Vet Res Commun (2013) 37:187–196
DOI 10.1007/s11259-013-9560-3

ORIGINAL ARTICLE

Co-expression of the Bcl-xL antiapoptotic protein enhances the induction of Th1-like immune responses in mice immunized with DNA vaccines encoding FMDV B and T cell epitopes

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Accepted: 28 February 2013 / Published online: 13 March 2013

Abstract Foot-and-mouth disease (FMD) is one of the most devastating animal diseases, affecting all cloven-hoofed domestic and wild animal species. Previous studies from our group using DNA vaccines encoding FMD virus (FMDV) B and T cell epitopes targeted to antigen presenting cells, allowed demonstrating total protection from FMDV homologous challenge in those animals efficiently primed for both humoral and cellular specific responses (Borrego et al. Antivir Res 92:359-363, 2011). In this study, a new DNA vaccine prototype expected to induce stronger and cross-reactive immune responses against FMDV which was designed by making two main modifications: i) adding a new B-cell epitope from the O-serotype to the B and T-cell epitopes from the C-serotype and ii) using a dual promoter plasmid that allowed inserting a new cistron encoding the anti-apoptotic Bcl-xL gene under the control of the internal ribosomal entry site (IRES) of encephalomyocarditis virus aiming to increase and optimize the antigen presentation of the encoded FMDV epitopes after in vivo immunization. In vitro studies showed that Bcl-xL significantly prolonged the survival of DNA transfected cells ($p<0.001$). Accordingly, vaccination of Swiss out-bred mice with the dual promoter plasmid increased the total IgG responses induced against each of the FMDV epitopes however no significant differences observed between groups. The humoral immune response was polarized through IgG2a in all vaccination groups ($p<0.05$); except peptide T_{3A}; in correspondence with the Th1-like response observed, a clear bias towards the induction of specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cell responses was also observed, being significantly higher ($p<0.05$) in the group of mice immunized with the plasmid co-expressing Bcl-xL and the FMDV B and T cell epitopes.

Keywords FMDV · B and T cell epitopes · Bcl-xL anti-apoptotic protein · Humoral immune responses · Cellular immune responses

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Introduction

FMDV is an highly contagious veterinarian threat which causes outbreaks among cattle, sheep, goats, pigs, and cloven-hoofed wildlife species. Although conventional inactivated virus vaccines are very efficient against FMDV, they possess several disadvantages including the short-time protection they confer (Sobrino et al. 2001) and the fact that

they only protect against homologous viruses (Sobrino and Domingo 2001; Knowles et al. 2005). Additionally, production of FMDV vaccines requires handling large amount of highly infectious live-virus in Bio-security Level 3 plus facilities, therefore increasing the chances of viral escapes to occur (Niborski et al. 2006; Sobrino and Domingo 2001; Knowles et al. 2005; Wang et al. 2002, 2007; Davies 2002; Grubman and Baxt 2004). Keeping in mind all of these facts, there is a clear need to develop broader and safer long-lasting vaccines against FMDV. DNA immunization appears to be a promising tool to fight against infectious pathogens, mainly attributed to its safety, easy manufacturing and its ability to induce strong humoral and cellular immune responses (Weiner 2008; Kutzler and Weiner 2008). DNA vaccines can encode full-length antigens but also smaller antigenic fragments or even individual B and T cell epitopes from specific pathogens (An and Whitton 1997). Thus, multiepitope DNA vaccines have been designed against many different pathogens, including FMDV (Wong et al. 2000, 2002; Cedillo-Barron et al. 2003; Wang et al. 2006; Borrego et al. 2006; Su et al. 2007). In the last years, novel strategies have been developed aiming to increase the immune responses elicited by DNA vaccines, such as: using electroporation devices in vivo to increase DNA uptake (Wang et al. 2008; Lin et al. 2011; Sardesai and Weiner 2011), co-expressing cytokines, chemokines or other molecular adjuvants (Wang et al. 2002; Su et al. 2008; Murtaugh and Foss 2002; Xiao et al. 2007; Shi et al. 2007; Mingxiao et al. 2007) or targeting antigens to professional antigen presenting cells (APCs) (Borrego et al. 2011; Rodriguez et al. 2001; Rush et al. 2010; Gil et al. 2011). Despite the large variety of experimental DNA vaccine prototypes tested in animal models, only a few of them induced full protection in swine (Wang et al. 2002; Wong et al. 2000, 2002). Therefore novel DNA vaccination strategies are still required to increase the immune response induced and the protection afforded against FMDV.

