Antiviral Research 92 (2011) 262–270

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Role of macrophages in early protective immune responses induced by two vaccines against foot and mouth disease

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ARTICLE INFO

Article history:
Received 2 March 2011
Revised 7 August 2011
Accepted 10 August 2011
Available online 24 August 2011

Keywords:
FMDV
Early protection
Macrophages

ABSTRACT

Foot and Mouth Disease (FMD) is an acute disease of cloven-hoofed species. We studied the protection and early immune response induced in the murine model by vaccines formulated with inactivated virus and two different adjuvants.

The presence of IMS12802PR or ISA206VG adjuvants yielded protection against viral challenge at early times post vaccination and induced FMDV-specific, but non neutralizing, antibody titers. *In vivo* macrophage depletion in vaccinated mice severely decreased the protection levels after virus challenge, indicating a central role of this cell population in the response elicited by the vaccines. Accordingly, opsonophagocytosis of FITC-labelled virus was augmented in 802-FMDVi and 206-FMDVi vaccinated mice. These results demonstrate the ability of the studied adjuvants to enhance the protective responses of these inactivated vaccines without the increase in seroneutralizing antibodies and the main role of opsonization and phagocytosis in the early protective immune responses against FMD infection in the murine model.

1. Introduction

Foot and mouth disease virus (FMDV) belongs to the *Picornaviridae* family and infects cloven-hoofed animals (House and Mebus, 1998; Doel, 2005). It is the causative agent of the highly contagious foot and mouth disease (FMD) and the most economically important ailment of livestock worldwide (Grubman and Baxt, 2004).

Because of the rapid spread of the virus it is necessary to implement control measures immediately after an outbreak occurs. These measures include the vaccination of nearest cattle with an emergency vaccine able to induce rapid protection, in addition to the slaughter of infected animals.

Many efforts have been devoted to the development of FMD emergency vaccines, in order to decrease the time between vaccination and development of a protective immune response. In several cases, this type of work is based on the use of new adjuvants together with higher dose of inactive virus than that used in conventional vaccines (Barnett et al., 2004; Doel et al., 1994; Golde et al., 2005).

Montanide ISA206VG is a water–oil–water (w/o/w) formulation which has been used to formulate emergency vaccines against FMDV and was tested in cattle and swine showing an increase in protection, especially in bovines (Barnett et al., 2002).

Montanide IMS12802PR is an aqueous composition that was never tested in FMD vaccines previously. Another aqueous adjuvant IMS1313NVG, similar to IMS12802PR, yielded increased protection against viral challenge when it was incorporated into an experimental FMD vaccine (Quattrocchi et al., 2004).

Knowledge on the immunity against FMDV in natural hosts is limited due to the impossibility of using inbred animals, the lack of specific reagents and the high costs involved in the use of large experimental animals such as cattle and swine. For these reasons, the mouse model has been widely used with FMD. Adult mice are not susceptible to natural infection with FMDV O1C, but they can be experimentally infected if the virus is inoculated intraperitoneally (ip). This infection is well characterized and includes viral replication in the pancreas without clinical symptoms (Collen et al., 1989; Fernandez et al., 1986).

Despite the differences regarding infection and symptomatology between mice and natural hosts, many similarities have been established: the main role of antibodies in resolving the infection happens in mice (Borca et al., 1986; Fernandez et al., 1986) as well as in swine and cattle (Bautista et al., 2003; Alexandersen et al., 2003), immunity after infection in mice last for life as in natural hosts (Lopez et al., 1990), the T-independence of the immune

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doi:10.1016/j.antiviral.2011.08.007

response against FMDV has been proved in mice (Borca et al., 1986) and in bovines (Juleff et al., 2009) and the inhibition of T functionality during the infection happens in mice (Ostrowski et al., 2005, 2007) as well as in swine (Bautista et al., 2003; Diaz-San Segundo et al., 2009). It has also been proved in mice that IL10 secreted by DCs, is a key in the FMDV-mediated immunosuppression (Ostrowski et al., 2005, 2007); the same phenomenon was observed in swine (Diaz-San Segundo et al., 2009). On the other hand, in our group we have obtained preliminary data indicating that protection against viral challenge obtained in this adult BALB/c mouse model correlates with protection in cattle (Decheneux et al., 2010).

The development of an acute disease, with clinical symptoms similar to that in natural hosts, produced by FMDV serotypes A22, C-S8c1 and SAT-1, in adult mice of the Swiss, BALB/c and C57/BL6 strains, was described by Salguero et al. (2005).

Although neutralizing Abs are key in resolving the infection, there are previous reports indicating that the importance of Abs might be more related to their ability to opsonize rather than neutralize viral particles (McCullough et al., 1986, 1988; Law and Hangartner, 2008).

This work was designed to assess the efficacy of IMS12802PR and ISA206VG in combination with inactive foot and mouth disease virus (FMDVi) serotype O1C, in raising early protection and to study the mechanisms involved in that protection in an adult BALB/c mouse model. Our data show that opsonizing Abs are associated with protection, that macrophages (MØs) are key for such protection at early times post vaccination in the murine model and that 802-FMDVi is a good candidate for an emergency FMD vaccine to be tested on natural hosts.

2. Materials and methods

2.1. Mice

BALB/c and N/NIH(S) nude 8–12-weeks-old male mice (School of Veterinary Sciences, La Plata, Argentina) were used. Experiments with animals were performed in accordance with the Institutional Committee for the care and use of experimental animals.

2.2. Virus

Binary ethylenimine (BEI)-inactivated FMDV serotype O1 Campos (FMDVi) was used to formulate experimental vaccines and ELISA assays. Infectious virus from the same serotype was used for viral challenge and was provided by the National Service of Animal Health, Argentina (SENASA). Infectious virus was obtained from vesicles of experimentally infected cattle with passages in BHK cells. All experiments involving infectious virus were performed in SENASA and INTA biosafety level3A facilities.

