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Foot-and-mouth disease virus causes a decrease in spleen dendritic cells and the early release of IFN-α in the plasma of mice. Differences between infectious and inactivated virus

C. Langellotti [a], V. Quattrocchi [a], C. Alvarez [b], M. Ostrowski [c], V. Gnazzo [a], P. Zamorano [a], M. Vermeulen [b,*]

[a] Instituto de Virología, Centro de Investigaciones en Ciencias Veterinarias, Instituto Nacional de Tecnología Agropecuaria (INTA)-Castelar, Buenos Aires, Argentina
[b] Laboratorio de Inmunología, Instituto de Inv. Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina
[c] Centro Nacional de Referencia del HIV (CNRS), Facultad de Medicina, Buenos Aires, Argentina

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ABSTRACT

Foot-and-mouth disease (FMD) is a highly contagious and acute viral disease of cloven-hoofed animals. From an economical point of view, it is the most important disease of livestock worldwide. It is known that the virus interacts with dendritic cells, both in the natural host and in mice, but the impact of this interaction on the adaptive immune response is controversial. Currently available vaccines are based on inactivated forms of the FMD virus. Little is known about the differences between infectious and inactivated virus, in terms of dendritic cell subsets involved in immune response activation. The present work, which was carried out in the murine model, shows that live virus infection induces a reduction in splenic dendritic cell subsets. In addition, lymphocyte proliferation is inhibited in the early stages of infection associated with IFN-α induction, but is restored to normal values 5 days post-infection when pro-inflammatory cytokines was produced. In contrast, the inactivated virus increases the percentage of plasmacytoid dendritic cells in the spleen and the production of IL-10, which triggers the activation of a T regulatory response.

1. Introduction

Foot-and-mouth disease (FMD) represents one of the most devastating diseases of cloven-hoofed animals around the world due to the large economic losses it causes (Zhang and Alexandersen, 2004). FMD is caused by FMD virus (FMDV), a highly contagious member of Picornaviridae family. FMDV causes fever and formation of vesicles in soft tissues such as tongue, lips, and teats and also around the coronary bands (Alexandersen et al., 2003; Díaz-San Segundo et al., 2006; Domingo et al., 2002; Grubman and Baxt, 2004). The infection in the natural host induces a quick neutralizing humoral response detectable in serum 3–4 days after infection, which is followed by the clearance of immune virus–antibody complexes through phagocytes (Summerfield et al., 2009).

In a similar way as what happens in natural hosts, FMDV experimental infection in mice induces thymus-independent (TI) protection, with the production of neutralizing antibodies, which rapidly eliminate the virus from the body (Borca et al., 1986; López et al., 1990). A remarkable feature of this response is the high expression of IgG3 isotype neutralizing antibodies (Batista et al., 2010). In contrast, inoculation of mice with inactivated virus elicits a typical thymus-dependent (TD) response, which requires T cell collaboration to induce an antibody response. Consequently, the time required to trigger a protective response is longer for inactivated than for infectious virus (Ostrowski et al., 2005).

Dendritic cells (DC), as antigen presenting cells (APC), catch the antigen (Ag) in peripheral tissues and migrate to lymph nodes to present it and to activate naïve T-lymphocytes, such as mature DC (Merad and Manz, 2009). The mouse spleen DC consist of two populations $CD11^+B220^+$ plasmacytoid DC (pDC) and $CD11c^+B220^-$ conventional DC (cDC). The cDC are subdivided into $CD4^+$ cDC, $CD8\alpha^+$ cDC, and $CD8\alpha^-CD4^-$ cDC subtypes (Liu and Nussenzweig, 2010; Steinman and Banchereau, 2007) in the steady state. These DC have different immune functions. $CD4^+$ cDC do not produce cytokines, but they effectively present antigens (Ags) to CD4 T cells (Liu and Nussenzweig, 2010). The $CD8\alpha^+$ cDC perform cross-presentation of

Abbreviations: FMDV, foot-and-mouth disease virus; iFMDV, inactivated FMDV; pDC, plasmacytoid dendritic cells; Treg, T regulatory lymphocytes; IFN-α, interferon-alpha; MFI, mean fluorescence intensity.

* Corresponding author. Address: Departamento de Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina.
E-mail address: mvermeulen@hematologia.anm.edu.ar (M. Vermeulen).

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foreign Ags to CD8 T cells and are the major producers of IL-12, while $CD8\alpha^{-}CD4^{-}$ cDC produce IFN-γ (Belz et al., 2002; León et al., 2004).

It is known that FMDV can infect DC leading to the synthesis of viral proteins, although this interaction is usually abortive in terms of virion production (Bayry and Tough, 2006; Ostrowski et al., 2005; Summerfield and McCullough, 2009). Despite this, the interaction of the virus with DC both in mice and pigs (Díaz-San Segundo et al., 2009; Guzylack-Piriou et al., 2006; Ostrowski et al., 2005) leads to the modification of the DC maturation status, reflecting its ability to modulate the adaptive immune response. Several authors have reported that FMDV infection of murine DC inhibits or does not affect the expression of key molecules involved in the activation of naïve T-lymphocytes (Bautista et al., 2005; Ostrowski et al., 2005; Summerfield et al., 2003), thereby preventing their maturation. In disagreement with this view, Guzylack-Piriou et al. (2006) reported that FMDV induces the activation of porcine pDC, through the recognition of virus–antibody complexes by pDC Fc gamma receptor II (RFcγIIR), leading to the secretion of large amounts of interferon alpha (IFN-α).

It has been previously demonstrated that DC are susceptible to infection with FMDV 01 Campos in vitro, although the viral replication is abortive, inducing a suppressive phenotype associated with a down-regulation of CD40 and CD86 molecules and the induction of IL-10 production (Ostrowski et al., 2005).

According the above evidence, in this work we decided to assess whether FMDV, depending on their activation status was able, in the first place, to modulate splenic DC subsets after challenge of live of inactivated virus and finally how this interaction would influence the subsequent T lymphocyte responses.

2. Materials and methods

2.1. Mice and viruses

All experiments were carried out using 2-month-old virgin male BALB/c mice purchased from Universidad Nacional de La Plata (Buenos Aires, Argentina). Mice were kept at 20 ± 2 °C under an automatic 12 h light–dark schedule. Animal care was in accordance with institutional guidelines.

Infectious FMDV, 01 Campos serotype, was provided by the National Service of Animal Health, Argentina (SENASA). FMDV was obtained from vesicles of experimentally infected cattle with passages onto BHK-21 cells. Inactivated FMDV (iFMDV) was provided by Biogénesis-Bagó, Argentina. The binary ethylendiamine (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.

