceptible cell line BHK-21. Viral stocks were kept at −80 °C and thawed immediately before use. Infection with FMDV or incubation with iFMDV of DCs was performed at a multiplicity of infection (MOI) of 10 at 37 °C. FMDV titers were determined in baby hamster kidney-21 (BHK-21) cells and were expressed as TICD50. The iFMDV recovered after BEI-inactivation of a virus suspension has a theoretical TCID50 and the MOI was calculated from this TCID50. All experiments involving infectious virus were performed in INTA biosafety level 3A facilities. After being subjected to any of these treatments, DCs were washed twice with PBS, pH 5.5 (1-min incubation), to inactivate non-internalized virus, followed by six washes with RPMI 1640 medium supplemented with 5% FCS. In some experiments, DCs were pre-incubated with PD98059 (5 μM) (Sigma–Aldrich) for 5 min, Epoxomicin synthetic (2 μM) (Calbiochem) or ALLN calpain inhibitor (10 μM) (Calbiochem) for 30 min at 37 °C before the different treatment with the viral particles.

2.4. Treatment of spleen tissues and dendritic cells isolation

Spleens were cut into small pieces and treated with a type I collagenase (250 U/ml) (Roche) for 30 min at 37 °C, and stopped by addition of phosphate buffer saline (PBS) supplemented with 5% FCS. Then, the fragments were incubated with DNase I (50 U/ml) (Invitrogen) for 40 min at 37 °C. Finally, the cell suspensions were collected through a gauze mesh and washed with cold PBS. DCs were purified by positive selection from spleen cell suspensions using magnetic beads coupled to anti-CD11c or anti-mPDCA-1 antibodies in accordance with the manufacturer's protocols (Miltenyi Biotec).

2.5. Western blot analysis

DCs were suspended in RPMI 1% FCS (4×10^6/ml) for 30 min at 37 °C and treated with or without FMDV or iFMDV (MOI, 10) for 30 min at 37 °C. To stop the reaction cold PBS was added and the mixture was centrifuged; 3 times. Lastly, pellets were resuspended in the Western sample buffer (100 mM

Tris–HCl pH 6.8; 4% SDS, 0.2% Bromophenol-Blue, 20% glycerol and 200 mM dithiothreitol), heated for 5 min at 95 °C and frozen at −80 °C. Extracts were separated onto 10% SDS–PAGE followed by electroblotting. The membranes were blocked in PBS + 5% milk powder for 1 h, and then incubated with the following primary antibodies in blocking buffer + 0.05% Tween-20 overnight at 4 °C: anti-phospho-ERK1/2 (Thr202/Tyr204, 1:1000; Santa Cruz Biotechnology); anti-phospho-p38K (1:1000; Santa Cruz), anti-phospho-Akt (1:300; Santa Cruz Biotechnology), anti-LC3 (1:2000; Santa Cruz Biotechnology) and anti-caspase-9 (1:500, Cell Signaling Technology). After washing, secondary antibodies were applied in blocking buffer for 1 h at room temperature: anti-rabbit, anti-mouse or anti-goat mAb-HRP (1:2000; Cell Signaling Technology). Specific bands were developed by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden). Membranes were stripped and reproved with a rabbit mAb against murine β-actin (1:3000; Cell Signaling Technology). The quantification of the bands expression was performed using the ImageQuant program. Having tried both ERK total and β-actin to normalize the bands, we observed that the same results were obtained. Consequently, we decided to use the latter along the repetitions.

2.6. Flow cytometry

The following monoclonal antibodies (mAbs) were used, conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC): anti-CD11c, anti-I-A^d, anti-B220, anti CD8α, anti-CD11b and anti-GR1, the lysosomal membrane proteins CD107a (Lamp-1) and CD107b (Lamp-2) (BD Pharmingen). Data were collected using a FACSCalibur and analyzed with CELLQUEST software (Becton Dickinson, San Jose, CA). To perform intracellular staining, DCs (incubated with or without inactivated or infective FMDV at the times indicated at 37 °C) were fixed in 0.5% paraformaldehyde and permeabilized with saponin (0.1% in PBS). Permeabilized cells were incubated with FITC-conjugated antibodies directed to Lamp-1 or Lamp-2, caspase-9 stained with anti-mouse-FITC (BD Pharmingen), LC3 stained with anti-goat-FITC (BD Pharmingen), or isotype-matched control antibodies for 30 min. Finally, cells were washed with saponin buffer twice, suspended in isoflow and analyzed the percentages of double positive DCs by flow cytometry. In most cases, DCs were labeled with anti-CD11c PE or FITC, previously their fixation and intracellular stained.