2.3. Vaccine formulations and vaccination

A dose of 0.05 μg FMDVi per mouse ($\approx 10^7$ $TCID_{50}$/dose) which elicited protection in less than 50% of vaccinated animals was chosen. The adjuvants used (Seppic, Paris, France) were ESSAIIMS D 12802 PR (named 802) and ISA206 (named 206), in combination with FMDVi in PBS (802-FMDVi or 206-FMDVi respectively). The formulations were prepared following the manufacturer's indications. The Berlin test, Oral LD 50, IP LD 50, ocular irritation test, dermal irritation test and pyrogenicity test were performed on MONTANIDE adjuvants. Montanide™ adjuvants and their components have been considered as safe by the Committee for Veterinary Medical Products (CVMP) for use in immunological products and are included as authorized substances in the annex of the European Council Regulation n° 470/2009. In addition the HET-CAM toxicity test was performed by the Centro de Toxicología y Biomedicina (TOXIMED, Santiago de Cuba, Cuba) in order to assess the safety of the vaccines formulated with adjuvant plus inactivated virus. All vaccines were classified as safe. Mice were intraperitoneally (ip) vaccinated using 0.2 ml of each formulation.

2.4. Viral challenge

Protection against FMDV was assessed as described previously with modifications (Carrillo et al., 1998; Lopez et al., 1990; Piatti et al., 1991). Briefly, mice were ip inoculated with 10^4 $TCID_{50}$ infectious FMDV. Animals were anesthetized and bled by retro orbital route 24 h later. Heparinized blood was spread on BHK-21 cell monolayers, after virus adsorption, monolayers were washed with sterile phosphate-buffered saline (PBS). Fresh DMEM with 2% FCS was added and the cells were kept for 48 h at 37 °C in a 5% CO_2 incubator. The animal was considered protected if the cell monolayer did not present cytopathic effect after a blind passage. Percentages of protection were calculated as (protected mice/challenged mice) × 100.

Viraemia titers were calculated by Reed and Muench. Briefly, blood was serially diluted and dilutions were spread onto BHK-21 cell monolayers. After 40 min incubation, blood dilutions were discarded and fresh DMEM with 2% FCS was added. Cells were kept at 37 °C in a 5% CO_2 incubator and after 72 h incubation cytopathic effect was recorded.

2.5. Seroneutralization assay

To measure anti-FMDV neutralizing Abs (sn-Abs), sera were serially diluted and dilutions were incubated with 100 $TCID_{50}$/well of infectious FMDV. The FMDV-serum mixtures were transferred onto BHK-2_z1 cell monolayers. Cells were kept for 48 h at 37 °C in a 5% CO_2 incubator. The appearance of cytopathic effects was recorded after 48 h of incubation at 37 °C.

2.6. Total anti-FMDV Abs measurement by ELISA

Immulon II plates were coated with anti-FMDV rabbit serum in 0.05 M carbonate–bicarbonate buffer, pH 9.6. After washing, FMDVi was added. Plates were blocked with polyvinylpirrolidone buffer (0.5 M NaCl; 0.01 M phosphate buffer; 0.05% Tween-20; 1 mM EDTA; 1% polyvinylpirrolidone 30–40 K, pH 7.2). Serum samples were incubated on the plates, followed by incubation with biotin-conjugated anti-mouse Ig (ebioscience, San Diego, USA). Horseradish peroxidase (HRP)-conjugated streptavidin was added and *o*-phenylenediamine-H_2O_2 was used as peroxidase substrate. Absorbance was recorded at 490 nm (A_{490}) in a MR 5000 microplate reader (Labsystems, MN, USA). The cut-off was established as the mean A_{490} of the negative sera plus two standard deviations. Positive control sera were included in every plate.

2.7. Isotype measurement by ELISA

Immulon II plates were coated with anti-FMDV rabbit serum in 0.05 M carbonate–bicarbonate buffer, pH 9.6. After washing, FMDVi was added. Plates were blocked with polyvinylpirrolidone buffer. Serum samples were incubated on the plates followed by incubation with biotin-conjugated anti-mouse isotype IgM, IgG1, IgG2a, IGg2b or IgG3 (Caltag, San Francisco, CA). Horseradish peroxidase (HRP)-conjugated streptavidin was added and *o*-phenylenediamine-H_2O_2 was used as peroxidase substrate. Absorbance was recorded at 490 nm (A_{490}) in a MR 5000 microplate reader (Labsystems, MN, USA). A positive control of each isotype was added and the A_{490} of each serum was expressed as a percentage

of the positive control. The cut-off was established as the mean of the values of negative sera plus two standard deviations.

2.8. Preparation of peritoneal and splenic cells

Mice were killed by cervical dislocation. Peritoneal cells were collected by lavage with 5 ml of serum-free 10 mM EDTA/PBS. Spleen cells were obtained by inoculation of 2 ml of serum-free 10 mM EDTA/PBS into the spleen to withdraw the cells from the capsule.

2.9. Flow cytometry analysis of cell surface molecules

The peritoneal and spleen cells were stained for surface markers using the following labelled monoclonal antibodies (Mabs): phycoerythrin (PE) anti-mouse F4/80 (pan macrophage cell marker),

