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The Early Protective Thymus-Independent Antibody Response to Foot-and-Mouth Disease Virus Is Mediated by Splenic CD9$^+$ B Lymphocytes$^\nabla$

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Infection of mice with cytopathic foot-and-mouth disease virus (FMDV) induces a rapid and specific thymus-independent (TI) neutralizing antibody response that promptly clears the virus. Herein, it is shown that FMDV-infected dendritic cells (DCs) directly stimulate splenic innate-like CD9$^+$ B lymphocytes to rapidly (3 days) produce neutralizing anti-FMDV immunoglobulin M antibodies without T-lymphocyte collaboration. In contrast, neither follicular (CD9$^-$) B lymphocytes from the spleen nor B lymphocytes from lymph nodes efficiently respond to stimulation with FMDV-infected DCs. The production of these protective neutralizing antibodies is dependent on DC-derived interleukin-6 (IL-6) and on CD9$^+$ cell-derived IL-10 secretion. In comparison, DCs loaded with UV-inactivated FMDV are significantly less efficient in directly stimulating B lymphocytes to secrete TI antibodies. A critical role of the spleen in the early production of anti-FMDV antibodies in infected mice was also demonstrated in vivo. Indeed, either splenectomy or functional disruption of the marginal zone of the spleen delays and reduces the magnitude of the TI anti-FMDV antibody response in infected mice. Together, these results indicate that in addition to virus localization, the FMDV-mediated modulation of DC functionality is a key parameter that collaborates in the induction of a rapid and protective TI antibody response against this virus.

Experimental infection of mice with serotype O of foot-and-mouth disease virus (FMDV), the prototypic member of the genus *Aphthovirus* of the family *Picornaviridae*, causes a subclinical infection characterized by viral replication in the pancreas and a viremia that lasts for 48 to 72 h (14, 17). At this time, concurrently with the appearance of serum neutralizing antibodies, the virus is cleared from circulation (8, 17).

The humoral immune response against infectious FMDV proceeds in two phases: an early thymus-independent (TI) phase, involved in viral clearance, and a late thymus-dependent (TD) memory phase (8, 60). A similar type of response has been described for other viruses such as vesicular stomatitis virus (VSV) (5, 58). We have recently shown that during the early phase of the response against infectious FMDV, there is a transient but generalized suppression of TD responses (51). This effect is mediated, at least in part, by the downregulation of major histocompatibility complex class II and CD40 molecules on dendritic cells (DCs) and by the production of interleukin-10 (IL-10) by splenocytes (51). In contrast, the antibody response against UV-inactivated FMDV (UV-FMDV) proceeds as a typical TD response. Thus, it exhibits a delayed induction (~1 week). Moreover, the antibody isotypes elicited by the inactivated virus are different from those induced by infectious FMDV (52).

DCs are potent stimulators of T-cell responses (56). In addition, DCs also have the ability to influence B-cell functionality by inducing isotype switching, differentiation towards the plasma cell, and antibody secretion (16). The stimulation of B cells by DCs has been described for both TD proteins and TI polysaccharide antigens (4, 13, 15, 62). Furthermore, the interaction of DCs and B cells has also been involved in the induction of the production of neutralizing antibodies against viruses. For example, after infection with VSV, DCs transport and release the virus in the secondary lymphoid organs, eliciting the rapid production of neutralizing immunoglobulin M (IgM) (35).

Marginal zone (MZ) B cells and B1 B cells are two subsets of B lymphocytes that are classified as "innate B lymphocytes" due to some peculiar developmental and functional characteristics (31). Both B-cell subsets are subjected to B-cell receptor (BCR)-mediated positive selection, resulting in the enrichment of clones with defined specificities. Moreover, these cells present an activated phenotype that allows their rapid proliferation and differentiation into antibody-secreting cells upon stimulation with TI antigens. Remarkably, both MZ and B1 cells preferentially secrete antibodies of the IgM and IgG3 isotypes. Phenotypically, innate-like B lymphocytes can be differentiated from conventional (follicular) B cells by the expression of the cell surface molecule CD9 (61).

Whereas B1 B lymphocytes are located mostly in the peritoneal and pleural cavities, MZ B lymphocytes are located in the MZ of the spleen, where they represent ~5% of total splenic B cells. The profuse irrigation of the MZ venous sinus

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allows MZ B cells to have immediate access to blood-borne particulate antigens (40). Thus, the lower threshold for activation due to their activated phenotype (38, 50), together with its privileged localization, allows MZ B lymphocytes, rather than follicular B cells located in the white pulp, to generate an early wave of TI antibodies against particulate antigens present in the bloodstream (38). For instance, early after infection with VSV, a virus that produces a high-titer viremia, the virus is trapped by MZ macrophages in a complement-dependent manner. Subsequently, the recruited virus stimulates MZ B cells and induces the secretion of TI neutralizing antibodies (47).

In the present study, the role played by FMDV-infected DCs in the induction of B-cell responses and the early production of anti-FMDV antibodies, as well as the cytokines involved in this interaction, were studied. In addition, the role of the different secondary lymphoid organs and distinct B-cell subsets involved in the early TI response to FMDV was investigated. Our data indicate that the spleen, but not the lymph nodes (LNs), is the main lymphoid organ involved in the generation of early TI antibodies against FMDV. Furthermore, we demonstrate that FMDV-infected DCs can directly stimulate splenic $CD9^+$ B lymphocytes to secrete anti-FMDV antibodies in a process that is dependent on DC-derived IL-6 and $CD9^+$ B-cell-derived IL-10.

MATERIALS AND METHODS

Mice. BALB/c mice used throughout the experiments were obtained from the Instituto Nacional de Tecnología Agropecuaria. In addition, N:NIH(S) *nu* and C57BL/6 mice were purchased from the Universidad Nacional de La Plata, La Plata, Argentina. Mice between 8 and 12 weeks of age were used. Animal care was performed in accordance with institutional guidelines.

DC preparation, infection, and lipopolysaccharide (LPS) stimulation. Bone marrow-derived DCs were obtained as previously described (51). Infection of DCs was performed with FMDV serotype O1 Campos, provided by the Servicio Nacional de Sanidad y Calidad Agroalimentaria, Argentina, at a multiplicity of infection (MOI) of 10 for 4 h at 37°C. The virus was isolated from vesicular lesions from infected cattle and was once amplified by infection of the susceptible cell line BHK-21.

Noninfectious UV-FMDV was prepared by the irradiation of the viral suspension with UV light as described previously (51). DCs were incubated with UV-FMDV at an MOI equivalent (measured before UV inactivation) of 10 for 4 h at 37°C. Mock-infected (control) DCs were incubated with the supernatant of uninfected BHK-21 cell cultures for 4 h at 37°C. After being subjected to any of these treatments, DCs were washed twice with phosphate-buffered saline (PBS) (pH 5.5) (1-min incubation) to inactivate noninternalized virus, followed by six washes with RPMI 1640 medium supplemented with 5% fetal calf serum.

In an additional set of experiments, DCs were stimulated for 6 h with 10 μg/ml of LPS from *Escherichia coli* O55:B5 (Sigma-Aldrich) in complete RPMI medium.