2.7. Quantitation of apoptosis by microscopy

Quantitation was performed as previously described [20] using the fluorescent DNA-binding dyes acridine orange (100 μg/ml) to determine the percentage of cells that had undergone apoptosis and ethidium bromide (100 μg/ml) to differentiate between viable and nonviable cells. The non-apoptotic cell nuclei show variations in fluorescence intensity that reflects the distribution of euchromatin and heterochromatin. By contrast, apoptotic nuclei exhibit highly condensed chromatin that is uniformly stained by acridine orange. In fact, the entire apoptotic nucleus is present as bright spherical beads. To assess the percentage of cells in apoptosis, at least 200 cells were scored in each experiment. Apoptosis was evaluated on a Zeiis Microscope (West Germany) with 58 Mm and UV polarizing filters.

2.8. Confocal microscopy

DCs were incubated with iFMDV, FMDV or medium (control group) for 4 h. Then cells were fixated in pre-treated glasses with L-polylysine and blocked with decomplemented normal mice serum 0.1% in RPMI medium for 10 min at 37 °C CO_2. Cells were washed twice with PBS and then fixed with 3% paraformaldehyde (Merck, Germany) for 10 min at 4 °C. Fixed cells were washed twice with PBS and treated with 0.1 M of glycine to blocked aldehyde groups for 5 min at room temperature. After two washes, cells were permeabilized with saponin (0.1% in PBS) plus BSA 0.5% for 20 min at 4 °C. Permeabilized cells were incubated with PE/FITC or Alexa 546 -conjugated antibodies directed to MHC class I, Lamp-1, Lamp-2 (BD Pharmingen) and anti-FMDV for 45 min at room temperature. Finally, after washing, cover-slips were mounted onto microscope slides using Fluoromount medium (Vector Labs; Southfield; MI, USA) Images were taken using a Philips CM120 Electron Microscope (FEI Company, Eindoven, Netherlands). Images were acquired using the ITEM program. Analyses were made using the Image J Program.

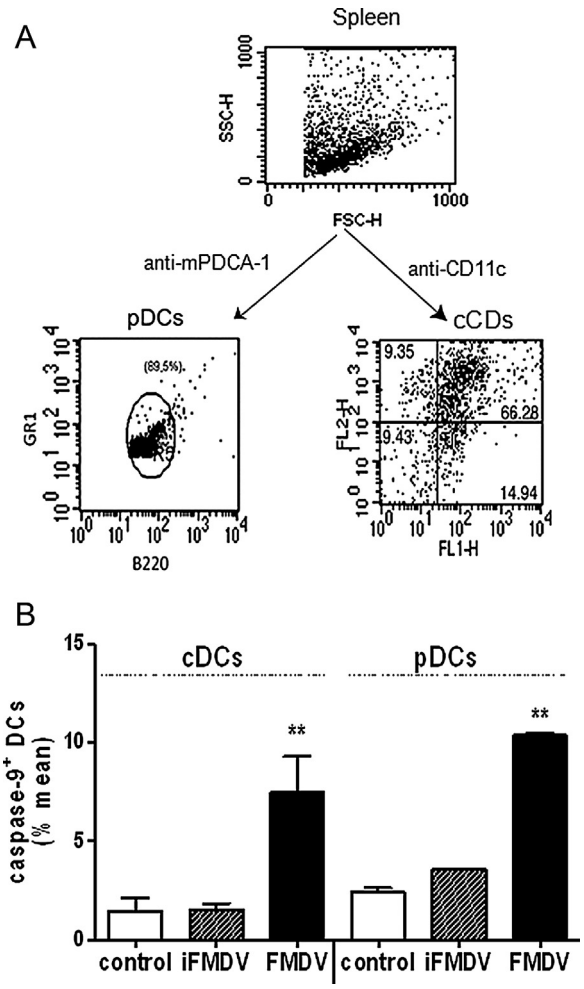

Fig. 3. Analysis of caspase-9 expression on splenic DCs by cytometry. (A) Representative dot plots of purified splenic DCs. (B) Bars represent percentage of positive cells expressing caspase-9 in purified splenic conventional CD11c+ DCs (cDCs) and in purified splenic plasmacytoid CD11c + CD45R+ DCs (pDCs). Results are expressed as means of positive DCs expressing caspase-9 of 3 individual experiments, $^{**}P < 0.01$.