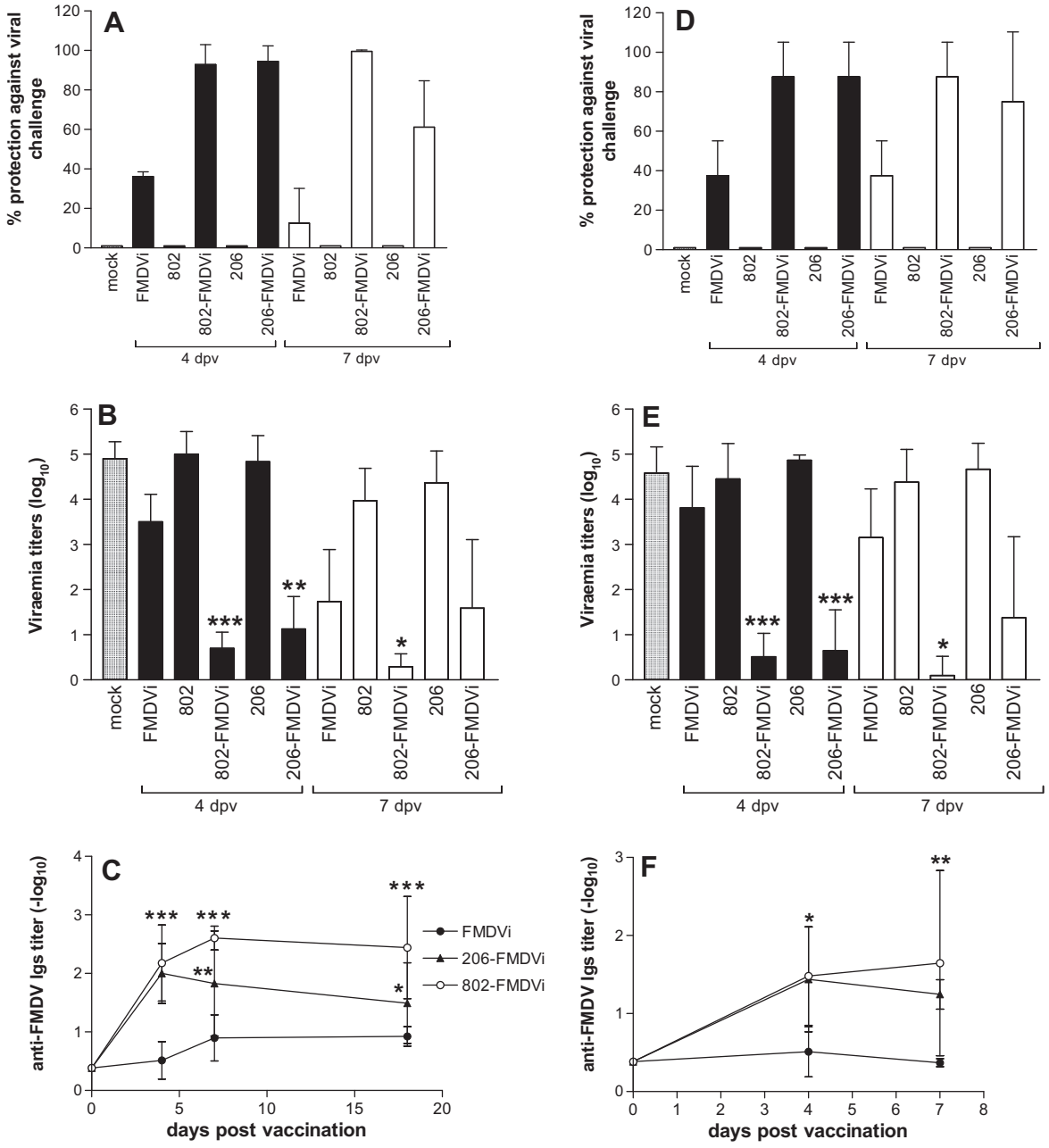

Fig. 1. Immune response in BALB/c and nude mice. (**A**) Percentages of protection against viral challenge at 4 dpv (black bars) or 7 dpv (white bars) in BALB/c mice. Mean percentages of 8 animals per group ± SD are shown. Groups 802 and 206 correspond to 4 animals vaccinated with adjuvants alone. The mock group corresponds to 4 animals inoculated with PBS. Protection was established as absence of viraemia after 24 h of ip inoculation of infective FMDV. Then the percentage of protection is calculated as (n° of animals without viraemia/n° of vaccinated) × 100. (**B**) Mean viraemia titers ± SD in BALB/C mice at 4 dpv (black bars) or 7 dpv (white bars). Titers were calculated by Reed and Muench. (**C**) Total specific IgG Abs against FMDV in BALB/c mice. Each point represents the mean titer ± SD of 8 sera measured by ELISA. (**D**) Percentages of protection against viral challenge at 4 dpv (black bars) or 7 dpv (white bars) in nude mice. Mean percentages of 8 animals per group ± SD are shown. Groups 802 and 206 correspond to 4 animals vaccinated with adjuvants alone. The mock group corresponds to 4 animals inoculated with PBS. Protection was established as absence of viraemia after 24 h of ip inoculation of infective FMDV. Then the percentage of protection is calculated as (n° of animals without viraemia/n° of vaccinated) × 100 (**E**) Total specific IgG Abs against FMDV in nude mice. Each point represents the mean titer ± SD of 8 sera measured by ELISA.. (**F**) Antibodies against FMDV elicited by vaccination. Each point represents the mean titer ± SD of 8 sera measured by ELISA. ***Significant differences ($p < 0.001$) **($p < 0.01$) *($p < 0.05$) with respect to the FMDVi group.

fluorescein isothiocyanate (FITC) anti-mouse CD3 (T lymphocyte marker), PE anti-mouse CD94 (activated T-cells and NK cells marker), FITC anti-mouse Gr1 (granulocytes marker) and FITC and PE-conjugated rat Ig isotype controls. All Mabs and isotype controls were purchased from Pharmingen. Briefly, cells were incubated for 20 min at 4 °C with Mabs, washed with PBS containing 1% FCS and fixed with 0.2% paraformaldehyde. Flow cytometry was performed in a BD FacsCalibur and analyzed with Cell Quest software. (BD Biosciences, San José, CA, USA).

2.10. In vivo depletion of cell populations

BALB/c mice were ip inoculated with rabbit anti-asialo GM1 Ab (one day before vaccination and one day before viral challenge) for NK depletion (Wako, Osaka, Japan) or with clodronate liposomes (one day after vaccination) for MØs depletion, as described previously (van Rooijen and Sanders, 1994, 1997; Nishikado et al., 2011). Depletion, assessed by flow cytometry, was ⩾90% in the peritoneal cavity and the spleen for NK and ⩾80% in the peritoneal cavity and ⩾60% in the spleen for MØ. Mice inoculated with rabbit normal serum or PBS-charged liposomes were included as mock-depleted controls. Clodronate liposomes are vesicles of phosphatidylcholine and cholesterol, with clodronate entrapped in the aqueous phase. Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany. Liposomes were prepared as described earlier (Van Rooijen and Sanders, 1994).

2.11. Opsonophagocytosis assay

FMDVi was labelled with FITC (Sigma, St. Louis, MO) and opsonophagocytosis of FITC-labelled FMDVi was analyzed by a previously described technique with minor modifications (Huber et al., 2001). Briefly, serum from vaccinated animals was mixed with FITC-labelled FMDVi. Peritoneal cells from the same animals were then incubated with the opsonized FITC-FMDVi (moi 10). Extracellular fluorescence was quenched with a 0.2 mg/ml solution of Trypan Blue. Flow cytometry was performed in an BD FacsCalibur and analyzed with CellQuest software. (BD Biosciences, San José, CA, USA).