Cocultures of DCs with splenocytes or LN cells. Cocultures of DCs (5×10^4 cells/well) and either splenocytes or LN cells (2.5×10^5 cells/well) were performed in RPMI 1640 medium containing 10% fetal calf serum, 10 mM HEPES buffer, and 5.5×10^{-5} M 2-mercaptoethanol (complete medium). When indicated, double amounts of DCs and effector cells were used. Cell-free supernatants were collected at day 2, 3, or 7 after the onset of the cocultures to evaluate either cytokine or antibody secretion.

Infection and vaccination of mice. Mice were infected or vaccinated with 10^5 50% tissue culture infectious doses ($TCID_{50}$) of either infective or UV-inactivated FMDV O1 Campos, respectively, by the intraperitoneal (i.p.) route. Mock-infected (control) mice were inoculated with supernatant of uninfected BHK-21 cell cultures.

To determine the presence of virus in the blood of FMDV-infected mice, blood was collected in heparin-containing tubes and diluted 1:100 in complete medium. The mix was then added onto BHK-21 cells previously seeded in 96-well plate. The presence of virus was evaluated by observations of typical cytopathic effects 48 h later.

Cytokine ELISA. Cytokine concentrations were determined in cell culture supernatants. Whereas the concentration of IL-6 was determined at 48 h, the concentration of IL-10 was determined at 72 h after the onset of the cultures by using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's directions (eBioscience). The absorbance at 450 nm was measured in a Multiskan EX spectrophotometer (Labsystems). Cytokine concentrations were calculated based on the optical densities obtained with the standards.

Detection of FMDV-neutralizing antibodies. To measure the anti-FMDV neutralizing antibodies, sera were inactivated by incubation at 56°C for 30 min, diluted 1/25 in complete Dulbecco's modified Eagle's medium, and incubated with serial 10-fold dilutions of FMDV for 1 h at 37°C. The FMDV-serum mixture was transferred onto confluent BHK-21 cells and incubated for 1 h at 37°C. The mixtures were then aspirated and replaced with fresh medium. The appearance of a cytopathic effect was recorded after 48 h of incubation at 37°C. Neutralizing indexes were calculated as the reciprocal $\log_{10}$ of the difference between the viral titer obtained after incubation with negative control serum minus the viral titer obtained with the experimental serum.

Detection of anti-FMDV antibodies by ELISA. To measure antibody isotypes against FMDV, a sandwich ELISA was used. Briefly, Immulon 2HB plates (Thermo Electron) were coated overnight at 4°C with rabbit anti-FMDV O1 Campos serum diluted to the optimum concentration in carbonate-bicarbonate buffer (pH 9.6). After washing three times with PBS-Tween, the plates were blocked with PBS-Tween containing 1% gelatin (blocking buffer) for 30 min at 37°C. Inactivated diluted FMDV was then added to PBS. After washing, serial dilutions of either mouse sera or cell culture supernatants were added and diluted in blocking buffer containing 2% normal rabbit serum for 2 h at room temperature. Biotin-labeled goat anti-mouse IgM or IgG subisotypes antibodies (Caltag Laboratories) were then added to blocking buffer containing 2% normal rabbit serum and incubated for 1 h at room temperature. After washing, alkaline phosphatase-labeled streptavidin was added to blocking buffer and incubated for 30 min at room temperature. Finally, *para*-nitrophenyl phosphate was added as the substrate for alkaline phosphatase, and the absorbance at 405 nm was measured using a Multiskan EX spectrophotometer (Labsystems). Positive and negative control sera or supernatants were included in each test. Antibody titers were determined as the reciprocal dilution that gave an absorbance higher than the mean plus 3 standard deviations (SD) of the mock-infected samples.

Splenectomy and sham operation. Mice were anesthetized i.p. with ketamine (80 mg/kg) and xylazine (5 mg/kg). The spleen afferent artery was tied off with a 4-0 suture, and the spleen was removed. The peritoneal cavity and skin were closed with 4-0 sutures. For sham-splenectomized mice, the peritoneal cavity was opened and surgically closed. Mice were inoculated 12 days after surgery.

Functional depletion of MZ B lymphocytes. For in vivo depletion of MZ B cells, mice were injected intravenously with 100 ng of pertussis toxin (PTx; List Biological Laboratories) as previously described (25). Depletion of MZ B cells was evaluated 2 days after inoculation of PTx by flow cytometry analysis. Briefly, splenocytes were subjected to negative selection using magnetic microbeads coated with anti-CD90 and anti-CD11c antibodies (MACS; Miltenyi Biotech) to deplete T lymphocytes and DCs, respectively. The unselected fraction (flowthrough) was then doubly stained with fluorescent anti-B220 and anti-CD9 antibodies and analyzed by flow cytometry.

The functionality of nondepleted B lymphocytes after PTx treatment was evaluated by analyzing the production of total IgM upon LPS stimulation of splenocytes. Briefly, splenocytes from PTx-treated or PBS-treated control mice were stimulated with 15 μg/ml of LPS from *Escherichia coli* O55:B5 during 5 days. The production of total IgM was assessed in a direct ELISA using horseradish-conjugated anti-mouse IgM antiserum as a secondary antibody.

At day 2 after inoculation, mice were either infected with FMDV or vaccinated with inactivated FMDV. Antibody titers in the sera of inoculated mice were evaluated 1 week after infection or vaccination.

Isolation of $CD9^+$ spleen cells. Spleen cells from naïve athymic N:NIH(S) *nu* mice were separated according to their expression of the cell surface molecule CD9. Briefly, splenocytes were incubated for 30 min with a rat anti-CD9 antibody at a 1-μg/ml dilution (kindly provided by J. F. Kearney), followed by incubation with biotinylated goat anti-rat IgG antibodies (1 μg/ml). After extensive washing, avidin-coated magnetic beads (MACS; Miltenyi Biotech) were added and incubated for an additional 30 min. Separation was performed according to the manufacturer's directions.

Statistical analysis. Differences among mock infection, FMDV infection, and UV-FMDV vaccination were determined by one-way analysis of variance, followed by post-analysis of variance comparisons using the Bonferroni test. When