2.9. In vivo cytotoxicity assay

Cytotoxicity was performed as described previously [21]. In brief, naïve syngeneic splenocytes were infected with FMDV (MOI, 10) (2 h, 37 °C), washed with cold acid PBS (pH 5.5) and then of washed extensively with RPMI and labeled with a low concentration (0.5 μM) of fluorescent dye carbox-fluorescein succinimidyl ester (CFSE, Invitrogen). A non-infected control population was labeled with a high concentration (3 μM) of CFSE. Then, CFSElow- and CFSEhigh-labeled cells were mixed at a 1:1 ratio (1×10^7 cells of each population) and injected i.v. into infected or control mice.

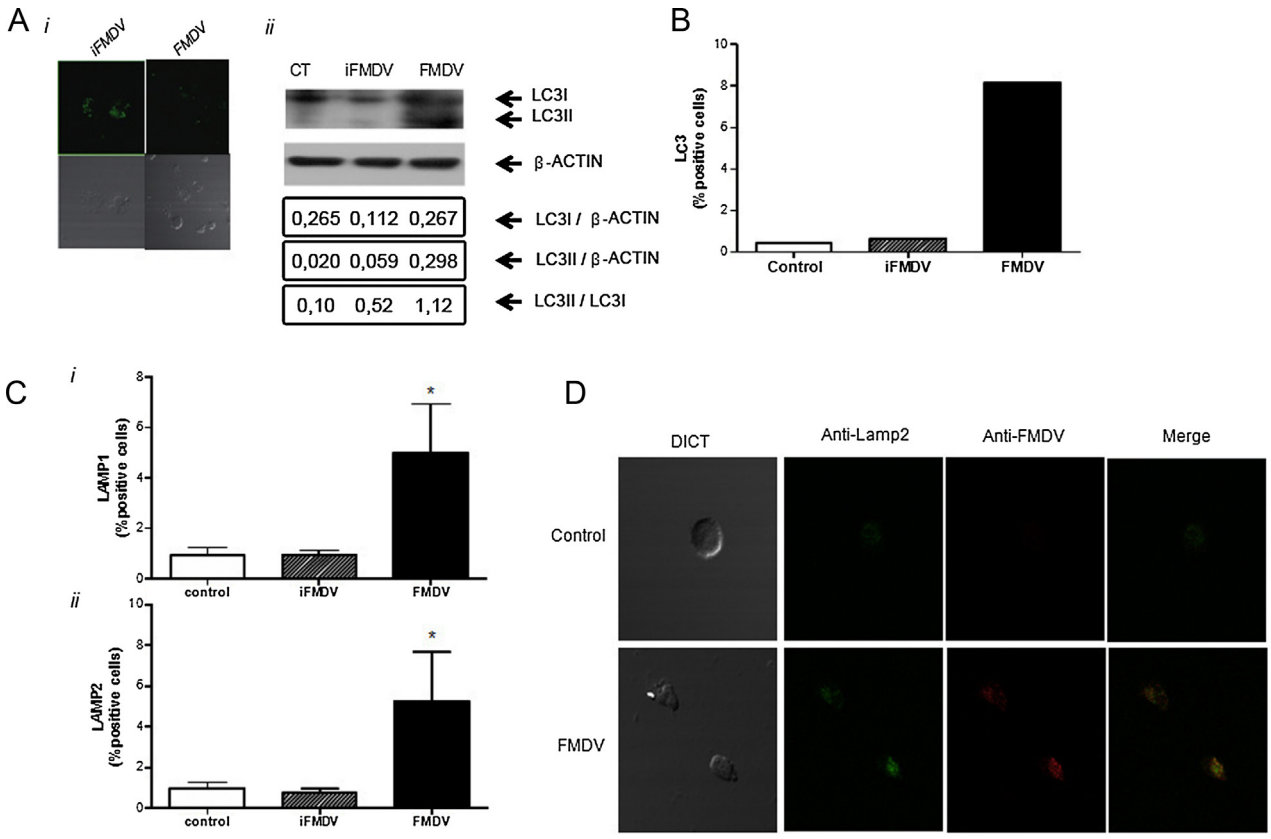

Fig. 4. Analysis of LC3I/II autophagic protein and lysosomes on DCs. (A*i*) Endocytosis of iFMDV and FMDV by confocal microscopy is shown. (A*ii*) A representative western blot to LC3I/II from lysates of DCs treated for 2 h with medium, iFMDV or FMDV (MOI, 10). In the table below the figure are shown the relativization of LC3 I and II proteins densitometric normalized with β-actin as loading control. (B) Bars show the percentage of cells positive to LC3 analyzed by flow cytometry after 2 h of treatments. (C) The bar graph shows the percentage of positive DCs to Lamp-1 (*i*) and Lamp-2 (*ii*) after treatment with medium, iFMDV and FMDV (MOI, 10) for 2 h at 37 °C. Bars represent the mean ± SE, $^*P < 0.05$, $n = 6$. D, confocal analysis of DCs treated or not treated with FMDV for 2 h. Cells were fixed, permeabilized and stained with Lamp-2 (green) and anti-FMDV (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The number of CFSE+ cells remaining in the spleen after 3 or 20 h was determined by flow cytometry. Cytotoxicity was expressed as the percentage of lysis, calculated from $[1\text{-}(r_{immune}/r_{control})] \times 100$, where r is given by the expression of %CFSElow/%CFSEhigh cells for immune and non-immunized (control) mice, respectively.

2.10. Statistical analysis

Differences among treatments were determined by one-way ANOVA, followed by post-ANOVA comparisons using the Dunnett test. Differences between two means were analyzed using Student's t-test. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. FMDV activates ERK1/2 MAPK pathway