2.12. Cytokine measurement

Spleen or peritoneal cells were incubated in vitro with culture media (without antigen) for 48 h. Cytokine concentrations (pg/ml) were determined in the supernatants of cell cultures, by commercial sandwich ELISA kits (BD-pharmingen and ebioscience). The A_{450} was measured in a MR 5000 microplate reader (Labsystems). Cytokine concentrations were calculated based on the A_{450} obtained with the standards.

2.13. Statistical analysis

ANOVA and Bonferroni post ANOVA tests were used to compare data among groups. Log_{10} transformation was done when necessary. P value <0.05 was considered as an indicator of significant difference.

3. Results

3.1. 802-FMDVi and 206-FMDVi increase the protection against viral challenge

Mice were vaccinated with FMDVi, 802-FMDVi and 206-FMDVi and challenged at 4 and 7 dpv (Fig. 1A). Formulations containing FMDVi alone protected 35% and 10% of the animals at 4 and 7 dpv, respectively. The inclusion of adjuvant 802 increased the protection levels to 90% at 4 and 7 dpv. The inclusion of adjuvant 206 increased the protection levels to100% at 4 and 60% at 7 dpv. Additionally, groups of mice were vaccinated with adjuvants 802 or 206 alone. No mice were protected in these groups, indicating that the protection elicited in 802-FMDVi and 206-FMDVi groups is dependent on the inactivated virus included in the vaccine.

Consistent with protection percentages, at 4 and 7 dpv, viraemia titers were significantly lower in group 802-FMDVi than in group FMDVi. Group 206-FMDVi had significantly lower viraemia than FMDVi at 4 dpv but not at 7 dpv (Fig. 1B).

Table 1
Neutralizing antibodies in BALB/c and Nude mice at 4 and 7 dpv. The seroneutralizing titer was calculated using the fixed virus – variable serum method.

	Vaccine	4 dpv	7 dpv
BALB/c mice	FMDVi	1.37 ± 0.13[a]	1.52 ± 0.17
	802-FMDVi	1.12 ± 0.13	1.70 ± 0.17
	206-FMDVi	1.30 ± 0.20	1.39 ± 0.15
Nude mice	FMDVi	0.93 ± 0.35	1.19 ± 0.34
	802-FMDVi	0.93 ± 0.23	1.53 ± 0.37
	206-FMDVi	0.88 ± 0.18	1.44 ± 0.34

[a] Mean of antibody titers ± standard deviation.

3.2. Total specific, but not neutralizing, Abs are related with protection

At 4 and 7 dpv, sn-Abs from mice vaccinated with 802-FMDVi or 206-FMDVi were not significantly different from those in the FMDVi group (Table 1). However, there were significantly higher titers of total specific Abs in groups 802-FMDVi and 206-FMDVi than in group FMDVi at 4 and 7 dpv. At 18 dpv the antibody titers in 802-FMDVi and 206-FMDVi groups remain significantly higher than those in FMDVi group (Fig. 1C).

The isotype profiles were analyzed by ELISA. Both 802-FMDVi and 206-FMDVi vaccines significantly increased the IgM and IgG2b isotypes at 4 dpv compared to the FMDVi group (Fig. 2A and D). Both vaccines induced significantly higher levels of every studied isotype at 7 dpv than the FMDVi vaccine (Fig. 2A–E).

In addition, 206-FMDVi vaccine significantly increased IgG3 and IgG1 at 4 dpv (Fig. 2B and E).

3.3. The protective immune response is T-cell-independent

To investigate the role of T-cells, vaccination and challenge experiments were carried out on nude, athymic mice. It has been reported that these mice can be experimentally infected with FMDV O1C and that infection takes place in the same manner as in BALB/c mice (Borca et al., 1986; Fernandez et al., 1986). Fig. 1D shows that inclusion of 802 or 206 adjuvants in the vaccine formulation also yielded increased protection in nude mice. Accordingly, viraemia titers were significantly lower in groups 802-FMDVi and 206-FMDVi at 4 dpv. At 7 dpv group 802-FMDVi had lower viraemia than FMDVi (Fig. 1E).

Seroneutralizing Abs from mice vaccinated with 802-FMDVi or 206-FMDVi were not significantly different from those in the FMDVi group (Table 1). As in BALB/c mice, the total specific-FMDV antibodies were significantly increased in 802-FMDVi and 206-FMDVi groups compared to the FMDVi group at 4 and 7 dpv (Fig. 1F).

These results indicate that protection and increase in Abs response induced by vaccination with 802-FMDVi and 206-FMDVi at 4 and 7 dpv do not depend on T-cell collaboration.