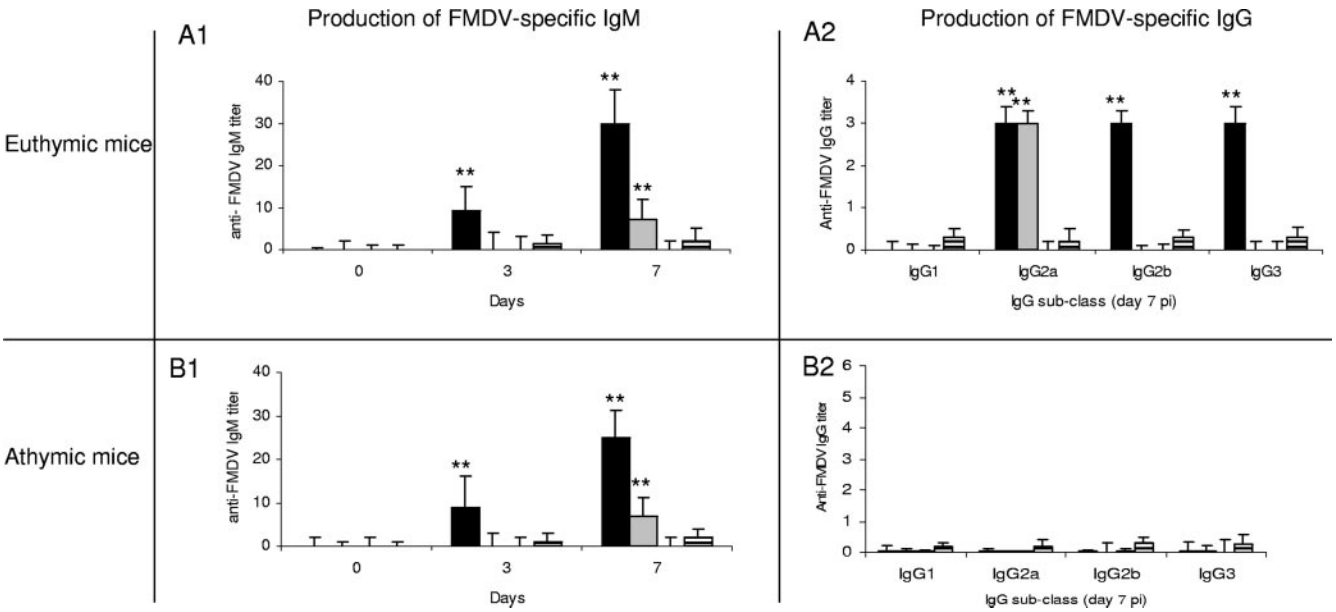

FIG. 1. Secretion of anti-FMDV antibodies in cocultures of FMDV-infected or UV-FMDV-loaded DCs and splenocytes. DCs were either infected with FMDV (■) (MOI of 10), loaded with UV-FMDV (▨) (MOI of 10), mock infected (□), or LPS stimulated (hatched bars). Four hours later, cells were washed and cocultured with either autologous splenocytes from BALB/c mice (A1 and A2) or autologous splenocytes from athymic mice (B1 and B2) (5×10^4 DCs versus 2.5×10^5 splenocytes). At 3 and 7 days after the onset of the cocultures, cell-free supernatants were collected, and the presence of FMDV-specific IgM antibodies was determined by sandwich ELISA (A1 and B1). Anti-FMDV IgG subclasses were determined at day 7 (A2 and B2). Results are expressed as mean antibody titers ± SD of four independent experiments (*, $P < 0.05$; **, $P < 0.01$).

indicated, a *t* test was performed. A *P* value of <0.05 was considered to be a significant difference.

RESULTS

FMDV-infected DCs induce a strong and rapid TI antibody response in vitro upon coculture with splenocytes. The abilities of either FMDV-infected or UV-FMDV-loaded DCs to interact with B lymphocytes and to trigger the production of anti-FMDV antibodies were evaluated in vitro. FMDV-infected DCs or UV-FMDV-loaded DCs were cocultured with autologous splenocytes from either euthymic or athymic mice. The production of IgM and the different IgG subisotypes was evaluated at 3 and 7 days after the onset of the culture. FMDV-infected DCs stimulated both euthymic (Fig. 1A1) and athymic (Fig. 1B1) splenocytes to produce FMDV-specific antibodies. Whereas the secretion of IgM was detected as early as day 3, the secretion of IgGs was not detected until day 7. At this time, in addition to IgM, low levels of IgG2a, IgG2b, and IgG3 (Fig. 1A2) were detected in the cultures of FMDV-infected DCs and splenocytes from euthymic mice. In contrast, only IgM was detected at this time in the cultures of DCs and splenocytes from athymic mice (Fig. 1B1 and B2). These results indicate that FMDV-infected DCs can directly stimulate B lymphocytes, even in the absence of T-cell collaboration, to induce the secretion of FMDV-specific IgM. However, T-cell help is required to induce class switching towards IgG.

The ability of UV-FMDV-loaded DCs to stimulate the production of anti-FMDV antibodies was significantly lower than that of FMDV-infected DCs. Low levels of FMDV-specific IgM were detected only at day 7 (Fig. 1A1 and B1). Moreover, the only IgG subclass elicited by UV-FMDV-loaded DCs in the cultures with splenocytes from euthymic mice was IgG2a. In contrast, none of the IgG subclasses was produced in the cocultures containing splenocytes from athymic mice (Fig. 1B1 and B2).

To determine whether the higher secretion of anti-FMDV antibodies elicited by FMDV-infected DCs, compared to UV-FMDV-loaded DCs, was due to unspecific stimulation of DCs, the production of virus-specific antibodies in the supernatants of splenocytes cocultured with LPS-stimulated DCs was determined. In this case, the secretion of anti-FMDV antibodies was not detected (Fig. 1). This result indicates that both FMDV-infected DCs and UV-FMDV-loaded DCs elicit the production of anti-FMDV antibodies by stimulating FMDV-specific B-cell clones. Moreover, this observation rules out the possibility that the secretion of anti-FMDV antibodies in these cultures is due to the polyclonal activation of B cells caused by the unspecific stimulation of DCs.

In all the cases in which anti-FMDV antibodies were detected by ELISA, they were also shown to be able to neutralize the virus in vitro (data not shown), indicating a potential protective role of these antibodies.

Altogether, these results indicate that FMDV-infected DCs stimulate a faster and stronger secretion of TI IgM antibodies by B lymphocytes than UV-FMDV-loaded DCs.

IL-10 and IL-6 are required to induce a rapid anti-FMDV TI antibody response. To investigate whether the differences in the kinetics, magnitude, and isotype profile of the antibody responses elicited by FMDV-infected or UV-FMDV-loaded DCs were due to a differential pattern of cytokine secretion, the production of the B-cell-stimulating cytokines IL-10 and IL-6 in cell culture supernatants was evaluated (Fig. 2).

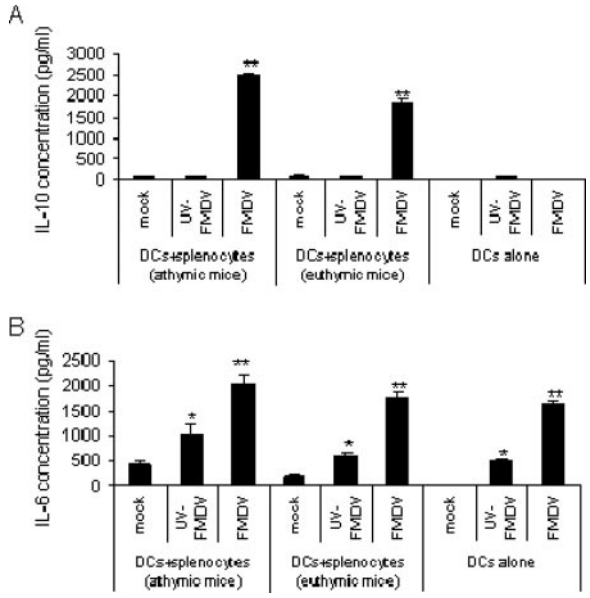

FIG. 2. Cytokine profile produced by FMDV-infected or UV-FMDV-loaded DCs and by cocultures of DCs and splenocytes. DCs were infected with FMDV (MOI of 10), loaded with UV-FMDV (MOI of 10), or mock infected. Four hours later, cells were washed and cultured (5×10^4 DCs/well) in complete medium. When indicated, DCs were cocultured with autologous splenocytes from either euthymic or athymic mice (5×10^4 DCs versus 2.5×10^5 splenocytes). Cell-free supernatants were collected, and the presence of IL-10 (A) and IL-6 (B) was evaluated at 48 and 72 h after the onset of the cultures, respectively, at a 1:3 dilution. Results are expressed as mean cytokine concentrations (picograms per milliliter) ± SD of four independent experiments (*, $P < 0.05$; **, $P < 0.01$).