Attending that activation of PI3K/Akt and MAPK exerts a central role in DCs function [22,23] as during interaction of RNA virus with host cells [24,25]. We decided to study signaling differences between inactivated or infective FMDV virus. To evaluate this point, lysates from DCs untreated (control) or treated (iFMDV, FMDV; MOI 10), were assayed by western blot with specific antibodies against MAPK. Fig. 1 shows that FMDV triggered the activation of ERK1/2 (A and B); while no differences were observed in Akt phosphorylation (C and D) and p38 phosphorylation (E and F) with any viral particle. Finally, in the presence of an inhibitor ERK1/2 phosphorylation (PD98059), the activation was completely abrogated in DCs incubated with the infective virus (A and B). This result suggests that functional modulation of both particles could depend on the activation of distinct signaling pathways.

3.2. FMDV activates the intrinsic pathway of apoptosis

Since FMDV induces apoptosis of DCs [12] and that ERK1/2 is associated to the activation of caspase-9 [26], we decided to analyze this fact on murine conventional DCs differentiated from murine bone marrow (Fig. 2A). As shown in Fig. 2B, the culture of DCs with FMDV activated caspase-9 tested by western blot. Also, we evaluated the percentages of cells caspase-9 positive at 24 h (i) and the mean fluorescence intensity of caspase-9 (ii) by cytometry; we observed that it was activated in infected DCs (Fig. 2C), contrary to what was observed in the presence of inactivated particles. Interestingly, when DCs were incubated with PD98059, the activation of caspase-9 was prevented (Fig. 2D).

Previously we demonstrated that plasmacytoid DCs (pDCs) die after FMDV infection; we decided to study this point on conventional DCs (cDCs) or pDCs purified from spleens of mice using the antibodies anti-CD11c or anti-PDCA-1, respectively (Fig. 3A). The expression of caspase-9 was analyzed in both subtypes of DCs after their incubation with iFMDV or FMDV. As shown in Fig. 3B, the percentages of positive cells for caspase-9 were increased in both subtypes of FMDV infected DCs being greater for pDCs. This activation correlated with an increase number of apoptotic cells; (cDC: 2% vs 5.2%; pDC: 2.3% vs 11.3%; Ct vs FMDV, respectively, $^*P < 0.05$, $N = 3$) determined by fluorescence microscopy using the acridine orange/ethidium bromide dyes (see Section 2).