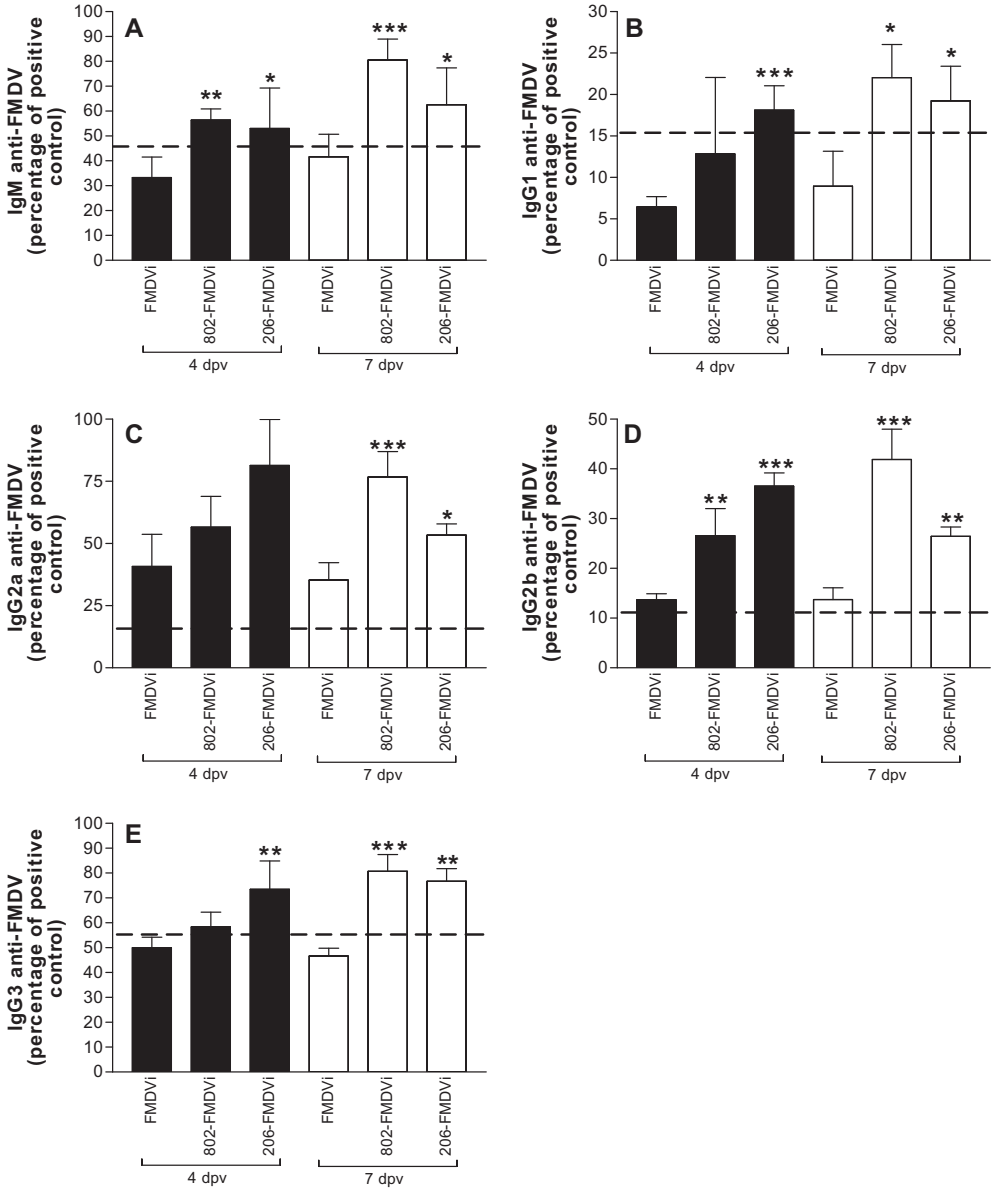

Fig. 2. Isotype profile of BALB/c mice. Data are expressed as the mean titer ± SD. Titers were calculated as percentage of the positive control. The dotted line represents the cut – off point calculated as the mean values for negative sera plus two standard deviations. (**A)** IgM titers, (**B)** IgG1 titers, (**C)** IgG2a titers, (**D)** IgG2b titers and (**E)** IgG3 titers.***Significant differences ($p < 0.001$) **($p < 0.01$) *($p < 0.05$) with respect to the FMDVi group.

3.4. 802-FMDVi and 206-FMDVi induce an inflammatory response

Since protection against challenge did not correlate with higher titers of neutralizing Abs, peritoneal cavity and spleen cells were studied by flow cytometry to investigate which immune cell populations could be related with such protection.

Granulocytes are among the first recruited to the inflammation sites. They are able to engulf and destroy microorganisms and to secrete a wide range of cytokines and chemokines which play a relevant role in recruiting other cells and in modulating the later adaptive immune response.

As shown in Fig. 3A–C, both 802-FMDVi and 206-FMDVi increase significantly the total number of peritoneal granulocytes (GR1+) from 2 to 7 dpv with respect to FMDVi. Spleen granulocytes (Fig. 3D–F) were increased in 802-FMDVi and 206-FMDVi groups from 2 up to 4 dpv as compared to the FMDVi group. At 7 dpv the number of granulocytes in spleen is significantly lower than in FMDVi group and similar to mock-vaccinated group. In FMDVi group, these cells only increased at 7 dpv as compared to the mock-vaccinated group. These results indicate that an inflammatory process is taking place in mice inoculated with 802-FMDVi and 206-FMDVi.

3.5. NK cells are modulated by vaccination but are not essential for protection

As shown in Fig. 3A–C, peritoneal NK cells (CD94+/CD3−) were significantly increased by inoculation of 206-FMDVi from 2 to 7 dpv. Inoculation of 802-FMDVi increased the number of peritoneal NK cells at 2 and 4 dpv, while the group inoculated with FMDVi was not different from mock-vaccinated mice. Spleen NK cells from 802-FMDVi or 206-FMDVi groups were not different from those coming from the FMDVi group (Fig. 3D–F).

The role of NK population in early protection was evaluated. Mice were vaccinated and NK-depleted with anti-asialo-GM1 or mock-depleted with normal rabbit serum. Then the animals were assayed by viral challenge. The protection levels induced in depleted animals were similar to those obtained in mock-depleted