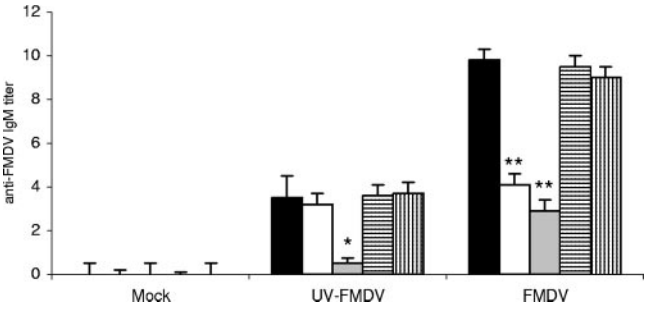

FIG. 3. Functional role played by IL-10 and IL-6 in the secretion of anti-FMDV antibodies induced by FMDV-infected or UV-FMDV-loaded DCs upon culture with splenocytes. DCs were mock infected, infected with FMDV (MOI of 10), or loaded with UV-FMDV (MOI of 10). Four hours later, cells were washed and cultured with autologous splenocytes from BALB/c mice (5×10^4 DCs versus 2.5×10^5 splenocytes). Culture medium (■), anti-IL-10 (□), anti-IL-6 (▨), or anti-IFN-γ (horizontal hatched bars) blocking antibodies, or their corresponding isotype control antibodies (vertical hatched bars), were added at the beginning of the culture (10 μg/ml). Cell-free supernatants were collected at 7 days after the onset of the coculture, and the presence of anti-FMDV IgM antibodies was evaluated by sandwich ELISA. Results are expressed as mean antibody titers ± SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$).

As we have previously shown (51), the presence of IL-10 was not detected in the supernatants of FMDV-infected DCs. Nevertheless, IL-10 production was detected in the cocultures of FMDV-infected DCs and splenocytes from either euthymic or athymic mice (Fig. 2A). These results indicate that FMDV-infected DCs do not produce IL-10 but that they induce the secretion of this cytokine upon an interaction with splenocytes. The production of comparable amounts of IL-10 by splenocytes from euthymic and athymic mice suggests that T cells are not the main producers of this cytokine and that other cell populations must be involved in its production.

The presence of IL-6 was detected in the supernatants of FMDV-infected DCs (Fig. 2B). The production of this cytokine was not significantly increased upon the addition of splenocytes to the DC cultures (Fig. 2B), suggesting that FMDV-infected DCs are the main producers of IL-6 in the cocultures.

The pattern of cytokine secretion was modified when DCs were incubated with inactivated UV-FMDV. Consistently with our previous results (51), UV-FMDV-loaded DCs neither secreted IL-10 nor induced the secretion of this cytokine upon coculture with splenocytes (Fig. 2A). Moreover, secretion of IL-6 by UV-FMDV-loaded DCs was significantly lower than that by FMDV-infected DCs (Fig. 2B). These observations indicate the existence of qualitative and quantitative differences in the cytokine secretion stimulated by infectious FMDV and inactivated UV-FMDV.

The role played by IL-6 and IL-10 in the induction of the antibody response against FMDV in vitro was then evaluated. The functional role played by gamma interferon (IFN-γ), another cytokine detected in cultures of splenocytes stimulated with FMDV-infected DCs (51), was also assessed. Neutralizing antibodies directed against IL-10, IL-6, and IFN-γ were added to the cocultures to block their activity. The production of anti-FMDV antibodies was evaluated at day 7, when, as shown in Fig. 1, anti-FMDV antibodies were detectable in cultures stimulated with both FMDV-infected and UV-FMDV-loaded DCs. Neutralization of either IL-10 or IL-6 significantly inhibited the secretion of anti-FMDV IgM antibodies elicited by FMDV-infected DCs (Fig. 3). However, neutralization of IFN-γ did not impair the secretion of anti-FMDV antibodies. To the contrary, in the cultures stimulated with UV-FMDV-loaded DCs, only the addition of neutralizing antibodies against IL-6, but not against IL-10, impaired the secretion of anti-FMDV antibodies. Taken together, these results indicate that IL-6, produced by DCs upon an interaction with either infectious or inactivated FMDV, and IL-10, secreted by splenocytes only upon an interaction with FMDV-infected DCs, stimulate B lymphocytes to rapidly produce anti-FMDV antibodies.

The TI antibody response against infectious FMDV is produced in the spleen. Previous reports have shown that the production of TI antibodies against polysaccharide antigens is more efficiently induced in the spleen than in LNs (21). To determine whether the production of TI antibodies against FMDV was also preferentially induced in the spleen, splenocytes or inguinal LN cells from athymic mice were cocultured with DCs. Both FMDV-infected and UV-FMDV-loaded DCs induced significantly more anti-FMDV IgM upon coculture with splenocytes than did inguinal LN cells (Fig. 4A1 and B1) or axillary LN cells (data not shown). Even when double amounts of LN cells were used, the secretion of IgM was still

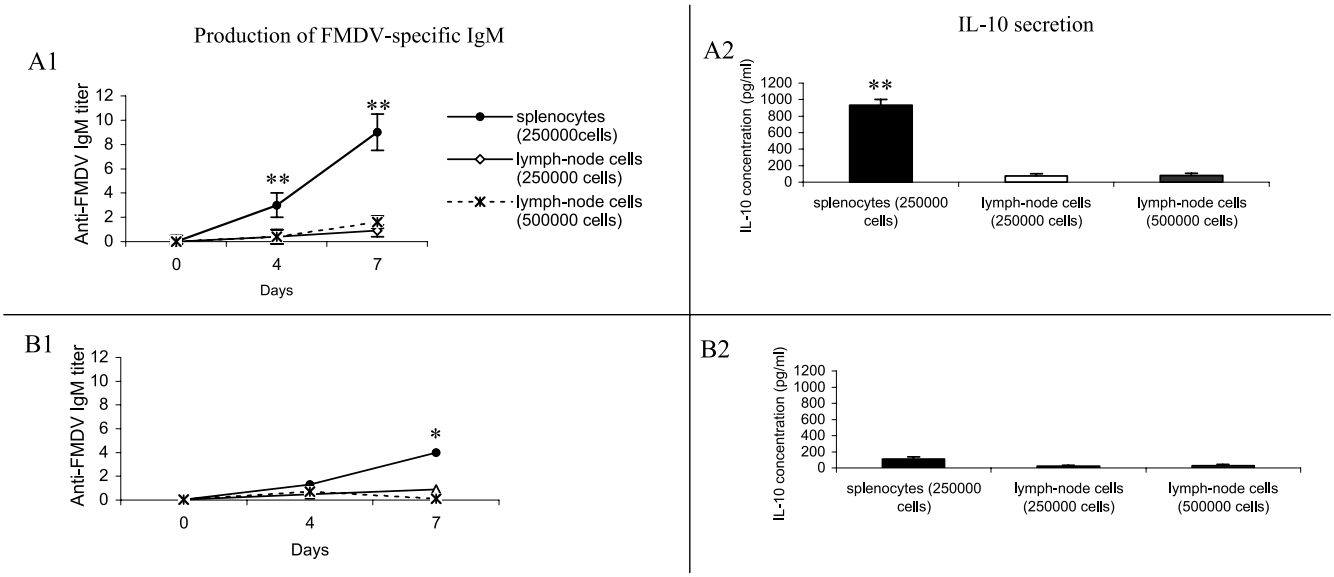

FIG. 4. Production of TI anti-FMDV IgM and IL-10 by splenocytes or LN cells from athymic mice stimulated with FMDV-infected DCs. DCs were either infected with FMDV (MOI of 10) (A1 and A2) or pulsed with UV-FMDV (MOI of 10) (B1 and B2). Four hours later, cells were washed and cocultured with splenocytes or inguinal LN cells from athymic mice (the number of effector cells added is indicated). The effector-to-stimulator ratio was 5:1. The production of anti-FMDV IgM (A1 and B1) was evaluated at 4 and 7 days. Results are expressed as mean antibody titers ± SD of three independent experiments. Secretion of IL-10 (A2 and B2) was evaluated at 3 days after the onset of the cocultures at a 1:3 dilution. Results are expressed as mean concentration values (picograms per milliliter) ± SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$).

significantly low in these cultures (Fig. 4A1 and B1), indicating that these results are not due to a lower percentage of B cells in LNs than in the spleen.