The mechanisms involved in protection against FMDV are not entirely understood (Joshi et al. 2009). Specific $CD4^+$ cell responses were detected both after vaccination and natural infection with FMDV with their helper role in neutralizing antibody production in cattle (Glass et al. 1991; McCullough and Sobrino 2004; Joshi et al. 2009) and pigs (Blanco et al. 2001; McCullough and Sobrino 2004). Similarly, $CD8^+$ T cell responses were also detected in cattle (Childerstone et al. 1999; Joshi et al. 2009; Guzman et al. 2010) and pigs (Blanco et al. 2001; Garcia-Briones et al. 2004). It is well long known that neutralizing antibodies play an important role in the protection however cattles with high neutralising antibody titers were not protected against the FMDV challenge (McCullough et al. 1992). Vice versa cattles with low or no detectable titers, protected from the disease (McCullough et al. 1992; Sobrino et al. 2001). In the FMDV vaccinated mice model, protection was also conferred in the absence of neutralizing antibody titers (Borrego et al. 2006). It was recently demonstrated that the $CD4^+$ T cells producing IFN-γ leading Th1 responses are the major proliferating phenotype after vaccination in cattle (Oh et al. 2012).

We have recently demonstrated that DNA vaccines encoding B and T cell epitopes from the C-serotype of FMDV could confer partial protection against FMDV challenge in pigs (Ganges et al. 2011) and that the protection afforded against homologous viral challenge could be exponentially improved by targeting the FMDV epitopes to the APCs by fusing them to a single chain antibody that recognizes the SLAII (Swine leukocyte antigen) molecules (Borrego et al. 2011). The fact that complete protection was afforded only in those animals showing specific T cell responses before challenge and an accelerated induction of neutralizing antibodies immediately after challenge, confirmed the important roles that both arms of the immune response played in protection.

We decided to modify the above mentioned construct by: i) adding a new B-cell epitope from the O-serotype to the B and T-cell epitopes from the C-serotype and ii) including the Bcl-xL anti-apopototic signal under the control of a second promoter aiming to prolong the survival of the DNA transfected cells, thus increasing the antigen presentation of the vaccine encoded epitopes to enhance the immune response elicited by the FMDV epitopes. It has been demonstrated that this strategy increased both the humoral and cellular responses induced against other antigens (Kim et al. 2004, 2005). The cell survival effect of the dual promoter-plasmid was first analyzed in vitro and later on, its effect on the immune responses induced against co-expressed FMDV B and T-cell epitopes was evaluated in vivo using the Swiss out-bred mouse model (Borrego et al. 2006). Our results clearly demonstrated that the inclusion of the anti-apoptotic Bcl-xL molecule in our DNA vaccines enhanced the induction of Th1-response in mice, therefore opening new expectations to be used in large animals.

Materials and methods

DNA vaccine construction

A dual promoter pIRES2EGFP vector (Clontech, USA) was used as the universal backbone to design three new vectors: pBcl-xL; encoding the Bcl-xL anti-apoptotic protein gene (Gene bank no: AAC53459.1), pFMDV; encoding the FMDV B and T cell epitopes fused to the APCH1 molecule (Borrego et al. 2011; Argilaguet et al. 2011) and pFMDV/Bcl-xL; encoding both polypeptides at the same time.

pBcl-xL plasmid construction was described elsewhere (Gulce Iz et al. 2012). Briefly, the Bcl-xL ORF was amplified from B6 MC57 mouse kidney mRNA by RT-PCR using the Bcl-xL forward: 5′-CCACAACCATGGTGTCT CAGAGCAACCGGGAGC-3′ and the Bcl-xL reverse:5′-CCATGGTTGTGGCCTTCCGACT GAAGAGTGAG CCC-3′ primers, both flanked by the Bst-XI restriction site and cloned within the unique Bst-XI site of the pIRES2EGFP vector. The final pBcl-xL plasmid encodes Bcl-xL gene in frame with the EGFP under the control of the IRES promoter. In order to increase the serotype coverage of the DNA vaccine, plasmid pFMDV, encoding the two B and two T cell FMDV epitopes fused to the APCH1 molecule, was obtained. The B cell epitope of FMDV O1K [B_O (VP1; 131–157 aa)] was PCR amplified from a FMDV O1K infectious clone (Saiz et al. 2001) with the following primers: B_O-forward: 5′-GCGCGCCATTTGCCAA GGTACAACAGAAATGCTGTGCCC-3′ and B_O-reverse, containing the BssHII restriction site; and then cloned in frame with the BTT epitopes within the unique BssHII site of the pCMV-APCH1BTT plasmid (Borrego et al. 2011), that contains the APCH1 molecule fused to the FMDV B [B_C (VP1; 133–156 aa)] and T cell epitopes of 3A and VP4 proteins [T_{3A} (3A; 11–40 aa), T_{VP4} (VP4; 20–34 aa)] of the FMDV C-S8c1 isolate.

To finally obtain pFMDV/Bcl-xL, the ORF encoding the two B and two T cell FMDV epitopes fused to the APCH1 molecule was then PCR amplified with the FMDV-forward: 5′- AGATCTCATGGACTTCGGGTTGAGCTTGG-3′ and FMDV-reverse: 5′- AGATCTCTACATG GAGTTTTGG TACTGC -3′ primers, both containing the BglII restriction site to be cloned within the unique BglII site of the pIRE2EGFP. pFMDV and the pFMDV/Bcl-xL plasmids encode the FMDV minigenes under the control of the CMV promoter.