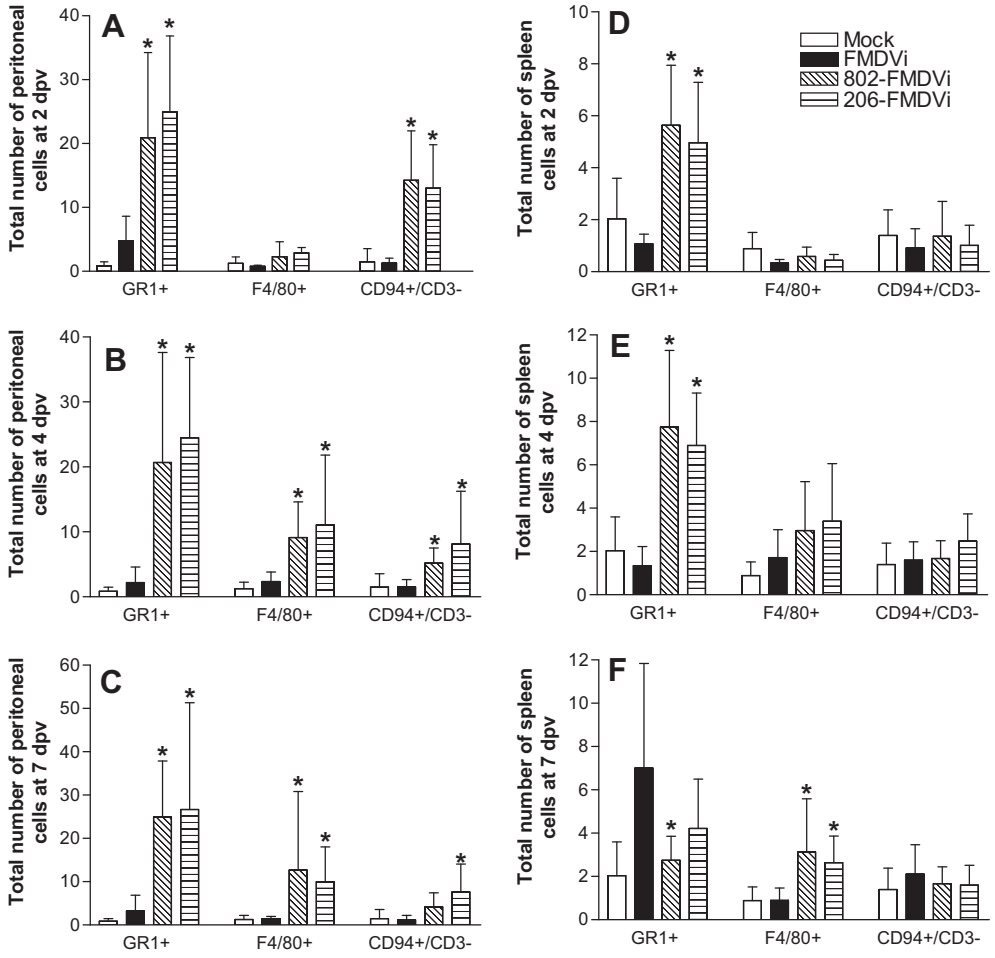

Fig. 3. Flow-cytometry analysis of peritoneal (A–C) and spleen (D–F) GR1+ cells (granulocytes), F4/80+ cells (macrophages) and CD93+/CD3− cells (NK). Mean numbers ± SD from 6 mice mock-vaccinated or vaccinated with FMDVi, 206-FMDVi or 802-FMDVi are shown at 2, 4 or 7 dpv. Numbers are expressed as cells × 10^6 except for NK in peritoneal cavity that are expressed as cells × 10^5. *Significant differences ($p < 0.05$) with respect to the FMDVi group.

Table 2
Protection against viral challenge in mock or NK-depleted mice. Animals were vaccinated, inoculated with anti-asialo-GM1 or rabbit normal serum and challenged. Protection was established as absence of viraemia after 24 h post challenge. Then the percentage of protection is calculated as (n° of animals without viraemia/n° of vaccinated) × 100.

Vaccine	BALB/c mice protected/challenged[a]			
	4 dpv		7 dpv	
	Mock[c]-depleted	NK-depleted	Mock depleted	NK-depleted
Mock-vaccinated[d]	0/2 (0%)[b]	0/2 (0%)	0/2 (0%)	0/2 (0%)
FMDVi	3/8 (37.5%)	2/8 (25%)	2/8 (25%)	1/8 (12.5%)
802-FMDVi	7/8 (87.5%)	6/8 (75%)	8/8 (100%)	6/8 (75%)
206-FMDVi	7/8 (87.5%)	7/8 (87.5%)	4/8 (50%)	5/8 (62.5%)

[a] Number of protected mice over number of vaccinated animals.
[b] The numbers in brackets represent the percentage of protection in each group.
[c] Mock depleted mice were inoculated with normal rabbit serum.
[d] Mock-vaccinated mice were inoculated with PBS.

mice (Table 2) indicating that NK cells are not relevant in the protection induced by 802-FMDi or 206-FMDVi vaccines.

3.6. Macrophages play a central role in protection induced by 802-FMDVi and 206-FMDVi

As shown in Fig. 3A–C, peritoneal MØs (F4/80+) significantly increased upon inoculation with 802-FMDVi or 206-FMDVi at 4 and 7 dpv as compared to the FMDVi group. Inoculation with FMDVi alone did not show differences in total peritoneal MØ numbers, as compared to the mock-vaccinated animals.

Spleen MØs (Fig. 3D–F were significantly increased at 7 dpv upon inoculation with 802-FMDVi or 206-FMDVi with respect to the FMDVi and mock-vaccinated group.

The importance of MØs in the early protection against FMDV was analyzed. Mice were vaccinated and MØ-depleted with clodronate-charged liposomes or mock-depleted with PBS-charged liposomes. Then animals were assayed by viral challenge. At 7 dpv, MØ depletion drastically decreased the protection levels when

Table 3
Protection against viral challenge in mock or MØ-depleted mice. Animals were vaccinated, inoculated with clodronate liposomes or PBS–liposomes and challenged. Protection was established as absence of viraemia after 24 h post challenge. Then the percentage of protection is calculated as (n° of animals without viraemia/n° of vaccinated) × 100.

Vaccine	BALB/c mice protected/challenged[a]	
	Mock[c]-depleted	MØ-depleted
Mock-vaccinated[d]	0/2 [0%][b]	0/2 [0%]
FMDVi	1/8 [12.5%]	0/8 [0%]
802-FMDVi	7/8 [87.5%]	1/8 [12.5%]
206-FMDVi	6/8 [75%]	2/8 [25%]

[a] Number of protected mice over number of vaccinated animals.
[b] The numbers in brackets represent the percentage of protection in each group.
[c] Mock depleted mice were inoculated with PBS-charged liposomes.
[d] Mock-vaccinated mice were inoculated with PBS.

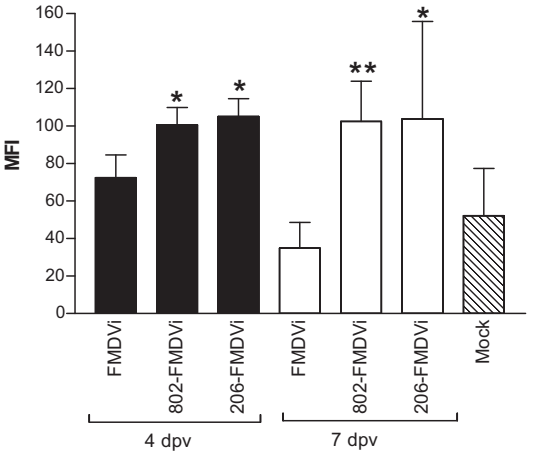

Fig. 4. Opsonophagocytosis assay in peritoneal cells. FITC-FMDV was incubated with serum from a mock-vaccinated mouse, an FMDVi vaccinated mouse, an 802-FMDVi vaccinated mouse or a 206-FMDVi vaccinated mouse. Then the mixtures were put in contact with peritoneal cells from the same animals. The mean numbers + SD of mean fluorescence intensity (MFI) from four assays are shown. *Significant differences ($p < 0.05$) **($p < 0.01$) with respect to the FMDVi group. Means were compared with the FMDVi group from the same date using the same serum dilution.

compared to mock-depleted groups (Table 3) indicating that MØs are playing a major role in protection.