The production of IL-10 in these cocultures was then evaluated. Whereas FMDV-infected DCs stimulated splenocytes to produce high amounts of IL-10, LN cells did not secrete this cytokine (Fig. 4A2 and B2). Finally, neither splenocytes nor LN cells produced IL-10 upon their culture with UV-FMDV-loaded DCs. Taken together, these results indicate that B lymphocytes from the spleen, but not from LNs, are stimulated by DCs to produce a TI antibody response against FMDV. This fact is associated with the observation that spleen cells, but not LN cells, produce the B-cell-stimulating cytokine IL-10 upon coculture with FMDV-infected DCs.

The role played by different secondary lymphoid organs in the induction of the early anti-FMDV neutralizing antibody response in vivo was then investigated in splenectomized mice infected with FMDV. Whereas control sham-splenectomized euthymic mice produced neutralizing antibodies that were detected as early as day 3 postinfection (p.i.), splenectomized euthymic mice did not produce neutralizing antibodies until day 6 after infection (Fig. 5 A1). In addition to delaying the induction of neutralizing antibodies, splenectomy also modified the profile of the IgG subclasses elicited after infection. While FMDV-infected sham-splenectomized mice produced IgM at day 3 p.i. (Fig. 5B1) and IgM, IgG1, and IgG3 anti-FMDV antibodies at day 6 p.i. (Fig. 5C1), only IgM and low levels of IgG1 antibodies, but not IgG3, were produced by splenectomized mice (Fig. 5C1).

The role played by T cells in the induction of the early neutralizing antibody response in splenectomized mice was then evaluated in athymic animals. As expected from previous reports (8), control sham-splenectomized athymic mice rapidly produced neutralizing antibodies that became detectable since day 3 after infection. In contrast, splenectomized athymic mice were unable to produce neutralizing antibodies, even at day 9 after infection (Fig. 5A2). The only isotype detected in control sham-splenectomized athymic mice was IgM, at both 3 (Fig. 5B2) and 6 (Fig. 5C2) days after infection. Taken together, these results indicate that the spleen is the main secondary lymphoid organ with the ability to generate a rapid TI antibody response against FMDV both in vivo and in vitro. In contrast, the production of anti-FMDV antibodies in LNs depends on T-cell collaboration. Furthermore, the quality of the response elicited, as measured by the antibody isotype profile, changes according to the secondary lymphoid organ in which the response is induced.

The delay in the induction of anti-FMDV neutralizing antibodies observed in both euthymic and athymic splenectomized mice resulted in long-lasting viremias. Thus, whereas viremia was eliminated by day 2 after infection in sham-operated infected mice, infectious FMDV ($TCID_{50}$ of $>10^3$) was detectable in the blood of euthymic and athymic splenectomized mice until days 4 and 7, respectively (data not shown). This observation is in agreement with the previously reported observation that viral clearance is mediated mainly by neutralizing antibodies (8).

$CD9^+$ cells are the effector cells producing anti-FMDV TI antibodies and IL-10 in the spleen. To determine the B-cell subsets involved in the production of TI antibodies against FMDV, splenic follicular B cells were separated from B1 and MZ B cells according to the expression of CD9 (61). The percentages of $B220^+$ $CD9^+$ cells in the unselected (total splenocytes), negatively selected, and positively selected frac-