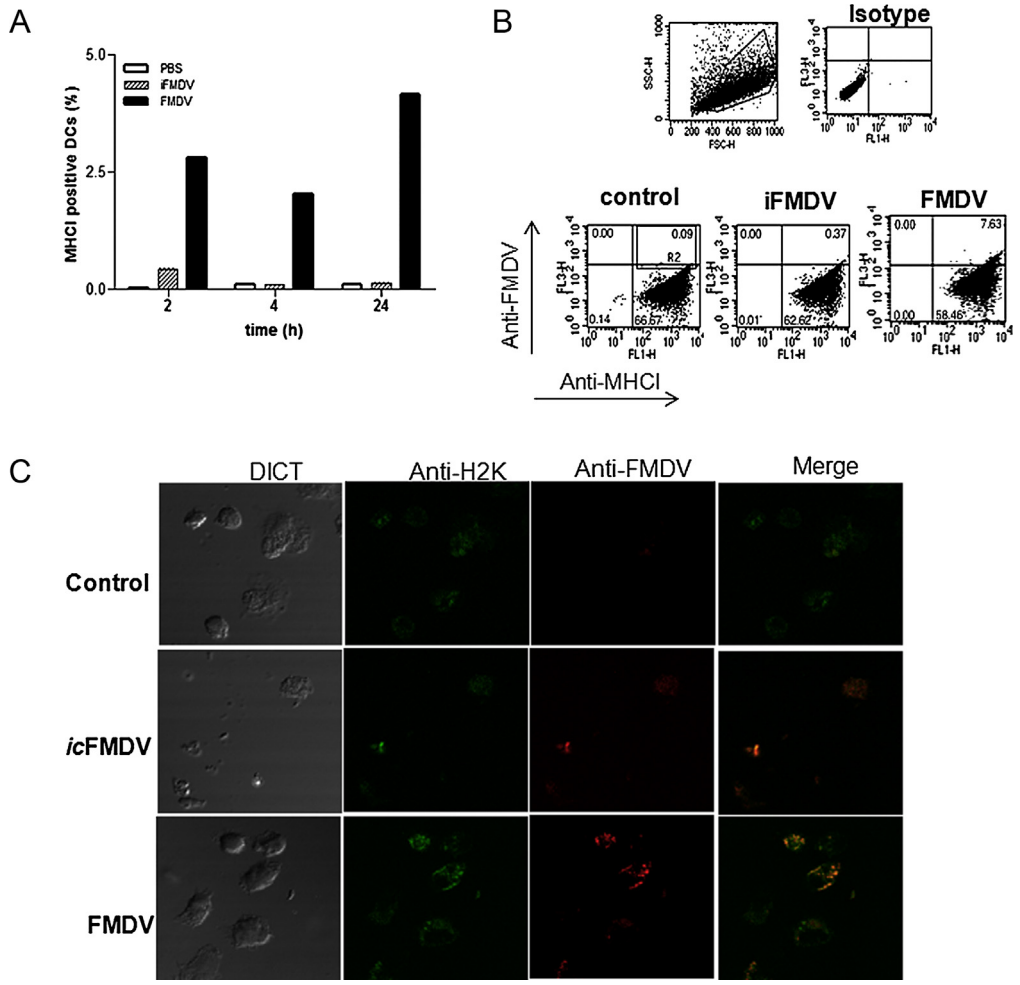

Fig. 5. FMDV increases the expression of MHC class I on DCs. (A) Kinetics of the expression of MHC class I on DCs treated with medium, iFMDV and FMDV by flow cytometry. Each bar represents the percentage of MHC class I associated with iFMDV or FMDV at the surface of DCs. (B) A representative dot plot of fluorescence at 24 h is shown. (C) Confocal analysis of DCs treated with or without FMDV (10 MOI) for 2 h. Cells were fixed and stained with $H2k^d$ (green) and with the anti-FMDV (red). In the middle panel, the intracitoplasmic colocalization at 4 h of $H2k^d$/anti-FMDV (icFMDV) in permeabilized DCs before staining is shown. Finally, in the lower panel, is observed the stained only on membrane surface (FMDV). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. FMDV internalization into DC involves LC3, LAMP-1 and LAMP-2 proteins