2.2. Infection or immunization of mice

BALB/c mice ($n = 6$ in each group) were intraperitoneally (i.p.) injected with 10^5 50% cell culture infective doses ($CCID_{50}$) of infective 01 Campos serotype or 3.5 μg/ml of iFMDV of the same serotype (corresponding to 10^5 $CCID_{50}$). Both particles were dissolved in phosphate-buffered saline (PBS).

All experiments involving infectious virus were performed in SENASA and INTA biosafety level 3A facilities.

Mock-infected control (CT) mice ($n = 6$) were inoculated intraperitoneally (i.p.) with the supernatant of uninfected BHK-21 cell cultures.

2.3. Treatment of spleen tissues to obtain a cellular suspension

At 1 or 3 days post-inoculation (dpi), six spleens per group (in each experiment) were harvested from mice and cut into small pieces, pooled and treated with Type I collagenase (250 U/ml) (Roche, Buenos Aires, Argentina) for 30 min at 37 °C. The reaction was stopped by addition of PBS supplemented with 5% fetal calf serum (FCS, GIBCO). Subsequently, spleen pieces were incubated with DNase I (50 U/ml) (Invitrogen, Buenos Aires, Argentina) for 40 min at 37 °C. Finally, cell suspensions were collected through a gauze mesh and washed with cold PBS.

2.4. Isolation of dendritic cells

DC were purified from spleen cell suspensions obtained as mentioned in Section 2.3 using MACS separator in accordance with the manufacturer's protocols (Miltenyi Biotec, Argentina). DC were purified by positive selection using magnetic beads coupled to anti-CD11c and anti-mPDCA-1 antibodies.

2.5. Flow cytometry

Cell staining was performed using the following monoclonal antibodies (mAbs), conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC): anti-CD11c, anti-CD8α, anti-GR1, anti-B220, anti-CD8, anti-CD4, anti-Foxp3, anti-CD25, anti-CD86 and anti-MHC II (BD Pharmingen, San Diego, CA). The data were collected using a FACSCalibur (Buenos Aires, Argentina) flow cytometer and analyzed with CELLQUEST program (BD Biosciences, Buenos Aires, Argentina).

2.6. Intracellular cytokine staining

Purified DC were stimulated or not for 18 h with FMDV in the presence of brefeldin A (5 μg/ml) (BD Golgi Plug, Pharmingen, Argentina). Cells were stained for cell surface markers with APC-conjugated anti-CD11c antibodies (BD Pharmingen, Argentina). After washing, cells were suspended in PBS and fixed with 0.5% paraformaldehyde, permeabilized with 0.1% saponin in PBS (Sigma, Argentina). Then, cells were incubated with PE-conjugated antibodies directed to IL-6, IL-10, TNF-α or isotype-matched control antibodies (BD Pharmingen, Argentina) for 30 min. Stained cells were washed twice with 0.1% saponin buffer, finally suspended in PBS, and analyzed by flow cytometry (BD Biosciences, Argentina).

2.7. Analysis of cell-death

Late apoptosis or necrosis of DC (Díaz-San Segundo et al., 2009) was determined using the fluorescent DNA-binding probe, 7-Amino-actinomycin D (7-AAD; a DNA intercalator) (BD Pharmingen, Argentina) following the manufacturer's instructions. Briefly, cells were centrifuged and resuspended in 100 μl of PBS in the presence of 5 μl 7-AAD (0.25 $\mu g/10^6$ cells). After 20 min of incubation at 4 °C in the dark, cells were collected by centrifugation, and washed once with ice-cold 1X PBS. Then, 1% paraformaldehyde solution containing 5 μl/ml Actinomycin D was added to the cell pellet to arrest further division. Cell samples were analyzed by flow cytometry.

2.8. Determination of cytokines

Cytokine levels in supernatants of DC cultures, co-cultures of DC and splenocytes or plasma were measured by ELISA. IFN-γ and IL-10, (Ebiosciences, San Diego, CA) and IFN-α (PBL Biomedical Laboratories, Leiden, Holland) were performed according to the manufacturer's protocols. The limits of detection were 4 pg/ml for IL-10, 10 pg/ml for IFN-γ and 12.5 pg/ml for IFN-α.

2.9. In vitro CFSE proliferation assay

DC-depleted splenocytes, were labelled with carboxylfluorescein diacetate succinimidyl ester (CFSE; 5 nM) (BD biosciences) for 40 min at 37 °C. Cells were washed four times and re-suspended in RPMI medium. Purified DC were incubated with splenocytes at a 1:5 ratio for 96 h. At different time points (24, 48, 72 and 96 h), total cells were washed in cold PBS, fixed by resuspension in 0.2% paraformaldehyde in isoflow (BD, Buenos Aires, Argentina) and analyzed by cytometry.

2.10. Statistical analysis

Differences between means were analyzed using Students' *t*-test, and values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Differential subsets of DC induced in spleen of mice by inactivated or infectious foot-and-mouth disease virus

FMDV is able to infect DC (Summerfield et al., 2009) affecting their maturation (Guzylack-Piriou et al., 2006; Harwood et al., 2008; Ostrowski et al., 2005; Summerfield et al., 2003). As the aim of this work was to assess whether FMDV is able to interact with DC in a differential way, BALB/c mice were inoculated with infectious or inactivated FMDV. Mock-infected mice were used as controls (CT). After 3 days, spleens were collected and DC were purified using magnetic beads coupled to anti-CD11c/anti-mPDCA-1 antibodies, as described in Section 2.4. The different DC subsets were analyzed by flow cytometry. As shown in Fig. 1(A and B), iFMDV injection increased the number of pDC ($CD11c^+/B220^+$), while the infection caused a small but significant decrease ($P < 0.5$) in this population, as compared to the CT. This effect was more pronounced when it was compared with the levels obtained with the inactivated virus. To determine whether the observed changes in the proportions of pDC between inactivated and infectious virus reflect differences in the time at which they are recruited from peripheral blood, pDC were analyzed at 1 and 3 days post-inoculation (dpi). As shown in Fig. 1C, at 1 dpi, the percentage of blood pDC, was slightly higher with infective than with inactivated virus. At 3 dpi, on the other hand, while the number of blood pDC was reduced in the iFMDV group, consistent with the observed increase in the spleen, the FMDV group showed similar values to those of the CT.