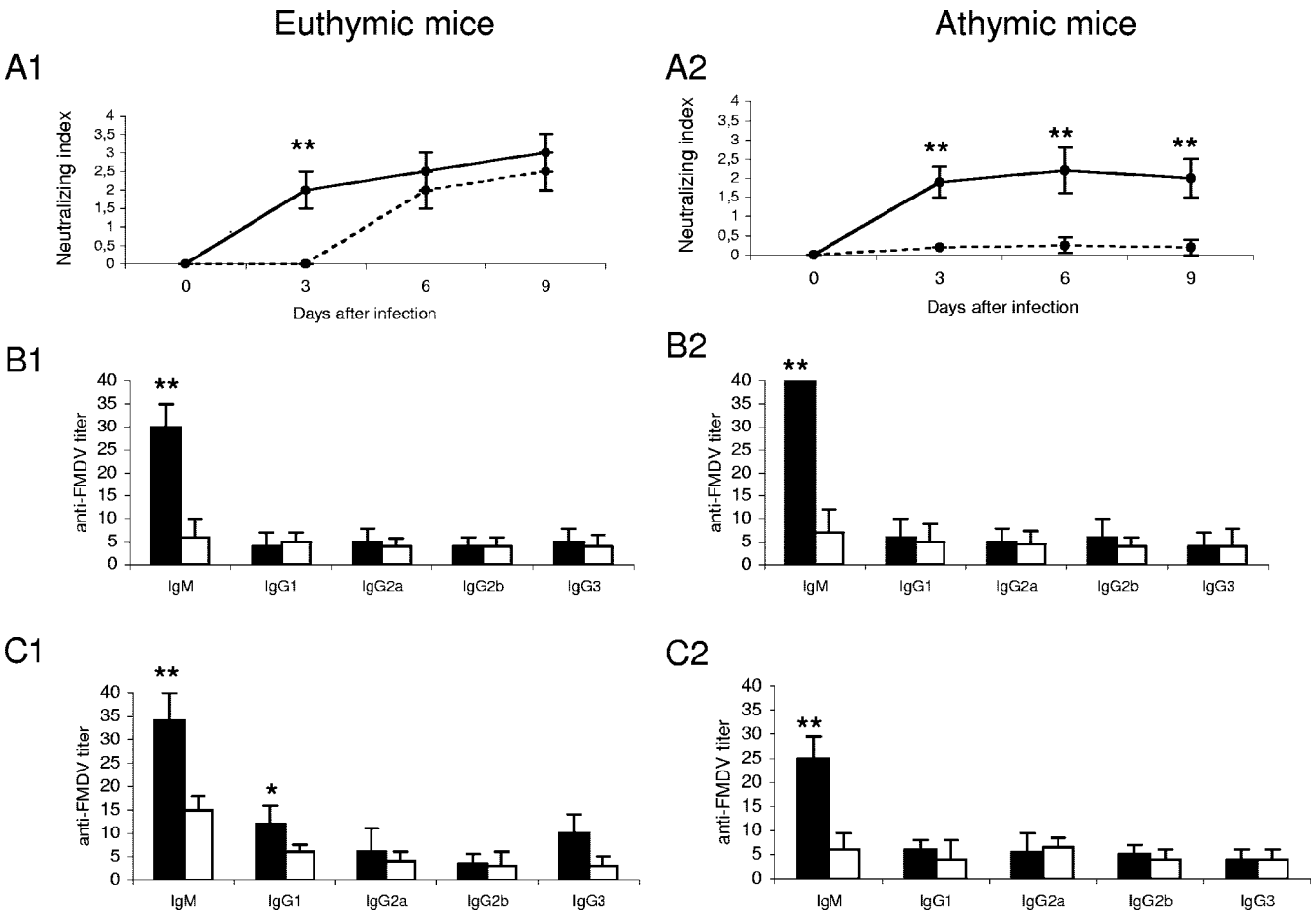

FIG. 5. Production of anti-FMDV neutralizing antibodies in splenectomized mice. Shown are kinetics of production of neutralizing antibodies in euthymic (left) or athymic (right) mice infected with 10^5 $TCID_{50}$ of FMDV i.p. Serum samples were collected at 3, 6, and 9 days after infection of control sham-splenectomized (solid lines) or splenectomized (broken lines) mice and analyzed for the presence of FMDV-neutralizing antibodies (A1 and A2). Antibody isotypes in the sera of control sham-splenectomized (■) and splenectomized (□) mice were evaluated at 3 (B) and 6 (C) days after infection by ELISA. Results are expressed as the reciprocal $\log_{10}$ of the mean neutralizing index (A1 and A2) or as mean ELISA titer (B and C) ± SD for five mice per group. Data from one representative experiment of two are shown (*, $P < 0.05$; **, $P < 0.01$).

tions were 3.5, 0.3, and 70%, respectively. Separated spleen cells were then stimulated in vitro with either FMDV-infected DCs or UV-FMDV-loaded DCs, and the production of anti-FMDV antibodies was evaluated (Fig. 6A). Following stimulation with FMDV-infected DCs, total splenocytes rapidly produced anti-FMDV IgM antibodies. In contrast, the cells in the CD9-depleted population (containing follicular B cells) did not respond to stimulation. Consistently, the $CD9^+$ selected cell population (containing B1 and MZ B cells) produced an anti-FMDV antibody response comparable that of to the unseparated cells. These results indicate that upon stimulation with FMDV-infected DCs, $CD9^+$ B cells are the main producers of antibodies in the spleen. The secretion of IL-10 by the different spleen cell populations was then analyzed (Fig. 6B).

This cytokine was also secreted by total spleen cells and by $CD9^+$-enriched cells but not by the CD9-depleted cells population.

To the contrary, the stimulation of $CD9^+$ selected cells with UV-FMDV-loaded DCs did not induce the secretion of either antibodies (Fig. 6A) or IL-10 (Fig. 6B), indicating that the infection of DCs is required to allow the stimulation of $CD9^+$ B cells.

Altogether, these results indicate that $CD9^+$ B lymphocytes, but not follicular B lymphocytes, participate in the early production of TI antibodies against FMDV. Moreover, $CD9^+$ cells are the main source of IL-10 in the spleens of FMDV-infected mice. Finally, the failure of UV-FMDV-loaded DCs to stimulate the secretion of antibodies and IL-10 by $CD9^+$ B lymphocytes suggests that infectious FMDV and inactivated FMDV differentially modulate the B-cell-stimulatory ability of DCs.

Functional depletion of MZ B lymphocytes reduces the generation of early IgM and IgG3. The role played by MZ B cells in the generation of early TI antibodies against FMDV in vivo was then assessed. To this end, MZ B cells were functionally depleted by intravenous inoculation of PTx as previously described (25). The depletion of MZ B cells was confirmed by a 95% reduction in the percentage of $B220^+$ $CD9^+$ cells analyzed by fluorescence-activated cell sorter (Fig. 7A). The functionality of nondepleted follicular B2 lymphocytes ($B220^+$

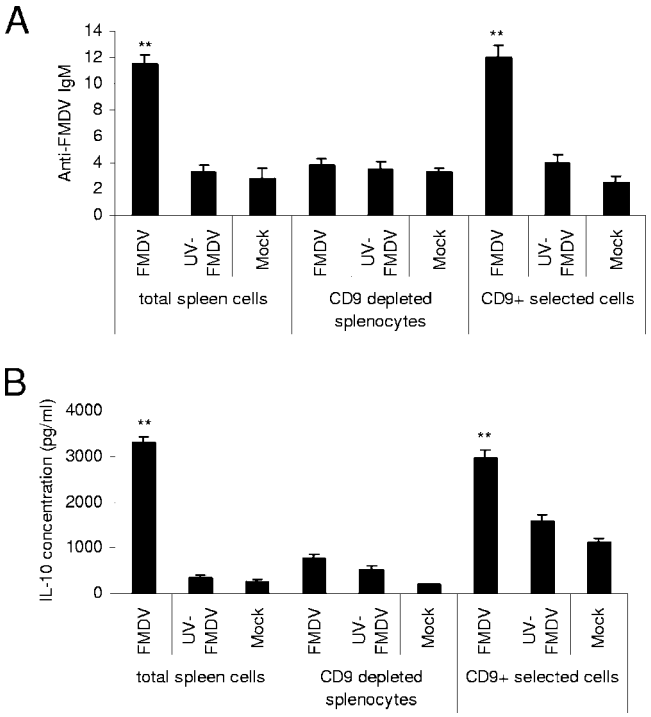

FIG. 6. Production of anti-FMDV antibodies and of IL-10 by different splenic B-cell subsets. Spleen cells from athymic mice were fractionated according to the expression of CD9. Production of FMDV-specific IgM (A) and IL-10 (B) at day 5 of culture by total spleen cells (prior to separation), splenocytes depleted of $CD9^+$ cells, and positively selected $CD9^+$ cells upon coculture with FMDV-infected, UV-FMDV-loaded, or mock-infected DCs (5×10^4 DCs versus 2.5×10^5 splenocytes) was evaluated in cell culture supernatants by ELISA. Results are expressed as either mean anti-FMDV IgM titers or mean cytokine concentrations ± SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$).

$CD9^-$) in PTx-injected mice was then assessed. Splenocytes from PTx-injected or PBS-injected control mice were stimulated with LPS, and the production of total IgM in these cultures was determined using a direct ELISA. Splenocytes from both PTx-treated mice and PBS-injected control mice produced similar IgM levels after LPS stimulation for 5 days (Fig. 7B). These observations indicate that treatment with PTx specifically depletes MZ B cells and that the functionality of follicular B lymphocytes is not altered by this treatment. At 48 h after PTx treatment, mice were either infected or vaccinated with inactivated FMDV, and the production of anti-FMDV IgM and IgG3, the two preferential isotypes secreted by MZ B lymphocytes (37), was evaluated 5 days later.

PTx-treated mice infected with FMDV produced significantly less seric IgM (Fig. 7C) and IgG3 (Fig. 7D) than infected mice that had not been subjected to the treatment with PTx. In contrast, no differences in antibody titers were observed between control and PTx-treated mice vaccinated with inactivated FMDV (Fig. 7C and D), confirming that the follicular B-lymphocyte subset is functionally active in PTx-treated mice. These results suggest that MZ B cells are the main B-cell subset involved in the production of early anti-FMDV antibodies after infection of mice with FMDV. Furthermore, they demonstrate that the early antibody response against infectious and inactivated viruses is produced by different B-cell subsets.