It was extensile demonstrated that FMDV interacts with autophagic LC3 protein in certain cellular lines [17,18]. However, the mechanisms involved on DCs entry are not well defined. We analyzed this fact on DCs. In the first place, the internalization of both particles was confirmed by confocal microscopy in purified DCs (Fig. 4A*i*). Then, we evaluated the expression of LC3 by western blot. As shown in Fig. 4A*ii*, the expression of LC3 protein was increased in cells infected with FMDV, which was corroborated by flow cytometry (4B). Furthermore, we studied the proteins related lysosomes Lamp-1 and Lamp-2. As observed in Fig. 4C, both Lamp-1 (C*i*) and Lamp-2 (C*ii*) were significantly increased in DCs infected with FMDV. Notably, iFMDV was not associated to any of both lysosomal membranes. Interestingly, when we assessed late lysosomal vacuoles by confocal microscopy, we showed an important colocalization of FMDV with Lamp-2 (Fig. 4D).

3.4. MHC class I presentation of FMDV by DCs

Previously, we demonstrated that infected DCs with FMDV induce production of IFN-γ by syngeneic lymphocytes [3,12]. It is known that IFN-γ is mainly responsible for sustaining the presentation through MHC class I. We decided to evaluate the membrane colocalization of viral particles and class I molecules at different times of DCs culture. As shown in Fig. 5(A) and (B) the percentages of MHC class I increased in infected DCs, being major the effect at 24 h post-infection. On the contrary, inactivated virus showed a moderate increase in the expression of MHC class I at DCs membrane only after 2 h of their co-culture. Finally, in confocal assays, we observed the colocalization of FMDV at membrane level and also in intracellular compartment of DCs 4 h after their co-culture (Fig. 5C).

Next, to study whether ERK1/ERK2 pathway was involved in this modulation, the presentation of MHC class I in presence of PD was analyzed. As shown in Fig. 6, DCs infected with FMDV in presence of the inhibitor abrogated the presentation of MHC class I, both in their expression by cell (A) as in the percentage of positive cells (B).

Finally, MHC class I presentation pathways were studied in splenic purified DCs. As shown, the inhibitors tested, ALLN and Epoxomicin, had not decrease MHC class I presentation by infected DCs (Fig. 6C). This observation supported the idea that proteasome would not participate in the generation of FMDV peptides.