All PCR products were directly cloned within the pGEMT-Easy plasmid (Promega, USA) to facilitate the excision of the amplicons with the corresponding restriction enzymes and their subsequent cloning in the final plasmids. The correct sequences were confirmed by automatic sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA) and the BioEdit genetic analyzer (Ibis Biosciences, USA).

In vitro transfection and Western blotting

Baby Hamster Kidney (BHK) 21 cells, obtained from American Type Culture Collection (ATCC No: CCL-10), were transfected with either: pBcl-xL, pFMDV, pFMDV/Bcl-xL or pGFP (control) plasmids, using the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol and 48 h after transfection the cells were harvested to evaluate the in vitro expression of the plasmid-encoded antigens by Western blotting.

Bcl-xL expression was evaluated with an anti-Bcl-xL Mab (Santa Cruz Biotech, USA). Briefly, an equal amount of transfected BHK-21 cells (approximately 5×10^5 cell per each sample) were separated by 12 % sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The separated proteins were transferred to a polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-P, Millipore, Germany). Thereafter, the membranes were probed with a 1:1000 dilution of the monoclonal anti-Bcl-xL Mab and next, the membranes were probed with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) antibody (Bio-Rad, USA). The blot was developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro-BT (Fisher Scientific, USA) diluted in alkaline phosphatase developing buffer (0.1 M Na_2CO_3, pH9.5, 0.1 M NaCl, 5 mM $MgCl_2$) (Döşkaya et al. 2007).

The expression of B_O (VP1; 131–157 aa) was detected by B2 monoclonal antibody recognizes the site 1A in the VP1 protein from the FMDV O (McCahon et al. 1989; Burman et al. 2006); the expression of B_C (VP1; 133–156 aa) was detected by SD6 monoclonal antibody recognizes against the site 1A in the VP1 protein from the FMDV C (Mateu 1987) and the expression of T_{3A} (3A; 11–40 aa) was detected by 2C2 monoclonal antibody recognizes FMDV 3A (De Diego et al. 1997). Briefly, BHK-21 cells (5×10^5 cells) were transfected with pFMDV and pFMDV/Bcl-xL plasmids, there after separated by 12 % SDS-PAGE. The separated proteins were transferred to PVDF transfer membrane and the membranes were probed with B2, SD6 and 2C2 monoclonal antibodies (1:50 diluted in PBS). Then the membranes were probed with alkaline phosphatase-conjugated goat anti-mouse IgG and developed as previously described.

Determining the cell survival effect of Bcl-xL protein

To determine the cell survival effect of Bcl-xL protein, Bcl-xL inserted plasmid pBcl-xL, and control plasmid, pGFP which was not carrying Bcl-xL anti-apoptotic protein were used to transfect BHK-21 cells. 48 h after transfection, cells were serum deprived (Kim et al. 2004). Cell survival was determined by measuring GFP expression in pBcl-xL and pGFP transfected cells (Gulce Iz et al. 2012). Briefly, BHK-21 cells were transfected with 2 μg of DNA vaccine plasmids using Lipofectamine 2000 (Invitrogen, USA) for 4 h using OPTIMEM (Invitrogen, USA). After that, cells were cultured in 10 % FBS supplemented GMEM (Biochrome, Germany) overnight. The media was discarded; cells were washed 3 times with PBS and were maintained for 48 extra hours in media without serum. Finally, cells were harvested, washed 3 times with PBS and fixed with Cytofix/Cytoperm solution (BD, USA). Cells were finally washed three times with Perm/Wash buffer and resuspended in Ca^{+2}-Mg^{+2} free

PBS with 1 % inactive FCS, 0.09 % (w/v) sodium azide (pH7.4) for flow cytometry to analyze the expression of GFP.

Vaccination

Animal experimentation was done as approved by the Ege University, Animal Experimentation Research and Ethics Committee by the protocol number 2010-112. Four groups of 6 week old, Swiss out-bred female mice (four per each group) were vaccinated thrice at 3 week intervals with either: pGFP (negative control), pBcl-xL, pFMDV, or pFMDV/Bcl-xL plasmids. A fifth group of mice was primed with two doses of the pFMDV/Bcl-xL plasmid and boosted with adjuvanted peptide mix and a sixth group was three times immunized with this same adjuvanted peptide mix (Table 1). The peptide mix containing the synthetic peptides B_C, B_O, T_{3A} and T_{VP4} (provided by AnaSpec, USA), corresponding to the sequences encoded by the DNA vaccines (Table 2). 100 μg of endotoxin-free purified plasmid (Qiagen, USA) was injected per dose and mouse into the right and the left anterior tibial muscle of anesthetized mice [100 mg/kg ketamine (Parke-Davis, USA), 3 mg/kg xylazine (Alfasan Internatioanl BV, Holland) diluted in physiological saline]. 100 μg of the synthetic peptide mix (25 μg each) was adjuvanted with Montanide ISA 50 V (Seppic, France) and administered intraperitoneally per dose and mouse.