DISCUSSION

After acute infection with viruses that rapidly spread through systemic circulation, the rapid induction of a protective immune response is of the utmost importance to ensure survival of the host (2). Early production (e.g., 2 to 4 days) of TI antibodies is a common feature of the immune response against many cytopathic viruses such as FMDV (8), coxsackievirus, encephalomyocarditis virus, Sindbis virus (11), polyomavirus (57), influenza virus (32), VSV (5), and rotavirus (19). The capacity of both enveloped and nonenveloped viruses to induce TI responses is associated, at least in part, with the high organization of viral surface antigens (1). Indeed, the threshold of activation of B cells is lower for repetitive and organized proteins than for monomeric, soluble proteins (2). Antigen localization is another key parameter that determines the T-cell independency of an immune response. For example, after inoculation with either highly ordered polysaccharides or some of the above-mentioned viruses, the spleen is the main organ involved in the production of TI antibody responses. In contrast, the response in LNs is inefficiently induced (21, 24, 48). In the present study, we report that in addition to antigen organization and localization, the FMDV-mediated modulation of DC functionality also plays a key role in determining the independency of the anti-FMDV immune response from T cells.

In initial experiments, we compared the ability of FMDV-infected or UV-FMDV-loaded DCs to induce antibody production by splenic B lymphocytes in an in vitro culture system (Fig. 1). Consistent with the responses observed in vivo in either infected or vaccinated mice (8, 34), FMDV-infected DCs elicited a faster and stronger antibody response than did UV-FMDV-loaded DCs. Moreover, FMDV-infected DCs and UV-FMDV-loaded DCs drove antibody isotype switching towards different IgG subclasses (Fig. 1). Considering that the viral input for FMDV-infected and UV-FMDV-loaded DCs was the same (10 $TCID_{50}$/cell) and that FMDV does not productively replicate in DCs (7, 51), it is unlikely that the enhanced antibody response observed upon stimulation with FMDV-infected DCs could be attributed to differences in viral load. Instead, these differences likely reflect the triggering of different B-cell activation pathways. Along these lines, the exclusive production of IL-10 in the cocultures of splenocytes stimulated with FMDV-infected DCs, but not in those stimulated with UV-FMDV-loaded DCs (Fig. 2), could account for the differences in kinetics and isotypes of antibodies elicited by these DCs. It was previously reported that the preferential production of IL-10 by B1 and MZ B lymphocytes but not by conventional mouse B2 cells (10, 33, 49) may indicate that innate B cells are specialized in the preferential production of this cytokine (10). Although the in vivo role of IL-10 in the function of murine B2 lymphocytes is generally immunosuppressive (44), this cytokine plays an important stimulatory role in the function of innate-like B lymphocytes. Among these functions, the promotion of self-renewal of B1a cells and the retention of this population in the peritoneal and pleural cavities (3, 26, 27) are remarkable. Moreover, a clear stimulating

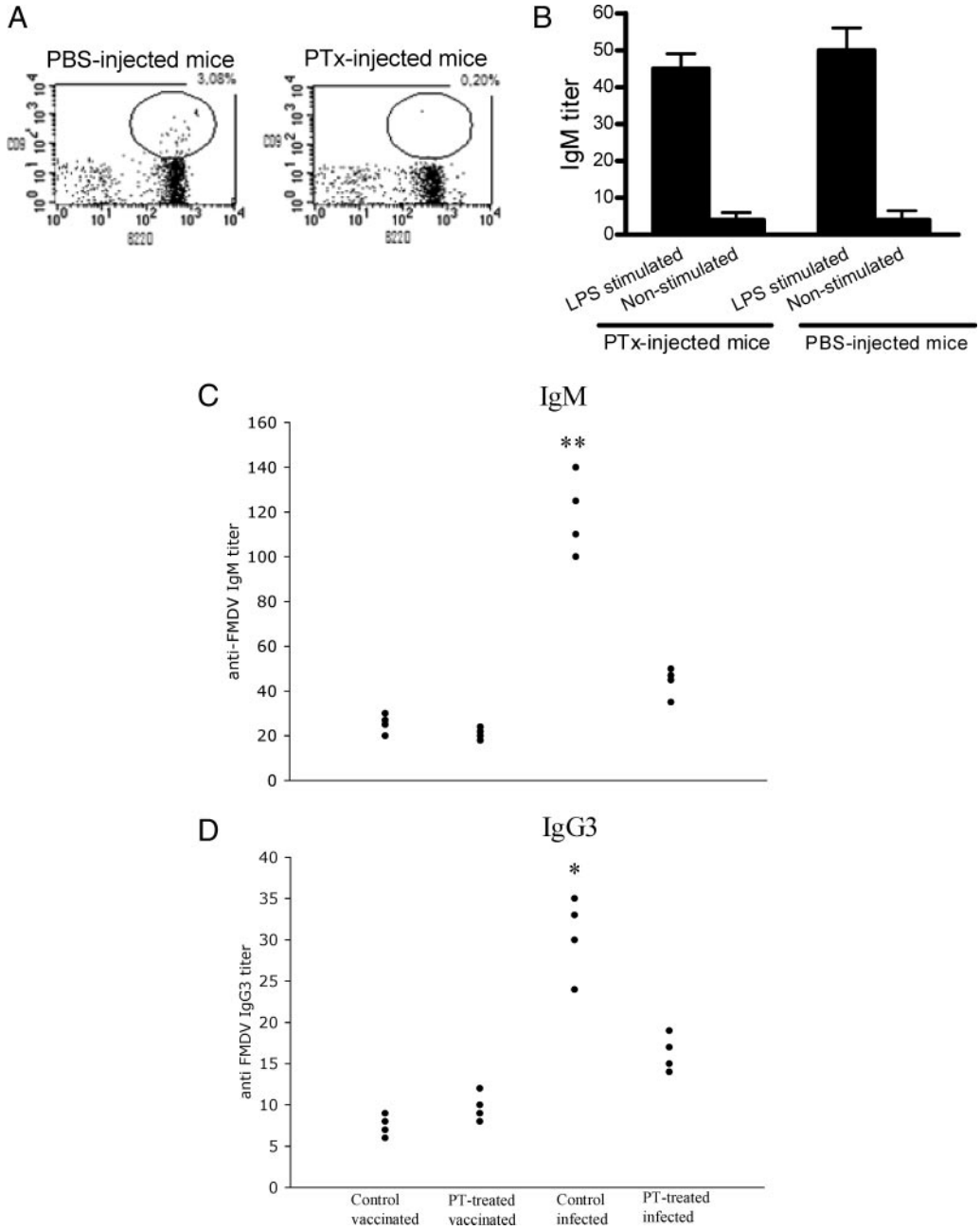

FIG. 7. Role of MZ B cells in the production of anti-FMDV antibodies in vivo. Mice were inoculated with PTx to functionally deplete MZ B cells or with PBS as a control. Forty-eight hours later, the percentage of B220/CD9 double-positive cells in the spleen was analyzed by flow cytometry to confirm the depletion of MZ B cells (A). The functionality of the nondepleted B lymphocytes in PTx-treated mice was assessed by titrating total IgM production in splenocyte cultures stimulated with LPS during 5 days (B). At 48 h after PTx treatment, mice were either infected or vaccinated with inactivated FMDV. The production of seric IgM (C) and IgG3 (D) was evaluated at 5 days after inoculation. Results are expressed as mean antibody titers. Data from one representative experiment of four are shown (*, $P < 0.05$; **, $P < 0.01$).