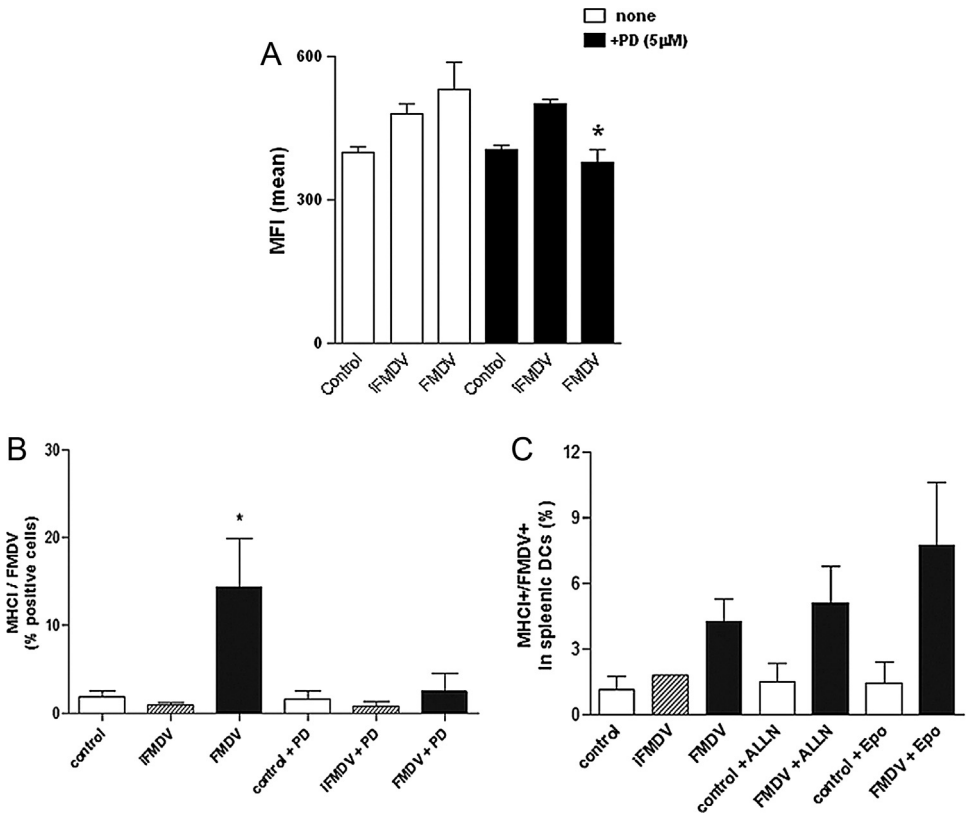

Fig. 6. Infection increases the presentation of FMDV on MHC class I on DCs. In DCs treated with medium, iFMDV and FMDV for 2 h and pre-treated with or without PD the expression of MHC class I was analyzed by flow cytometry. (A) The bar graphs represent the mean of fluorescence intensity (MFI). (B) Percentages of positive cells that co-express MHC class I and FMDV. Bars represent the mean ± SE of 11 experiments, $^*P < 0.05$. (C) Percentage of positive purified splenic DCs that co-express MHC class I and FMDV on DCs pretreated with or without ALLN or epoxomicin (Epo). Bars represent the mean ± SE of 4 experiments, $^*P < 0.05$.

3.5. FMDV induces the activation of in vivo cytotoxicity

Taking into account, that after FMDV infection their presentation on MHC class I was induced, we evaluated the modulation of a specific cytotoxicity (CTL). Splenocytes treated *in vitro* with or without FMDV and stained with CFSE at different concentrations, were injected to infected or naïve BALB/c mice. Finally, lysis of target cells was analyzed by cytometry. As shown in Fig. 7(A) and (B), FMDV induced a more vigorous specific CTL response *in vivo* compared to control mice. So, FMDV enables its presentation by DCs in MHC class I context which induces cytotoxicity against infected cells.

4. Discussion

It is known that FMDV interacts with DCs both in mice and in natural host [27,28]. However, the mechanisms involved in this interaction, are not well known. In the current study, we demonstrated that infectious virus induces phosphorylation of ERK1/2 MAPK in DCs. Notably, it was found for the first time that FMDV can induce DCs cellular death by the intrinsic pathway of apoptosis, through the activation of caspase-9 via ERK1/2 phosphorylation. Several stressors of leukocytes, such as virus, induced their apoptosis through the activation of PI3k/Akt and/or ERK1/2 [29,30]. Akt deactivation by the soluble VP1 protein of FMDV was demonstrated by Peng et al. [14]. Moreover, the authors demonstrated that this capsid protein acts as a potent pro apoptotic agent in a model of human cancer cells [31]. It should be note that, activation of Akt during viral infections is usually associated to type I IFNs secretion and viral replication [10,13]. By the contrary, any of these processes are produce in DCs after FMDV infection [12,32,33]. Robinson et al. [34] showed that bovine DCs are susceptible to infection with FMDV but their frequency is greater in the presence of neutralizing antibodies which correlates with induction of apoptosis, while inactivated virus did not induce it. We obtained similar results in relation to the low susceptibility to infection in DCs unlike inactivated particles, which could be due to the inactivation would modify certain epitopes facilitating their cellular entry [35]. However, this low viral frequency was sufficient to induce DCs apoptosis via the activation of caspase-9. We believe that induction of apoptosis would be responsible for the enhancement of cross-presentation and subsequent lytic activity [36].

Viruses, which use host machinery to replicate and to perpetuate, need the autophagy to entry cells. Several families of proteins are associated with autophagy, however only Atg and LC3 proteins were associated to FMDV [37]. In cellular lines was shown that FMDV utilizes autophagic pathway to replicate [38,39]. We demonstrated in DCs that FMDV are associated to protein LC3. Cellular viral infection with positive strand RNA causes reorganization of intracellular membranes in viral replication complexes. The origin of these membranes is not completely known, but the induction of the autophagy process is beneficial for the replication of poliovirus, and recently it has proved be advantageous for the replication of FMDV [38] in an epithelial line. This observation is based on the redistribution of molecules associated with autophagy, in particular, LC3 and Atg5, together with viral antigens. However, it is known that DCs internalization of FMDV progress to a non-replicative state [3]. This could indicate that autophagy is only required to virus entry, like as observed to negative-stranded viruses such as vesicular stomatitis and Influenza A viruses [40] which required the membrane remodeling to gain access to host. So, it was previously demonstrated by Berryman et al. [17] in CHO cells that FMDV induce the formation of