Detection of humoral immune response

Tail bleeds were performed 3 weeks after each immunization for the detection of specific antibodies induced by vaccination. Serum neutralization assay was performed as described (Borrego et al. 2006; Mateu 1987), using sera from vaccinated mice and both the FMDV C-S8c1 and O1K serotypes. B_C, B_O, T_{3A} and T_{VP4} synthetic peptides were used to develop specific ELISAs, following protocols previously described (Doel 2003) to detect the peptide-specific levels of total IgG response as well as IgG1 and IgG2a subtype antibodies. Briefly, each well of maxisorp microtiter plates (Nunc, USA) were coated with 100 μL of peptide suspension containing 2 μg synthetic peptide and incubated overnight at 37 °C. Next, serum samples at dilution of 1/33 in blocking buffer (0.5 % BSA and 0.1 % Tween 20 containing PBS, pH7.4) were added to each well and incubated for 1 h at room temperature. Thereafter, the wells were probed with anti-mouse IgG (Thermoscientific, USA), anti-mouse IgG1 (Jackson Immuno Research Labs, USA) or IgG2a (Jackson Immuno Research Labs, USA) conjugated with horse radish peroxidase at dilutions of 1:2500 for 1 h at room temperature. Thereafter, bound antibodies were visualized after adding 3, 3′, 5, 5′ tetramethylbenzidine (TMB) substrate. Reactions were stopped by adding 75 μL of 2 N sulfuric acid and the results were quantified in a microtiter plate reader (Bio-Tek, USA) at 450 nm.

Detection of cellular immune response

To determine the specific cellular immune response elicited by each vaccine, mice were sacrificed 3 weeks after the third immunization and their spleens were removed and used to prepare single cell suspensions in complete growth medium. 5×10^5 viable splenocytes were added to each well of 96 well round bottom plate (Nunc, USA) and stimulated with synthetic peptide mix (each peptide at a final concentration of 10 μg/mL) for 72 h at 37 °C and 5 % CO_2. As positive control, splenocytes were incubated with concanavalin A (Sigma, USA) at a final concentration of 10 μg/mL. Growth medium was used as negative control. During the last 4 h of incubation, monensin was added to the cultures to allow the intracellular accumulation of cytokines at a final concentration of 2 μM.

T cell populations were surface stained with Alexa flour 647 conjugated rat anti-mouse CD3 (Biolegend, USA), FITC conjugated rat anti-mouse CD4 (BD, USA), or FITC conjugated rat anti-mouse CD8a (Abcam, USA), permeabilized with Cytofix/Cytoperm (BD, USA) and labeled with an PE conjugated rat anti-mouse IFN-γ (BD, USA) or PE conjugated rat anti-mouse IL-4 antibodies (BD, USA) according to the manufacturer's protocol.

Surface staining was done diluting the antibodies in Ca^{+2}-Mg^{+2} free PBS with 1 % inactive FCS, 0.09 % (w/v) sodium azide (pH7.4) while intracellular staining was done diluting the antibodies in Perm/Wash solution (BD, USA). All antibodies were used at a final concentration of 0.5 μg/10^6 cells and incubated at 4 °C for 30 min. T cell populations, gated in the flow cytometer (FACS Aria, BD) by CD3 positive expression, were analyzed to quantify: the percentage of peptide-specific T cells double positive for IFN-γ and $CD4^+$ or $CD8^+$ markers, and the percentage peptide-specific $CD4^+$ T-cells that also expressed IL-4.

Specific secretion of IFN-γ and IL-4 were also determined with commercial ELISA kits (Pierce, Thermoscientific, USA). The supernatants of the splenocytes stimulated with the synthetic peptide mix (at a concentration of 10 μg/mL each) were collected after 72 h and analyzed for IFN-γ and IL-4 secretion following the manufacturer's protocol.

Statistical analysis

Data obtained during the study were processed using Prism 5 (GraphPad, USA). A two-tailed unpaired *t*-test or one-way analysis of variance with 95 % confidence interval was used to determine the significance between the vaccination groups.

Table 1 DNA vaccine and control plasmids doses and routes of administration used during vaccination of mice

DNA vaccine groups		Dose and route of administration		
		1st dose (day 0)	2nd dose (day 21)	3rd dose (day 42)
1	pGFP	100 μg (i.m.)	100 μg (i.m.)	100 μg (i.m.)
2	pBcl-xL	100 μg (i.m.)	100 μg (i.m.)	100 μg (i.m.)
3	pFMDV	100 μg (i.m.)	100μg (i.m.)	100μg (i.m.)
4	pFMDV/Bcl-xL	100 μg (i.m.)	100 μg (i.m.)	100 μg (i.m.)
5	pFMDV/Bcl-xL (+) peptide mix (B_C, B_O, T_{3A} T_{VP4})- Montanide adjuvant	100 μg (i.m.)	100 μg (i.m.)	Adjuvanted100 μg peptide mix (i.p.)
6	Peptide mix (B_C, B_O, T_C, T_C)- Montanide adjuvant	Adjuvanted100 μg peptide mix (i.p.)	Adjuvanted100 μg peptide mix (i.p.)	Adjuvanted100 μg peptide mix (i.p.)