When evaluating myeloid DC ($CD11c^+/CD8\alpha^-$), both infectious and inactivated virus significantly reduced its numbers compared to the CT. However, this effect was more pronounced in the FMDV group (Fig. 2A and B). Finally, as shown in Fig. 2A and C, FMDV caused a significant reduction of lymphoid DC ($CD11c^+/CD8\alpha^+$), which are responsible for the activation of cytotoxic T lymphocytes in mice (Idoyaga et al., 2009). These results suggest that the immune mechanisms triggered by the inactivated virus are significantly different from those elicited by infection.

Maturation of DC is associated with the up-regulation of costimulatory and MHC class II molecules, which are involved in

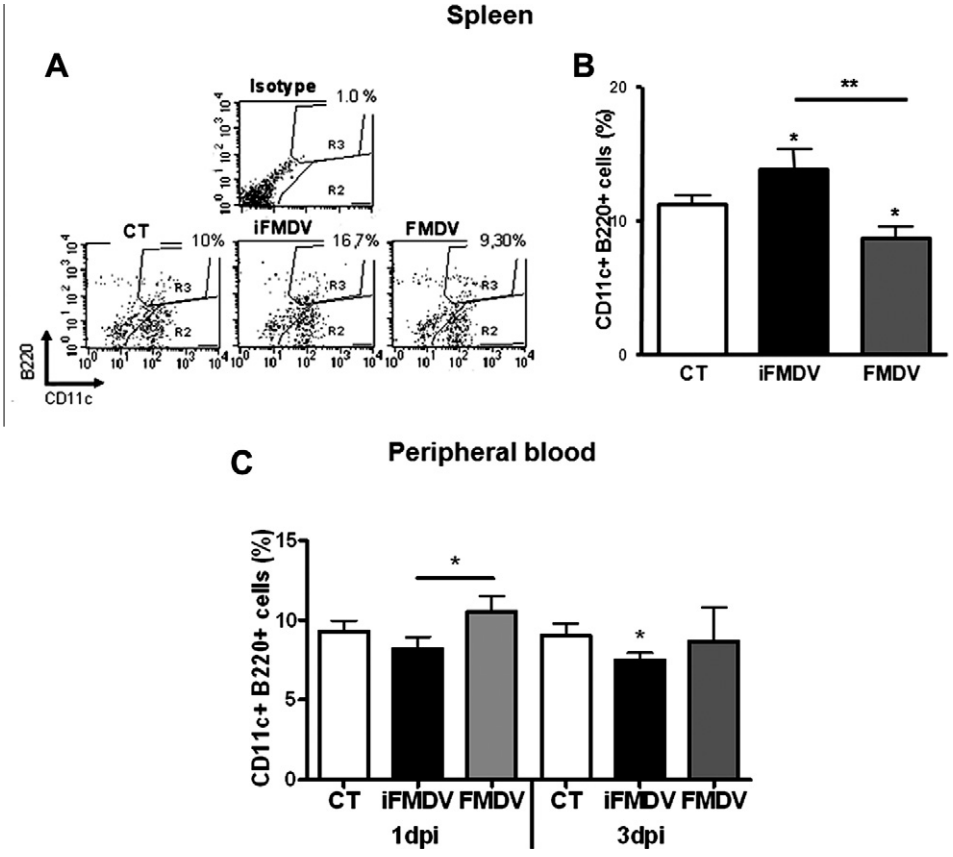

Fig. 1. Variations in the recruitment of plasmacytoid DC between mice injected with infectious or inactivated FMDV. Subsets of splenic DC from FMDV, iFMDV or mock-infected control (CT) mice were evaluated by cytometry. (A) A representative experiment for pDC is shown. (B) Results are expressed as the percentage of $CD11c^+$ $B220^+$ DC and represent the mean ± standard error of mean (SEM) of six experiments. In (C) results are expressed as the percentage of peripheral blood pDC ($CD11c^+$ $B220^+$ cells) at 1 and 3 days post-inoculation (dpi), respectively, and represent the mean ± SEM of six experiments. Asterisks indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$).

the activation of naïve T lymphocytes (Reis e Sousa, 2006; Steinman and Hemmi, 2006; Villadangos et al., 2005). In Fig. 2D, it can be observed that neither infectious nor inactivated virus significantly modified the levels of CD86 expression. In contrast, both viral particles induced the down-regulation of MHC class II molecules at 3 dpi.

3.2. Foot-and-mouth disease virus induces an early death of spleen dendritic cells

As shown above, the infection causes a rapid and significant decrease of spleen DC, and given that FMDV is highly cytopathic in many cell lines (Escarmís et al., 2008; Lay et al., 2010; Pejawar et al., 2005), the viability of purified DC was assessed after infection with FMDV by 7-AAD staining, an indicator of cell death. Fig. 3A shows that, the infection induced the death of spleen CD11c$^+$ cells both at 5 and 24 h post-infection (hpi). Interestingly, splenic pDC was the most affected subset with respect to viability upon FMDV infection at 1 dpi (B). Nevertheless, the virus did not induce death of CD11$^+$ cells in peripheral blood at this time point (C).

3.3. Cytokine induction by iFMDV and FMDV in spleen dendritic cells

pDC are the main source of IFN-α (Guiducci et al., 2009; Szabo and Dolganiuc, 2008), while conventional DC define the profile of polarization of T lymphocytes (Belz et al., 2002; Liu and Nussenzweig, 2010). Taking this into account, as well as the observed differences between infectious and inactivated virus in splenic DC, the pattern of cytokines induced in this population was evaluated. To this end, mice were injected with iFMDV, FMDV or CT. At 3 dpi, CD11c$^+$ DC were purified from spleens and cytokines were evaluated by intracytoplasmic staining. Fig. 4A shows that while the inactivated virus increased the production of the pro-inflammatory cytokines IL-6 and TNF-α, by CD11$^+$ DC the infection induced only poor levels of IL-6. An interesting fact was that iFMDV, but not FMDV, significantly induced the production of IL-10 by DC (Fig. 4A). Finally, production of IFN-α, the main cytokine involved in immunosuppression in acute viral infections, was evaluated in DC. As shown in Fig. 4B, the levels of IFN-α were not affected by the infection being similar to those observed for the control and inactivated virus. In contrast, levels of this cytokine were significantly increased in plasma of mice 1 dpi but not at day 3 (Fig. 4C). These results allow inferring that inactivated and infectious viruses differentially modulate the immune response. While iFMDV induces a rapid inflammatory response mediated by the release of the pro-inflammatory cytokines TNF-α and IL-6 by DC, the early immunosuppression resulting from the infection might be mediated by IFN-α.