role of innate-like B lymphocytes for IL-10 in the development of B1 lymphoproliferative disease (55) and the stimulation of the production autoantibodies in mouse models of lupus erythematosus (28) and autoimmune anemia (46, 59) has been demonstrated. IL-10 also plays an important stimulatory role in the production of antibodies by B1 lymphocytes during the development of an immune response against antigens from pathogens. For instance, a clear B-cell-stimulating role for IL-10 has been demonstrated in mice immunized with glutamate dehydrogenase from *Trypanosoma cruzi* (43). In this case, IL-10, together with IL-6 and BAFF, promote B-cell proliferation. In addition, IL-10, in combination with IL-6, drives B-cell terminal differentiation into antibody-secreting plasma cells by upregulating critical transcription factors (43). Consistent with these reports, data shown in Fig. 3 demonstrate that the neutralization of IL-10 considerably reduces anti-FMDV

TI antibody secretion. This result indicates that this cytokine plays a major role in the induction of this antiviral immune response, which, as shown in Fig. 6, is carried out mainly by innate-like $CD9^+$ B lymphocytes. Furthermore, infection of DCs results in the high secretion of IL-6 compared to UV-FMDV-loaded DCs. Consistent with the previously reported functions of IL-6 as a stimulator of antibody secretion (29, 30, 45), the addition of a neutralizing anti-IL-6 monoclonal antibody significantly decreases the production of antibodies in cultures stimulated with FMDV-infected DCs (Fig. 3). Hence, herein, it is demonstrated that both IL-6 and IL-10 are crucial for the induction of anti-FMDV antibodies after infection.

The weaker and slower antibody response elicited by FMDV-infected DCs upon an interaction with LN cells than the response produced by splenocytes is associated with the lack of IL-10 in these cultures (Fig. 4). The inability of LN cells to secrete anti-FMDV TI antibodies is consistent with previous reports that indicated that the antibody responses to type 2 TI antigens are produced exclusively by splenocytes but not by LN cells (21, 24), further reinforcing the concept that antigen localization is a central parameter that determines the fate of the immune responses (63). The localization of murine MZ B and B1 cells in the spleen but not in LNs (54) could explain this observation. Indeed, herein, it is demonstrated that the in vivo TI antibody response against infectious FMDV is produced exclusively in the spleen (Fig. 5) by $CD9^+$ innate-like B lymphocytes (Fig. 6). Moreover, functional depletion of MZ B cells by the pharmacological inactivation of the $G_{\alpha i}$ protein with PTx results in the inhibition of the early TI antibody response to infectious FMDV. In contrast, the response elicited by vaccination with UV-FMDV is not affected by the treatment with PTx, indicating that infectious FMDV, but not UV-FMDV, targets MZ B cells during early times after inoculation (Fig. 7). Since treatment with PTx does not completely abolish the production of early TI IgM and IgG3 antibodies in infected mice, the involvement of other B-cell subsets (e.g., B1 B lymphocytes) in the early production of antibodies against FMDV in infected mice cannot be ruled out. Regarding the results shown in Fig. 6, it was unexpected that after the enrichment of innate-like $CD9^+$ B lymphocytes, there was not an augmentation of the antibody and IL-10 response compared to that of total splenocytes. Nevertheless, at least two possible explanations could be envisaged. First, survival of isolated $CD9^+$ B cells could be lower than that in the absence of other splenic cells. Alternatively, the selection process through CD9 ligation could partially alter the physiology of the selected B lymphocytes, thus resulting in a lower response than expected. Nonetheless, the significant reduction in the response observed after the depletion of $CD9^+$ cells in total splenocytes indicates that cells expressing this molecule are the main responders to stimulation with FMDV-infected DCs (Fig. 6).

MZ B cells have been reported to be the main B-cell population involved in the generation of rapid TI responses to blood-borne bacteria (38). In addition, MZ B cells play an important role in the production of early antibody responses against infectious VSV (47) and against noninfectious virus-like particles (22). The easy accessibility of MZ B cells to blood-borne antigens, their lower threshold of activation, and the enrichment in clones specific for TI antigens might explain the critical role that this B-cell subset plays in the induction of these rapid TI antibody responses (4, 37). Nevertheless, in vitro experiments demonstrated that BCR cross-linking with anti-IgM antibodies is not sufficient to induce MZ B-cell proliferation. Therefore, to activate MZ B cells, signaling via the BCR needs to be associated with cosignals (38). This costimulation is probably induced by ligands for Toll-like receptors (54). Alternatively, cosignals provided by DCs also contribute to MZ B-cell activation and to their rapid differentiation into antibody-secreting plasmablasts (4). Herein, it is demonstrated that FMDV-infected DCs, but not UV-FMDV-loaded DCs, stimulate $CD9^+$ B cells to rapidly produce TI antibodies and IL-10 (Fig. 6), indicating that the activation status of DCs determines the outcome of the B-cell response.

In addition to the previously discussed innate B-cell-stimulatory effects of IL-10, this cytokine plays an important role in the prevention of inflammation and immunopathology due to the excessive activation of cellular responses (53). Thus, the secretion of IL-10 by $CD9^+$ B lymphocytes upon stimulation with FMDV-infected DCs could account for the impairment of TD responses observed early after infection with this virus (51). These observations are in agreement with previous reports that demonstrated a role of B lymphocytes in the regulation of inflammatory reactions (41). For instance, it has been reported that IL-10-producing regulatory B cells inhibit the progression of inflammation in several autoimmune inflammatory diseases including inflammatory bowel disease (42), experimental autoimmune encephalomyelitis (18), arthritis (39), and lupus (9, 33). Furthermore, secretion of IL-10 by B cells with a regulatory function during infection with *Schistosoma mansoni* (36) and *Brugia pahangi* (23) has also been described. Therefore, data contributed by other laboratories and the results presented in this study suggest that under certain conditions, MZ B cells could mediate the inhibition of TD responses.

The development of a rapid TI anti-FMDV antibody response concurrent with the downregulation of T-cell functionality is in agreement with data from previous reports in which the early anti-FMDV immune response in the natural hosts was studied. For example, it has been shown that during the viremic phase of FMDV infection of swine, the infected animals experience a transient T-cell immunosuppression (6), while a rapid neutralizing antibody response capable of clearing viremia is elicited (12). In addition, it has been reported that the low primary cellular response following infection of cattle with FMDV is in contrast to the vigorous antibody production during the initial priming response (20). Thus, it is possible that the mechanisms to regulate the rapid production of TI antibodies in a context of generalized impairment of TD responses postulated herein are similar to those that occur in natural FMDV hosts.

In summary, the data presented herein indicate that FMDV-infected DCs stimulate $CD9^+$ B lymphocytes from the spleen to rapidly secrete specific TI antibodies. This response depends on IL-6, secreted by FMDV-infected DCs, and on IL-10, autocrinously secreted by $CD9^+$ B lymphocytes. Moreover, our data demonstrate that splenectomized mice or mice lacking functional MZ B cells produce a delayed antibody response to FMDV that requires T-cell collaboration. Thus, innate-like B cells located in the spleen are the main B-cell subset involved in the early production of TI protective antibodies against

infectious FMDV. The involvement of different B-cell subsets and cytokines in the immune response elicited against UV-inactivated FMDV, compared to infection with FMDV, could explain the differences in the kinetics and antibody isotypes observed between both responses.

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