Results

Transient over expression of Bcl-xL in vitro protects from serum-deprived apoptosis allowing the co-expression of plasmid-encoded antigens

BHK-21 cells were transfected with pGFP, pBcl-xL, pFMDV or pFMDV/Bcl-xL plasmids and 48 h later, cells were harvested and analyzed to certify the correct expression of each one of the encoded antigens by Western blot. As expected, the anti-Bcl-xL monoclonal antibody did recognize a specific protein of 26.6 kDa of the plasmid used independently, in correspondence with the housekeeping Bcl-xL gene encoded-protein (Fig. 1a). An additional band of ~54 kDa was also evident in cells exclusively transfected with either pBcl-xL or pBcl-xL/FMDV, corresponding with the fusion of the GFP and Bcl-xL proteins (Fig. 1a). The correct expression of the FMDV epitopes was also confirmed using extracts from pFMDV and pFMDV/Bcl-xL transfected cells and using specific anti FMDV monoclonal antibodies (Fig. 1b). An expected band of ~40.5 kDa of molecular weight was observed, corresponding with the fusion of the APCH1 molecule and FMDV B and T cell epitopes.

In vitro cell survival effect of Bcl-xL inserted plasmids were determined under serum deprived conditions. The percentage of surviving transfected-cells was followed by detecting the plasmid-encoded GFP positive cells in a flow cytometer (Fig. 1c). As theoretically predicted, a significantly higher proportion ($P<0.0001$) of the cells scored positive after transfection with either pBcl-xL or pFMDV/Bcl-xL than with their plasmid counterparts not encoding the Bcl-xL protein; pGFP or pFMDV alone (Fig. 1c).

Peptide immunization equilibrates the Th1-bias induced by DNA immunization in a peptide-specific manner

After demonstration cell survival effect of Bcl-xL co-expression, mice were immunized with plasmids, peptides or plasmid plus peptides as indicated in Table 1. Specific ELISAs were developed for each one of the four FMDV peptides encoded within our vaccines with the objective of comparatively measuring the specific humoral responses induced. The fact that all animals specifically induced specific IgGs against each one of the peptides used (Fig. 2), clearly demonstrated the successful protocol of vaccination. Mice immunized with the pFMDV/Bcl-xL plasmid tended to show higher levels of specific IgGs (Fig. 2) and IgG2a (Fig. 3), albeit these differences were not statistically significant. Interestingly, immunization with adjuvanted peptide-mix alone or in a prime-boosting regime did not reflect any significant change on the total induction of specific IgGs (Fig. 2), neither on the specific IgG2a/IgG1 ratio (Fig. 3) against peptides: Bo, Bc or T_{VP4}. Conversely, mice immunized with peptide alone or with the pFMDV/Bcl-xL plasmid plus adjuvanted peptide-mix, induced significantly higher levels ($P<0.05$) of specific IgGs against the T_{3A} peptide than those receiving only the pFMDV/Bcl-xL plasmid (Fig. 3c), corresponding with an exponential increase in

Table 2 Amino acid sequences of synthetic peptides; VP1 protein B_C epitope, VP3A protein T_{3A} epitope, VP4 protein T_{VP4} epitope of FMDV C-S8c1 and VP1 protein B_O epitope of FMDV O1K infectious clone

Synthetic peptide sequences	aa sequence
B_O of VP1 (131–157 aa)	R Y N R N A V P N L **R G D** L Q V L A Q K V A R T L P
B_C of VP1 (133–156 aa)	T T T Y T A S A **R G D** L A H L T T T H A R H L
T_{3A} of VP3A (11–40 aa)	Y F L I E K G Q H E A A I E F F E G M V H D S I K E E L R P
T_{VP4} of VP4 (20–34 aa)	S I I N N Y Y M Q Q Y Q N S M