3.4. iFMDV and FMDV induce differences in lymphocyte populations

Since a differential pattern of cytokines was induced in DC by inactivated or infectious FMDV, the impact of these treatments

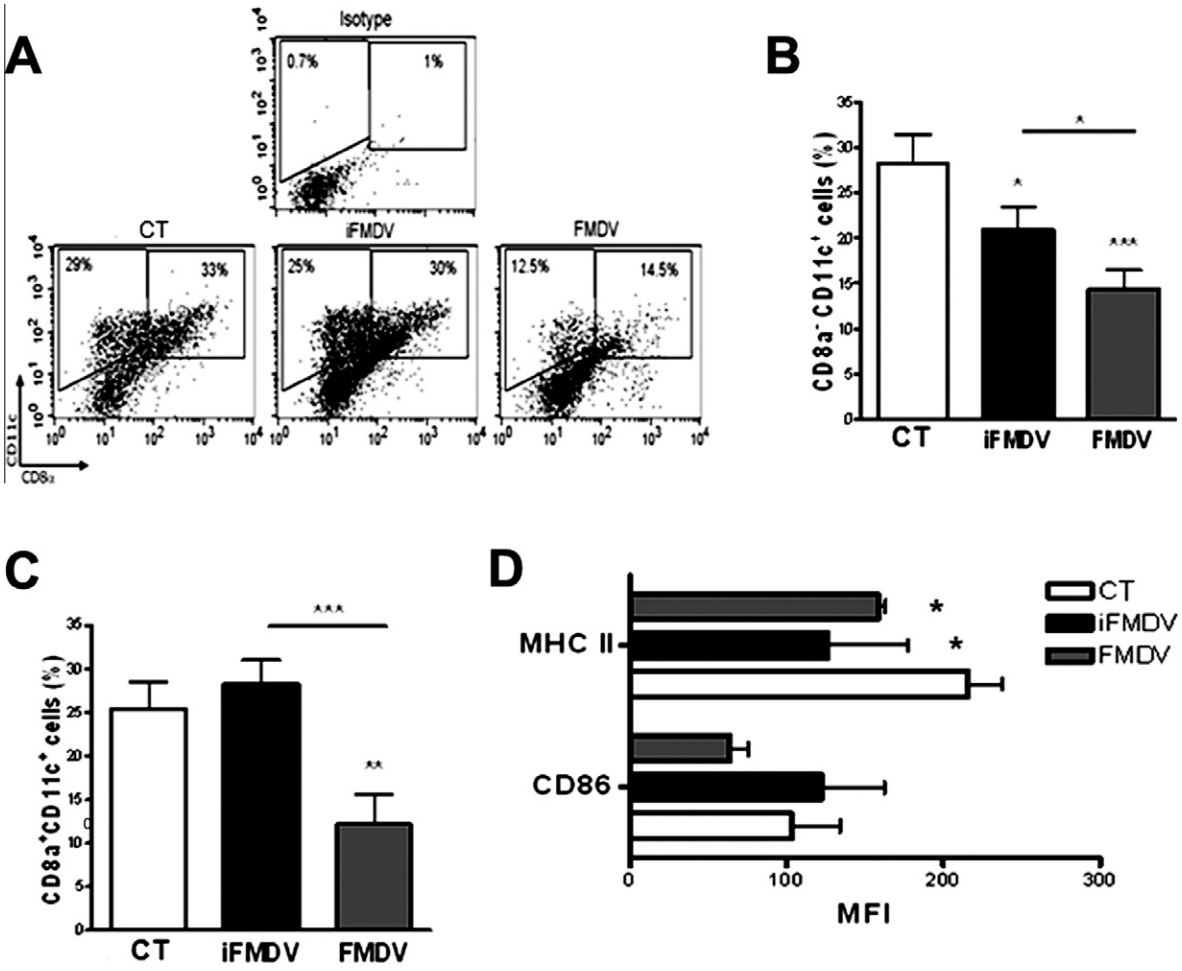

Fig. 2. FMDV diminished conventional DC in spleen of mice. (A) A representative experiment of splenic myeloid DC (mDC, CD11c$^+$ CD8α$^-$ cells) and lymphoid DC (LDC; CD11c$^+$ CD8α$^+$ cells) obtained by cytometry is shown. (B and C) Results are expressed as percentages of mDC and LDC, respectively, and represent the mean ± SEM of seven experiments. In (D) the surface expression of MHC class II and CD86 molecules on purified splenic DC were analyzed by cytometry. Results are expressed as the mean of fluorescence intensity (MFI) values and represent the mean ± SEM of seven independent mice. Asterisks indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

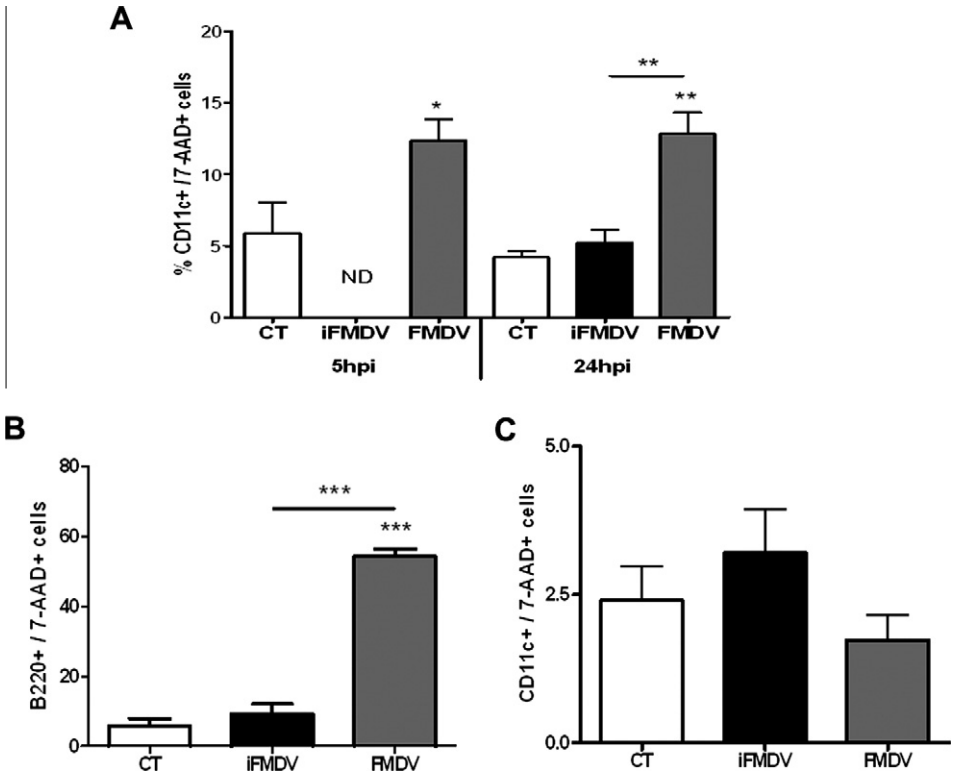

Fig. 3. Infection affects the viability of plasmacytoid dendritic cells. After 5 or 24 h post-infection (hpi) cellular death were measured in purified splenic DC from iFMDV, FMDV and CT mice by cytometry (A). In (B) splenic CD11c$^+$B220$^+$ 7-AAD$^+$ cells at 1 dpi are represented. Results are expressed as the percentages of 7-AAD$^+$ splenic DC (B) or peripheral DC (C) and represent the mean ± SEM of six or three experiments, respectively. Results represent the mean ± SEM, $N = 3$, $^*P < 0.05$.