At 4 dpv, both MØ-depleted and mock-depleted mice had much lower protection when compared to vaccinated, liposome-untreated animals (data not shown).

3.7. Opsonophagocytosis is increased by vaccination with 802-FMDVi and 206-FMDVi

Since MØs play a key role in the protection induced by vaccination with 802-FMDVi and 206-FMDVi, opsonophagocytosis was measured in peritoneal cells from vaccinated animals using flow cytometry.

As shown in Fig. 4, peritoneal cells from 802-FMDVi and 206-FMDVi vaccinated animals significantly incorporated more FITC-virus than those from the FMDVi or mock-vaccinated groups at 4 and 7 dpv.

3.8. Cytokine profile characterization

IL6 was significantly increased in peritoneal cells from 206-FMDVi-vaccinated animals at 2, 4 and 7 dpv, and in the 802-FMDVi group, at 2 and 7 dpv. The levels of IL6 were similarly low in the FMDVi group and the mock-vaccinated group (Fig. 5A). In the spleen, there were no significant differences (data not shown).

IL10 secretion was significantly lower in peritoneal cells from 802-FMDVi or 206-FMDVi groups than in those from FMDVi group but similar to mock-vaccinated group from 2 to 7 dpv, while peritoneal cells from FMDVi group, had higher IL10 secretion than those from mock-vaccinated group (Fig. 5C). In spleen cells the same phenomenon was seen (Fig. 5D).

IFNγ was significantly increased in peritoneal cells from the 802-FMDVi group at 2 dpv and in 206-FMDVi group at 2 and 7 dpv (Fig. 5B). Spleen cells showed no significant differences in IFNγ levels regarding FMDVi or mock-vaccinated groups (data not shown).

4. Discussion

FMD is one of the diseases which impose most restrictions on international trade of animals and their derivatives. After FMD outbreaks in Europe in 2001, ring vaccination of animals around the outbreak focus with emergency vaccines was incorporated as a measure to prevent the spread of FMDV in some countries.

Our data show that animals vaccinated with 802-FMDVi and 206-FMDVi were protected against viral challenge at 4 and 7 dpv, although at 7 dpv the protection percentages induced by 206-FMDVi were lower. Mice vaccinated with adjuvants alone were not protected, indicating that the protective response is specific for the virus and that it is not due to innate immune mechanisms induced by adjuvants.

Viraemia titers of vaccinated animals confirmed these data. Animals with high protection percentages had significantly lower viraemia than those with low protection levels.

It is generally accepted that effective protection against FMDV requires a response of specific and neutralizing Abs. We observed that sn-Abs were absent or very low in groups 802-FMDVi and 206-FMDVi and similar to those induced in the FMDVi group, indicating that the early protective response induced by our vaccines is independent from the viral neutralization capacity of the serum. This phenomenon has been observed by others using emergency vaccines (Barnett et al., 2002; Doel et al., 1994; Golde et al., 2005; Salt et al., 1998). However, the total anti-FMDV Abs levels were significantly higher in groups 802-FMDVi and 206-FMDVi than in the FMDVi group, suggesting that Abs are related with protection.

Since the risk of infection under an outbreak lasts for more than 7 days, we tested the antibody titers at longer times post vaccination and observed that they remained high at 18 dpv, suggesting that these vaccines could be useful for periods longer than 7 dpv.

Preliminary data obtained in our group, indicate that commercial vaccine currently used in Argentina, is capable of protect cattle against viral challenge at 7 dpv (Quattrocchi et al., 2008) and that is capable of induce antibody levels at 15 dpv, which are considered as partially protective (Maradei et al., 2008). Therefore, based on the fact that the use of fast-acting vaccine would not prevent the concurrent use of regular commercial vaccine, the protective immunity induced by 802-FMDVi vaccine could give time enough for the commercial vaccine to elicit long term protection. In this way, fast and long term immunity could be achieved.

Because of the rapid development of protection and Ab titers, we studied the T-cell collaboration in the immune response elicited by vaccination. The protection and the total anti-FMDV Abs response induced by vaccination in athymic nude mice was similar to that obtained in BALB/c euthymic mice, indicating that the protective immune response induced by vaccination is independent of T-cell collaboration. Additionally we observed no