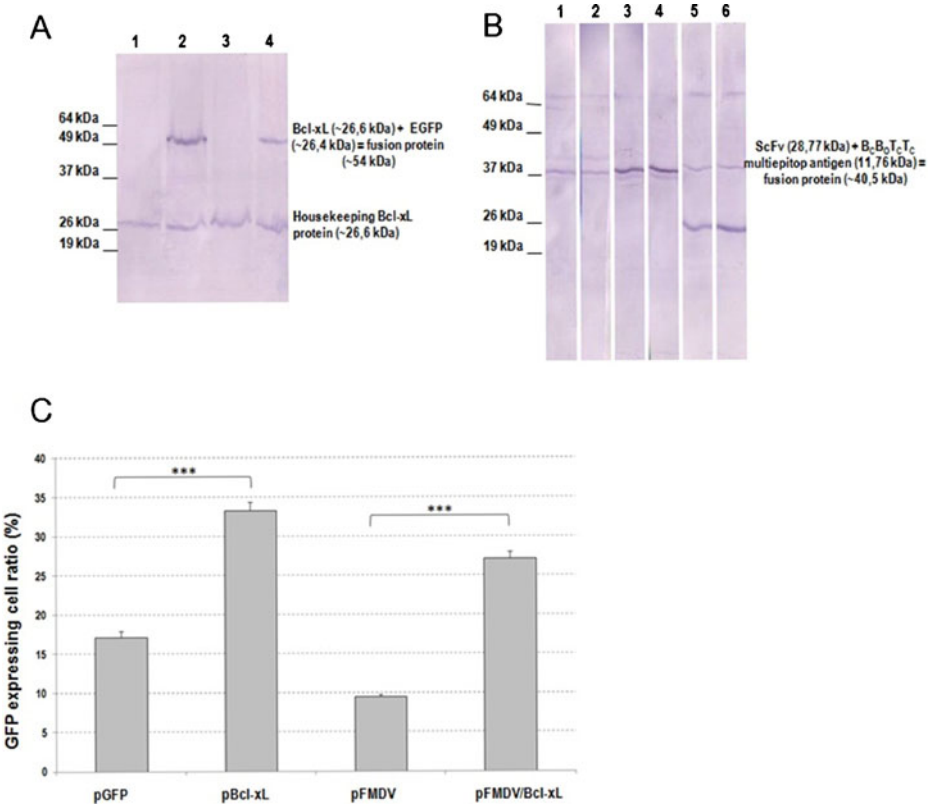

Fig. 1 **a** Western blot detection of Bcl-xL expression using protein extracts from cells transfected with the following plasmids: pGFP (*1*); pBcl-xL (*2*); pFMDV (*3*) and pFMDV/Bcl-xL (*4*). **b** Western blot detection of FMDV epitopes using protein extracts from cells transfected with the pFMDV (lanes *1*, *3* and *5*) and pFMDV/Bcl-xL (lanes *2*, *4* and *6*) plasmids and using monoclonal antibodies against: the B_O (lanes *1* and *2*) with Mab B2, the B_C (lanes *3* and *4*) with Mab SD6 and the 3A (lanes *5* and *6*) with Mab 2C2. **c** GFP detection after BHK-21transfection with plasmids: pGFP; pBcl-xL; pFMDV and pFMDV/Bcl-xL (4); (***, $P<0.001$): error bars represent the standard deviations ($n=3$)

the detection of specific IgG1 (Fig. 3c). Thus, with the exception of this equilibrated balance between IgG1/IgG2a, a clear polarization towards the induction of IgG2a immunoglobulin was observed in the rest of the cases ($P<0.05$), indicative of a Th1 like-response.

Finally, no neutralizing activity was found in sera from immunized mice with the exception of two animals: one belonging to the pFMDV and the other to the pFMDV/Bcl-xL vaccination groups that showed marginal neutralization activity against only C serotypes (data not shown).

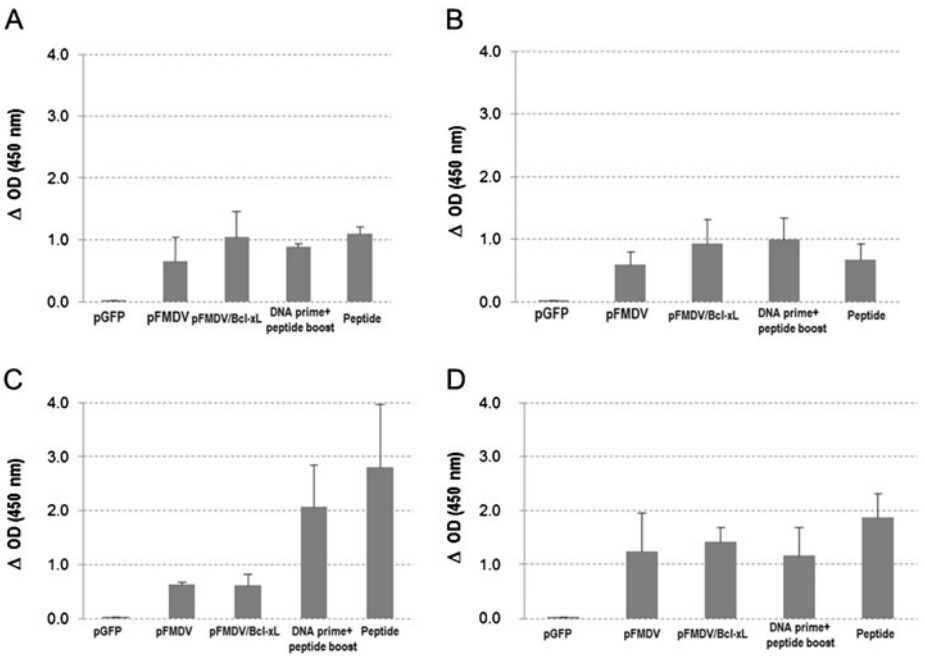

Fig. 2 Total IgG response against Bc (**a**); B_O (**b**); T_{3A} (**c**); and T_{VP4} (**d**) epitopes. ΔD (450 nm) results from subtracting the OD value obtained for each animal using serum at 1/33 dilution before vaccination and after the last immunization with: pGFP, pFMDV, pFMDV/Bcl-xL, DNA prime plus peptide boost or peptide alone