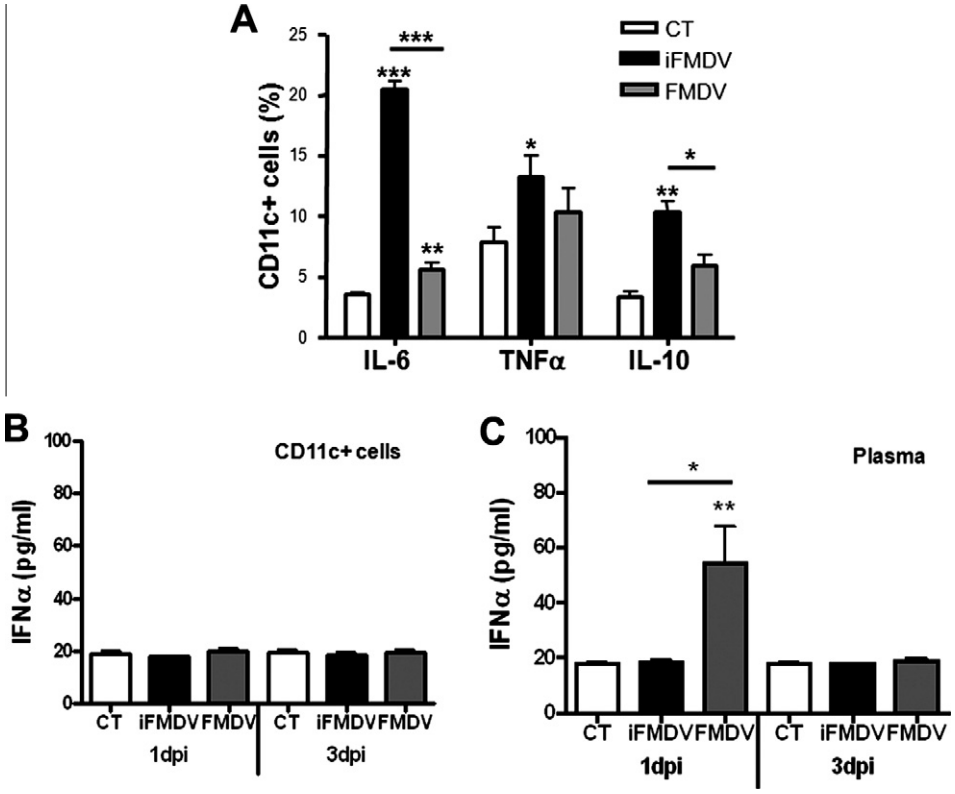

Fig. 4. Dendritic cells from infective or inactivated FMDV induce a different profile of cytokines. In (A) the percentages of IL-6, TNF-α and IL-10 in purified CD11c$^+$ cells were determined by cytometry. Data are expressed as the percentage of positive DC for the production of each of the cytokines evaluate. The production of IFN-α was analyzed in the supernatants of splenic CD11c$^+$ cells (B) or in plasma (C) from iFMDV, FMDV and CT mice. Histograms represent the concentration values (pg/ml) and correspond to the mean ± SEM of 4 (B) and 7 (A and C) experiments. Asterisks indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

was further evaluated on splenic T lymphocytes. At 3 dpi, spleens were harvested from mice and the negative population obtained after positive selection of $CD11c^+$ cells was stained and analyzed by cytometry. Fig. 5(A and C) shows that while in the FMDV group the percentages of T $CD8^+$ lymphocytes were slightly reduced, as compared to the CT group, this population was increased in the iFMDV group.

On the other hand, when the percentages of $CD4^+$ T cells were analyzed (Fig. 5B and C), no alterations were found in this population after treatment. Unexpectedly, regulatory T cells ($CD4^+CD25^+$ $Foxp3^+$) were increased in the spleens of mice injected with the inactivated form of the virus, as shown in Fig. 6(A and B). This could indicate that after a rapid activation of the inflammatory response, the inactivated virus causes inhibition of T effector response.

3.5. Infection increases lymphocyte proliferation

It has been reported that regulatory T cells can inhibit the proliferation of helper T lymphocytes (Campbell and Koch, 2011). Since, as mentioned above, both infectious and inactivated virus down-regulate the expression of MHC class II molecules, we assessed the ability to FMDV to prime T cell response. To this aim, purified DC obtained from the spleens of injected mice at 3 dpi were co-cultured with autologous splenocytes at a DC/splenocyte ratio of 1:5, stained with CFSE dye (5 nM) and finally incubated for several days as described in Section 2.9. As shown in Fig. 7A and B, DC from infected mice were able to induce proliferation of autologous lymphocytes after 72 h of co-culture, while no proliferation was observed during the first 48 h. In contrast, DC from mice injected with iFMDV inhibited the proliferation of splenocytes. Finally, cytokines induced in the supernatants (SN) were evaluated after 48 h of DC/splenocyte co-culture. Consistent with previous results, we demonstrated that infection strongly increased the release of IFN-γ and to a lesser extent of IL-10 (Fig. 7C). Moreover, IL-10 was only detectable in SN of cocultures of mice injected with iFMDV, resulting from both DC production and T regulatory lymphocytes. This result is consistent with the observed inhibition of the proliferative response and the proposed induction of a regulatory T profile by the inactivated virus.