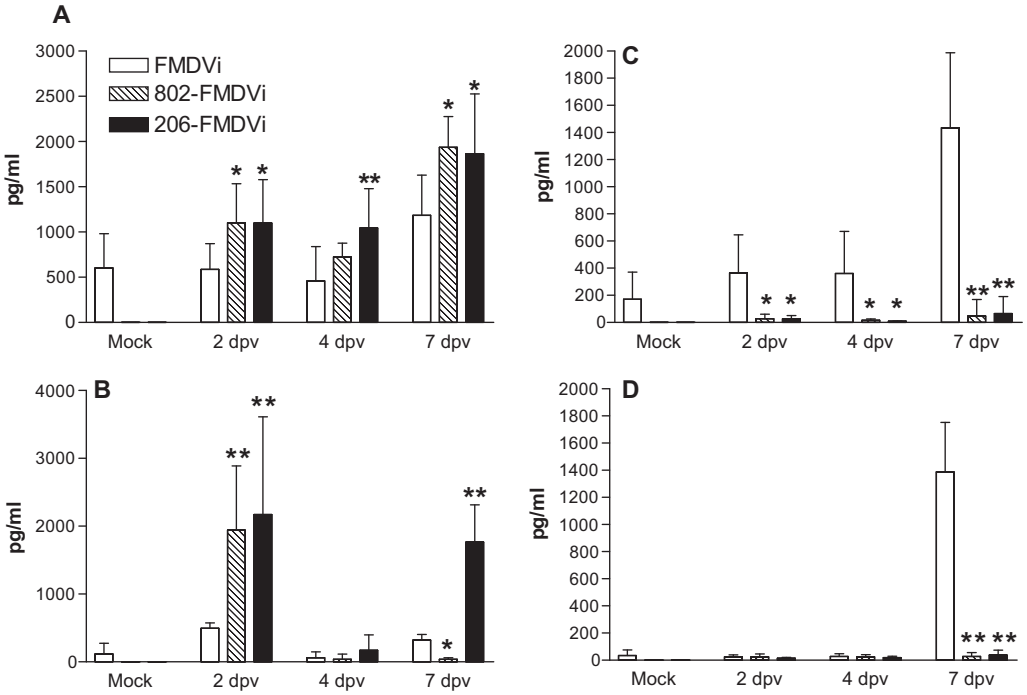

Fig. 5. Cytokines secreted by cells from peritoneal cavity or spleen. **(A)** IL6 secreted by peritoneal cavity cells. **(B)** IFNγ secreted by peritoneal cavity cells. **(C)** IL10 secreted by peritoneal cavity cells. **(D)** IL10 secreted by spleen cells. Mean numbers + SD from 6 mice mock-vaccinated or vaccinated with FMDVi, 206-FMDVi or 802-FMDVi are shown at 2, 4 or 7 dpv. *Significant differences ($p < 0.05$) **($p < 0.01$) with respect to the FMDVi group.

changes in CD4 + cells numbers and there was no secretion of IL4 in BALB/c mice (data not shown).

Emulsions (as ISA 206) and nanoparticles (as those included in IMS802) interact with the antigen to form multi molecular aggregates, improving the antigen uptake by MØs and DCs (Schijns and Lavelle, 2011; Singh et al., 2007). These aggregates are more efficiently filtered in the spleen, where they can be captured by MØs and B lymphocytes of the marginal zone, which would trigger the production of a TI Ab response (Martin and Kearney, 2000; Zandvoort and Timens, 2002). While it would be necessary to carry out more experiments to reach a conclusion, these mechanisms could explain why vaccines containing ISA206 and IMS802 produce an early specific Ab response, which is significantly higher than that induced by the viral antigen alone.

We also studied the cellular populations involved in the protection. It has been reported that adjuvants 802 and 206 induce local inflammation thus recruiting antigen presenting cells (Aucouturier et al., 2001). The inoculation of 802-FMDVi and 206-FMDVi increased the amount of granulocytes in the peritoneal cavity and spleen as well as the secretion of IL6 by peritoneal cells corroborating the occurrence of an inflammatory process. Also, the secretion of the inhibitory IL10 was increased in peritoneal cells from the FMDVi group but not in 802-FMDVi and 206-FMDVi groups and the same happened at 7 dpv in spleen cells, suggesting that these vaccines could prolong such inflammatory processes.

NK cells play a major role during viral infections (Vojvodić and Popović, 2010). They are capable of destroying infected IgG-coated cells by antibody-dependent cytotoxicity (ADCC), a key mechanism in protection against several viral infections (Chung et al., 2006; Jegerlehner et al., 2004). In addition, in a vaccination trial in pigs, the isolation of NK cells with cytotoxic activity against FMDV was reported, although the role of these cells in protection has not been elucidated (Amadori et al., 1992). Since NK cells were increased by vaccination with 802-MDVi and 206-FMDVi in the peritoneal cavity and IgG2b and IgG2a isotypes, major mediators of ADCC in mice (Denkers et al., 1985; Yokoyama and Riley, 2008), were detected in both vaccinated groups, the role of NK cells in protection was assayed. When NK cells were depleted there were no differences in protection, thus indicating that this population is not responsible for early protection and that its modulation could be a side effect of inflammation.

MØs have a high phagocytic capacity and it has been reported that they can engulf and destroy FMDV, especially if it is opsonized by specific Abs (McCullough et al., 1988, 1992). MØs were increased by vaccination with 802-FMDVi and 206-FMDVi as compared to FMDVi in the peritoneal cavity and spleen. These results are in agreement with previous results from Rigden et al. (2003), in which phagocytes from pigs vaccinated with an ISA206 formulation, showed increased chemotactic activity. IgG Abs capable of binding to FcR of MØs were significantly augmented in these groups and since the IFNγ secretion was increased in peritoneal cells at 2 dpv it possible that by 4 dpv, MØs were already activated. To test the importance of MØs in early protection, we depleted them in vaccinated animals and performed the viral challenge. Depletion of MØs drastically diminished the protection at 7 dpv, thus indicating that this population plays a key role in early protection elicited by 802-FMDVi and 206-FMDVi. At 4 dpv inoculation with PBS–liposomes also abolished protection. It has been reported that liposomes are easily incorporated by MØs and this can cause a saturation of their phagocytic capacity (Daemen et al., 1995; Lutz et al., 1995). Therefore, administration of PBS–liposomes only 3 days before challenge could be saturating the phagocytic capacity of MØs, abolishing the uptake of the Ab-virus complexes and avoiding the protective effect observed by vaccination. These results suggest that at 4 dpv, MØs are also playing a key role in the protection elicited by vaccination. Consistent with these data, cells from animals vaccinated with 802-FMDVi or 206-FMDVi, incorporated more FITC-FMDV than those from FMDVi or mock-vaccinated animals at 4 and 7 dpv, indicating that 802-FMDVi and 206-FMDVi improve the opsonophagocytosis mechanisms.

In conclusion, our results indicate that MØs play a central role in early protection and that opsonizing, not neutralizing Abs are

enough to induce early protection against FMDV. These data could explain why animals with low levels of sn-Abs are protected against viral challenge.

Both adjuvants have achieved high protection levels at 4 and 7 dpv, nevertheless the protection levels achieved by IMS802 at 7 dpv were higher than those elicited by ISA206.

For this reason we consider 802-FMDVi as a good candidate for an emergency FMD vaccine to be tested on natural hosts.

Acknowledgements

The authors wish to thank Osvaldo Zabal for the valuable assistance with cell cultures, to Dr. María José Gravisaco and Mariela Gammella for helping with flow cytometry assays, to National Service of Animal Health (SENASA, Argentina) for allowing us the use of their biosecurity facilities, to Seppic (Paris, France) for kindly provision of the adjuvants used in this work and to Dr. Mónica Florin-Christensen and Dr. Mariano Perez-Filgueira for correcting the manuscript. This work was supported by a grant from the National Institute of Agricultural Technology (AESA 1572).

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