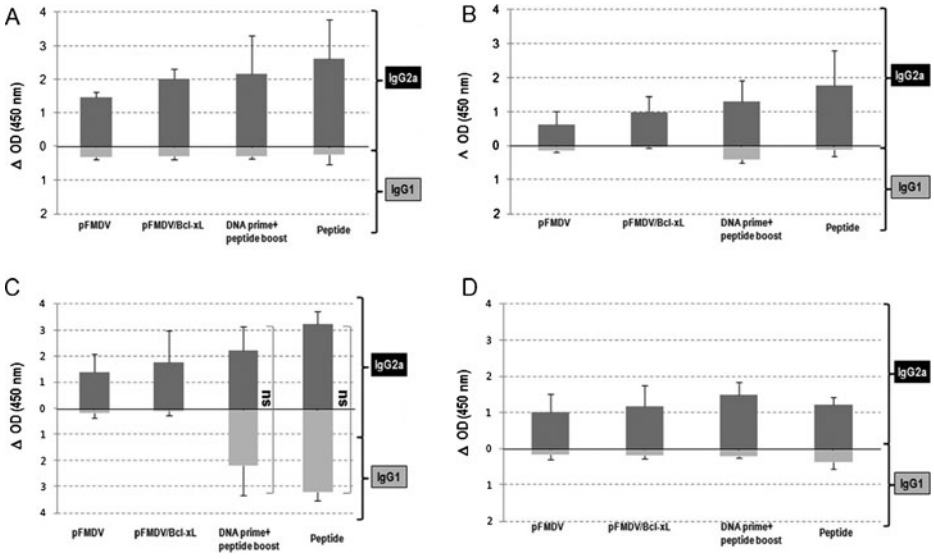

Fig. 3 IgG1 and IgG2a antibody response against Bc (**a**); B_O (**b**); T_{3A} (**c**) and T_{VP4} (**d**) epitopes. ΔD (450 nm)results from subtracting the OD value obtained for each animal using serum at 1/33 dilution before vaccination and after the last immunization with: pGFP, pFMDV, pFMDV/Bcl-xL, DNA prime plus peptide boost or peptide alone, (*ns* non-significant differences, $P>0.05$, the other groups in all graphs are significantly polarized through IgG2a, $P<0.05$)

Co-expression of Bcl-xL enhances the induction of cellular responses after DNA immunization

In order to characterize the cellular responses induced by each immunogen, the spleen cells obtained from each mouse 3 weeks after last immunization were in vitro stimulated with and without the specific peptides and 72 h later, supernatants were harvested to measure the specific secretion of IFN-γ and IL-4, signature cytokines of Th1 and Th2-like responses.

No specific extracellular IL-4 secretion was detected in all vaccination groups (Fig. 4d), however mice immunized with pFMDV/Bcl-xL showed significantly higher levels of extracellular IFN-γ secretion ($P<0.05$) in response to the specific peptide stimulation (Fig. 4d). Confirming these results, mice immunized with pFMDV/Bcl-xL showed higher percentages of peptide-specific INF-γ positive $CD4^+$ (Fig. 4a, $P<0.05$) and $CD8^+$ (Fig. 4b, $P<0.05$) T-cells than the rest of the immunization groups, as shown by INF-γ intracellular staining. In spite of the negative ELISA results, specific intracellular detection of IL-4 was exclusively detected in $CD4^+$ T-cells from mice primed with pFMDV/Bcl-xL and boosted with adjuvanted peptide (Fig. 4c, $P<0.05$), confirming the induction of a Th1/Th2 balance by this vaccine regime.

Discussion

The mechanisms involved in protection against FMDV are not entirely understood (Joshi et al. 2009) however it has been mainly attributed to the induction of neutralizing antibodies (Barteling and Vreeswijk 1991). Increasing evidences have demonstrated that cellular immune responses are also involved in protection (McCullough and Sobrino 2004; Garcia-Briones et al. 2004). Specific IFN-γ induction both from $CD8^+$ T cells (Childerstone et al. 1999; Joshi et al. 2009; Guzman et al. 2010) as a cytotoxic T cell response and $CD4^+$ T cells as a Th1-like response (Glass et al. 1991;