4. Discussion

The results presented in this work showed marked differences in the induced immune response elicited by infectious and inactivated forms of the FMD virus, in an experimental murine model. Thus, total spleen DC were reduced 3 days after injection with live FMDV, since both pDC and conventional DC decreased as compared to the control group. A possible explanation could be that FMDV induces the apoptosis of DC (Clarke and Tyler, 2009; Galluzzi et al., 2010). In many cases, viral infections (Influenza A, reovirus, dengue, hepatitis C, Ebola) trigger host cell death as an escape mechanism (Kaminskyy and Zhivotovsky, 2010; Shrivastava et al., 2011; Tomlins and Storey, 2010). We found that early infection induced a low but significant percentage of cellular death in $CD11c^+$ cells that particularly affected the splenic pDC. However, at 1 dpi, peripheral blood DC were not affected, while pDC values found in spleen and peripheral blood were similar at this time point. Accordingly, Nfon et al. (2010) previously showed a transient decrease of peripheral pDC in pigs infected with several serotypes of FMDV, which was associated with the induction of lymphopenia.

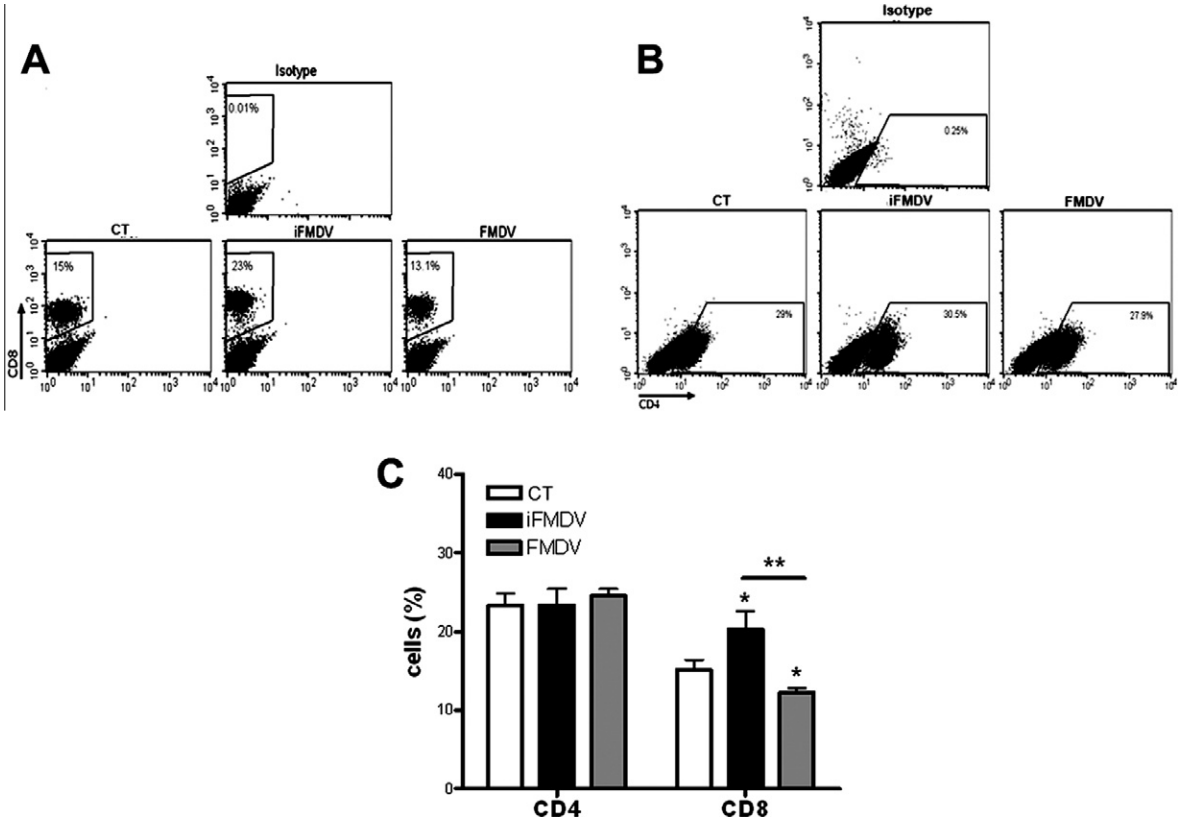

Fig. 5. T lymphocyte population is not affected by FMDV. Mice were injected with iFMDV (3.5 μg), FMDV (10^5 $CTCID_{50}$) or were mock-infected (CT). Three days later, spleens were removed and purified with an anti-CD11c/anti-PDCA-1. The percentages of T lymphocytes in the negative fraction were evaluated by cytometry. In (A and B), representative experiments ($n = 11$) corresponding to $CD8^+$ and $CD4^+$ T lymphocytes, respectively, are shown. In (C), results are expressed as the percentage of $CD4^+$ and $CD8^+$ cells and represent the mean ± SEM for 11 experiments. Asterisks indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^*P < 0.001$).