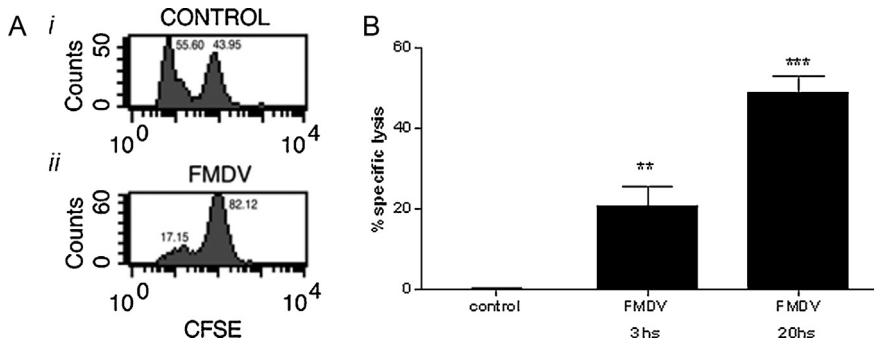

Fig. 7. Study of specific cytotoxicity. Briefly, two CFSE labeled cell populations (control and FMDV-infected splenocytes) in equal amount were inoculated on PBS immunized recipient mouse (control) or FMDV-infected recipient mice as was described in Section 2. After 3 or 20 h, CFSE labeled cells were recovered from spleens of treated mice. (A) Representative histograms indicate the intensity of CFSE recovered per treatment (control mouse histogram (i) and FMDV-infected mouse histogram (ii)). In each histogram all high intensity CFSE peaks (right) represent control target cells; while low intensity CFSE peaks (left) represent FMDV specific target cells. (B) The bar graph shows the percentage of specific cytotoxicity at indicated times. Results represent the mean ± SE, $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 6$.

autophagosomes to cell entry but this mechanism does not provide membranes for its replication. Interestingly, these authors showed that these structures could be induced also by UV-inactivated virus, contrary to observed for us. The infective virus progresses through the autophagic vesicles since it induces the accumulation of LC3-II, resulting of the lipid modification of cytosolic LC3-I [41] indicative of their maturation. The progression in the autophagy cycle could indicate the contribution of antigens to activate the adaptive immune response.

The MHC class I molecules reside in endosomal and lysosomal compartments of DCs [42]. FMDV association to early endosomes was demonstrated in epithelial cells and cellular lines [38,39,43], as its uptake by antigen presenting cells [3,34,44]. Our results demonstrated DCs internalization of both viral particles. Delivery of particles into early endosomal compartments was demonstrated by their association with Lamp-1, despite that infectious uptake is the minor grade, particles are observed also associated to Lamp-2 proteins, contrary to observe to iFMDV in correlation to demonstrate by Martín-Acebes et al. [44]. This delivery of FMDV in big percentages of DCs could offer a portion of antigen to couple to MHC class I molecules inducing the activation of a cytotoxic response. Reinforced by the fact, that both apoptosis and autophagy generate efficiently peptides to their delivery in MHC class I molecules [45], both functions were triggered by FMDV during its interaction with DCs. Furthermore, in our screening the use of proteasome inhibitors did not modify the expression of MHC class I molecules in the membrane of DCs after FMDV infection, indicating that viral antigens not result to the escape from autophagosomes to cytoplasm. Further experiments are needed to asses this point, as they were described proteasome independent mechanisms to generate peptides to class I molecules [46].

Finally, the potentiation by FMDV of class I presentation by DCs correlated with a specific cytotoxic response against virus, in agreement with different authors [8,47]. However, we did not showed induction of a specific TCD8+ response against inactivated virus. These contrasting results could be explained considering that the intracellular mechanisms that regulate the class I presentation in DCs involved the activation of ERK1/2, which was not activated by iFMDV.

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