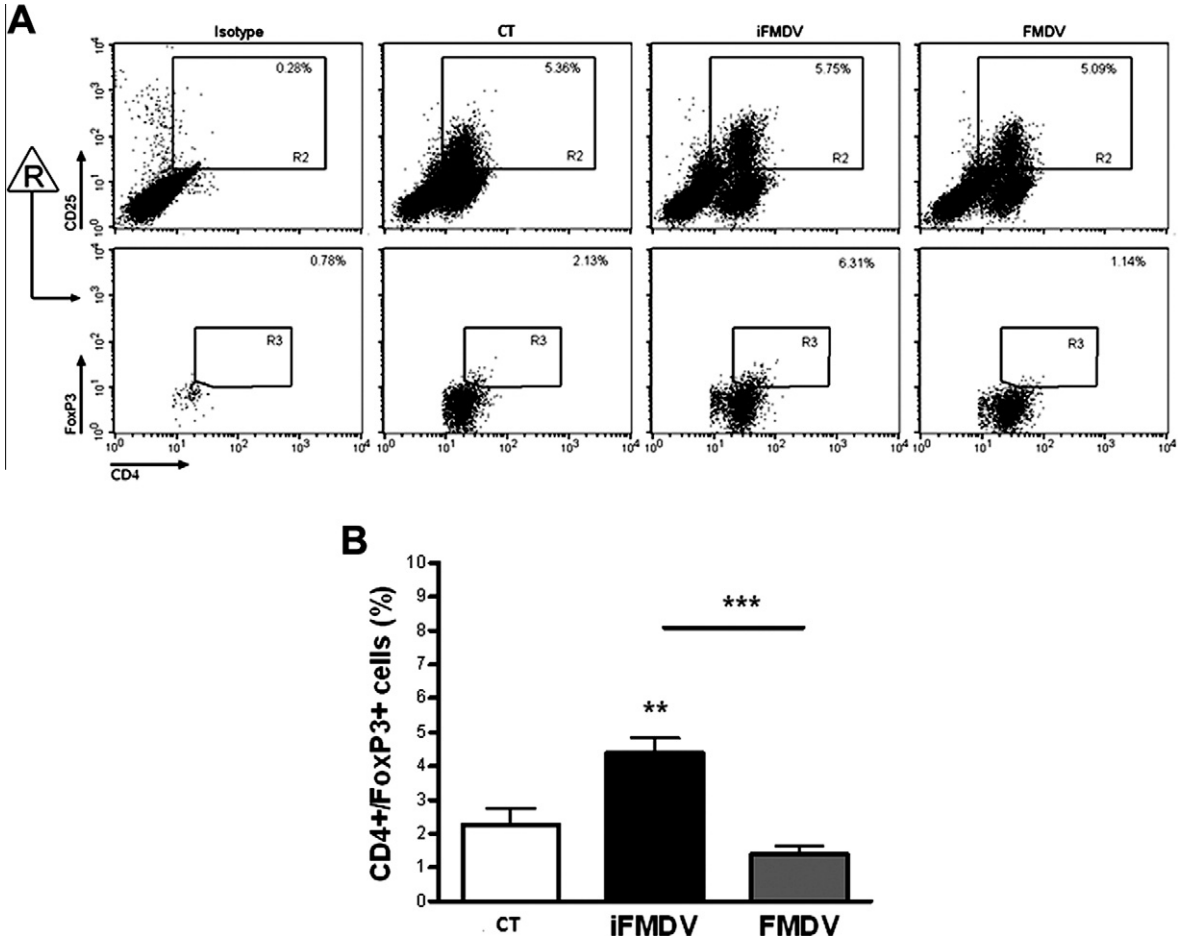

Fig. 6. Vaccination increases regulatory T lymphocytes. The proportions of regulatory T lymphocytes ($CD4^+CD25^+Foxp3^+$) were analyzed in the spleens of mice at 3 dpi. In (A), a representative experiment of 5 repeats is shown. (B) Percentages of $CD4^+CD25^+Foxp3^+$ cells, corresponding to the mean ± SEM of five experiments. Asterisks indicate statistical significance ($^*P < 0.05$, $^{**}P < 0.01$, $^*P < 0.001$).

By contrast, the present work shows that *in vivo* injection of inactivated virus caused a significant up-regulation of splenic pDC without modifying the amounts of conventional DC (myeloid and lymphoid subsets). In line with previous studies in natural hosts (Díaz-San Segundo et al., 2009; Golde et al., 2008; Reid et al., 2011), we could not detect IFN-α in spleen DC from infected mice. In this sense, it has been described that IFN-α induction requires the formation of immune-complexes (Guzylack-Piriou et al., 2006; Summerfield et al., 2009) or the presence of live viral particles (Bautista et al., 2005). We believe that, besides to the rapid elimination of virus in mice (Ostrowski et al., 2005), the most likely explanation for the lack of IFN-α production by murine pDC is the tropism displayed by FMDV that leads to selective death of this particular subset of DC, the main source of type I IFNs.

FMDV infection does not interfere with the ability of conventional DC to activate T lymphocytes, as evidenced by the allostimulatory reaction observed. However, consistent with previous results (Nfon et al., 2010; Ostrowski et al., 2005), we found a transient immunosuppression of T cell proliferation during the first 48 h of culture. These observations support the fact that the infection-induced production of IFN-α by cells other than pDC could be responsible for the suppression observed in the early stages of FMDV infection. Moreover, it is not possible to exclude that, after exposure of mice to the FMDV, splenocytes produced significant levels of IFN-γ and IL-10, cytokines with known immunosuppressive effects, also associated to FMDV infection (Díaz-San Segundo et al., 2009; Ostrowski et al., 2005, 2007). In our model, we assume these cytokines do not participate in immunosuppression since the peaks of IFN-γ and IL-10 appear at 48 h of DC/splenocyte co-culture and coincide with the activation of T cell response.

Indeed, the lymphoid DC subset is responsible for priming $CD8^+$ T cells against different viruses as well intracellular bacteria (Belz et al., 2004; Neuenhahn and Busch, 2007). Therefore, depletion of splenic $CD8\alpha^+$ DC induced by FMDV infection of mice could be associated with a reduction of $CD8^+$ T lymphocytes. Experiments are in progress to determine if increased IFN-γ levels could explain the late activation of this lymphocyte population. In this regard, the late activation of specific effector and memory cytotoxic T $CD8^+$ lymphocytes following FMDV infection has been demonstrated in cattle (Guzman et al., 2008; Childerstone et al., 1999). Unexpectedly, we found that FMDV does not interfere with the ability of conventional DC to activate T lymphocytes. After 48 h of in vitro culture, purified DC obtained from infected mice were able to present viral antigens to autologous splenocytes and activate the adaptive immune response, which strongly suggests that FMDV is internalized by DC (Díaz-San Segundo et al., 2009; Ostrowski et al., 2005; Summerfield et al., 2009). Taking into account that FMDV has cytopathic effects on murine pDC, it cannot be ruled out that conventional DC perceive this death as a "danger signal", resulting in the incorporation of cellular material and viral antigens through the cross-presentation pathway (Belz et al., 2004).

Because it was demonstrated that IFN-α is capable of activating myeloid DC to prime B lymphocyte and induce the acquisition of

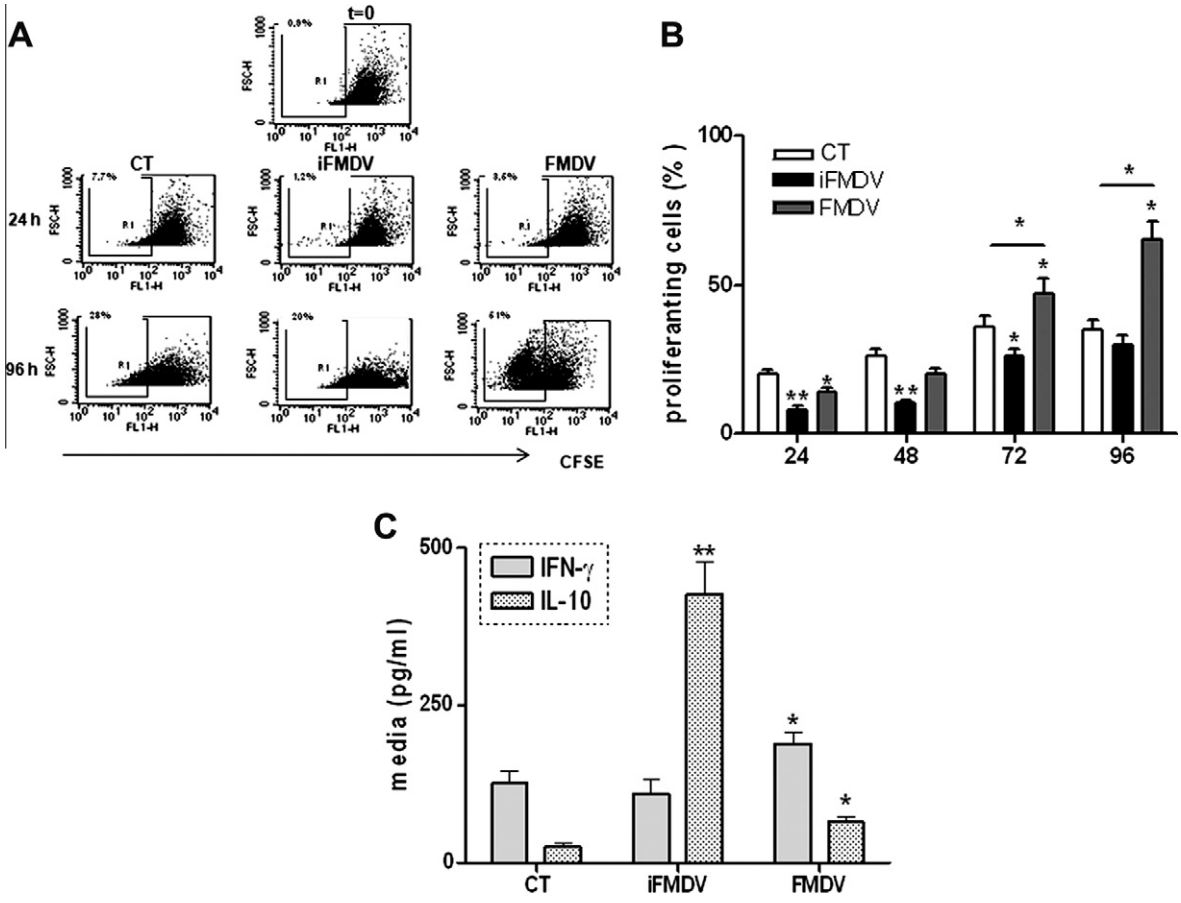

Fig. 7. FMDV stimulates the proliferation of lymphocytes. Purified DC of iFMDV, FMDV or CT mice were cultured with CFSE-stained splenocytes at a ratio of 1:5. (A) Representative dot plots at 24 and 96 h are shown. (B) Histogram bar represents the percentage of proliferating cells at different time points of culture. Results are expressed as mean ± SEM of six experiments. (C) IFN-γ and IL-10 production in co-cultures was analyzed in the supernatants by ELISA. The bars represent the mean ± SEM of four experiments. The figure shows mean concentration values (pg/ml). Asterisks indicate statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$).

the memory phenotype via inflammatory cytokines (Jego et al., 2005). It is likely that FMDV is rapidly eliminated by the activation of T-independent neutralizing antibodies. Then, at later stages of infection, the virus is capable of promoting the development of a T-dependent humoral response and immunological memory, through the induction of IL-6/IL-10/IFN-γ cytokines (Childerstone et al., 1999; Díaz-San Segundo et al., 2009).

The failure of BEI-inactivated FMDV to stimulate T lymphocyte proliferation can be associated with the production of IL-10 by DC, which mediates an immunosuppressive effect, resulting in a decreased maturation and of T lymphocyte activation (Frick et al., 2010; Rutella et al., 2004). It is known that T-cell primed in the presence of immature DC or IL-10 treated-DC (Moore et al., 2001) evoke a T regulatory response. Similarly, the increase in the percentage of pDC in the spleen of vaccinated mice could also elicit the development of tolerance mechanisms (Adema, 2009; Kuwana, 2002; Sakaguchi, 2004). We show for the first time that injecting mice with inactivated form of FMDV induces the expansion of $CD4^+CD25^+Foxp3^+$ T lymphocytes. Although splenic $CD8^+$ T lymphocytes were increased 3 days post-injection, they were unable to produce IFN-γ.

A common problem in the design of antiviral vaccines is their inability to elicit a protective response mediated by IFN-γ (Kimman et al., 2009). In our model, this inability to secrete IFN-γ, plus the fact that DC produced higher levels of IL-10 could favor the induction of a T regulatory profile and the subsequent inhibition of an effector T response. Recently, LeRoith et al. (2011), analyzed the effect of a FMDV vaccine against porcine reproductive and respiratory syndrome (PPRS) in pigs and found an induction of regulatory T cells associated with exacerbation of this pathology. Here, we demonstrated that iFMDV rapidly induces the genesis of an inflammatory response through the secretion of IL-6 and TNF-α by DC. Also, the inactivated virus increases pDC in spleen tissues and the production of IL-10. We propose that the induction of a regulatory profile observed in our model is a mechanism to prevent deleterious effects mediated by the activation of $CD8^+$ T lymphocytes. In this sense, we demonstrated the induction of $CD4^+$ T regulatory cells, which could mitigate the adverse effects of cytotoxic lymphocytes during viral infections (Fogle et al., 2010; Myers et al., 2009).

Our results demonstrate that modulation of the immune response is very different between infectious and inactivated virus in a mouse model. Infected murine DC induce early and transient immunosuppression. After this first encounter with the active virus, DC are able to induce the proliferation of T lymphocytes and activate the adaptive immune response, resulting in the development of immunological memory. In fact, the generation of high titers of specific antibodies for long periods is required for the induction of memory (López et al., 1990; Wigdorovitz et al., 1997).

In contrast, the inactivated virus induces a regulatory state that inhibits effector mechanisms. This could partially explain why vaccines based on inactivated particles are effective in virus neutralization, but antibody titers rapidly decline and animals need to be re-vaccinated to maintain high antibody levels (Piatti et al.,

1991). Comprehensive studies of the immune response against FMDV, such as the one presented in this work, will allow the development of suitable vaccines against the disease.

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Glossary

CT: mock-infected control
DC: dendritic cells
FMDV: live foot-and-mouth disease virus
iFMDV: inactivated foot-and-mouth disease virus
Ag: antigen
CCID: cell culture infective dose
CFSE: carboxylfluorescein diacetate succinimidyl ester
pDC ($B220^+CD11c^+$ cells): plasmacytoid dendritic cells
DC ($CD11c^+CD8\alpha^+$ cells): lymphoid dendritic cells
DC ($CD11c^+CD8\alpha^+$ cells): myeloid dendritic cells
Treg ($CD4^+CD25^+Foxp3^+$): T regulatory lymphocytes