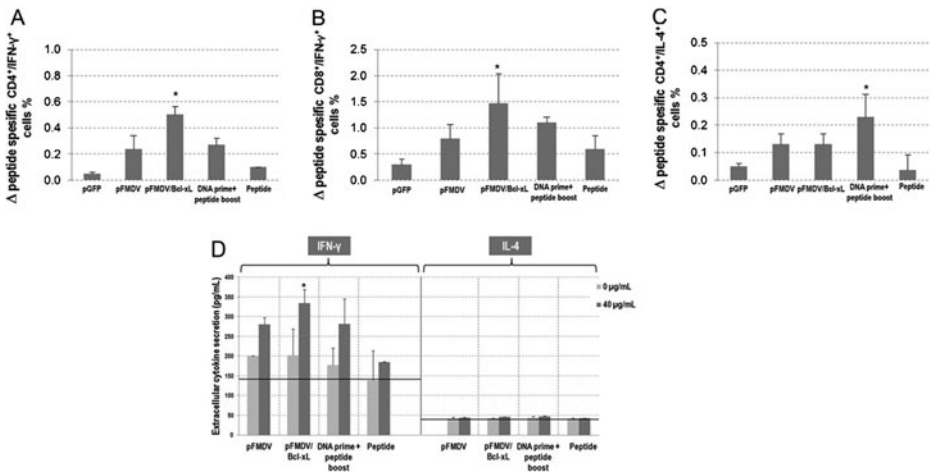

Fig. 4 Detection of specific T cell responses after vaccination. Peptide-specific intracellular detection of IFN-γ in $CD4^+$ (**a**) and $CD8^+$ T-lymphocytes (**b**); peptide-specific intracellular detection of IL-4 in $CD4^+$ T-lymphocytes (**c**); peptide-specific extracellular secretion of IFN-γ and IL4 (**d**) (*: $P<0.05$, indicates significant differences between the vaccination groups)

McCullough and Sobrino 2004; Joshi et al. 2009; Blanco et al. 2001; McCullough and Sobrino 2004), seemed to correlate with protection against FMDV in cattles and pigs. In addition, some studies have demonstrated the potential to induce solid protection against FMDV in the absence of neutralizing antibody titers (Wong et al. 2002; Barnard et al. 2005). On this regard, we have previously showed that DNA vaccines targeting FMDV B and T-cell epitopes to APCs with a single chain antibody driven against the SLAII molecules (named as APCH1 molecule), increased the protection induced against FMD in the absence of detectable antibodies prior to challenge (Borrego et al. 2011). In addition, total protection (no viremia, shedding, nor FMD clinical signs) was afforded in those animals showing Th1-like and SLA II-restricted T-cell responses prior to challenge and an accelerated induction of neutralizing antibodies immediately after FMDV challenge, therefore ratifying the relevance of both arms of the immune response in protection (Borrego et al. 2011).

Aiming to improve the vaccine potency, a dual plasmid were generated coexpressing the B and T cell determinants from FMDV together with the Bcl-xL anti-apopototic protein, a genetic adjuvant previously used against other pathogens to increase both the humoral and the cellular responses induced after DNA vaccination (Kim et al. 2004, 2005; Huang et al. 2007). As expected, in vitro co-expression of Bcl-xL dramatically increased cell survival after serum deprivation, thus confirming previous reports with other antigens (Gulce Iz et al. 2012; Kim et al. 2004; Blomer et al. 1998; Yang et al. 2005; Fiebig et al. 2006). It has been also demonstrated that pBcl-xL plasmid protected cells from serum deprived apoptosis (Gulce Iz et al. 2012).

All vaccination groups induced total IgG responses against each epitope however there were not a significant differences between the vaccination groups. Significant differences could not be determined after vaccination because all plasmids provided optimal amounts of antigen to induce peptide-specific B-cell responses. This result could be in accordance with the results obtained in vitro, with no evident differences on the FMDV epitope expression after transfection of the cells cultured with serum. Comparative DNA immunization experiments with suboptimal amounts of plasmid and one only vaccine dose might be more conclusive on this regard to see significant differences. Similar results were also obtained for the peptide-specific induction of IgG1 and IgG2a, with no significant improvement being observed after inclusion of the Bcl-xL antiapoptotic gene. Interestingly, a clear bias towards a Th1-like immune response ($p<0.05$) was found independently of the peptide specificity tested and/or the plasmid used (Borrego et al. 2006). Interestingly, peptide immunization and/or DNA vaccine priming and peptide boosting dramatically changed the balance of the immune response against the T_{3A} peptide, showing a significant increase in the total IgG and an almost perfect IgG2a/IgG2b balance, perhaps ideal for the future vaccines as demonstrated previously for emergency FMDV vaccines (Barnard et al. 2005).

After, in vitro peptide stimulation, cells in all vaccination groups were capable to secrete IFN-γ which was both detected by extracellular cytokine ELISA and flow cytometry. Both specific CD4 and CD8 T-cells were showed a significant increase those animals immunized with the pFMDV-Bcl-xL plasmid, co-expressing FMDV minigenes and the antiapototic Bcl-xL gene ($p<0.05$). Similar results were obtained in Bcl-xL encoding DNA vaccines against different pathogens such as human papilloma virus (Kim et al. 2004, 2005; Huang et al. 2007). IL-4 secretion was not detectable by extracellular cytokine ELISA however CD4 T-cells secreting IL-4 were detected by flow cytometry. The correlation of IgG1 polarization and IL-4 secretion in only DNA prime (+) adjuvanted peptide boost vaccination group confirms that this vaccine protocol induces Th2-like responses in addition to Th1 like-response against FMDV.

In vitro co-expression of Bcl-xL prolonged cell survival after serum deprivation. Bcl-xL co-expression in vivo was reflected in an improvement in the T-cell responses induced by DNA vaccines encoding FMDV B and T cell epitopes. Thus, immunization with the plasmid pFMDV/Bcl-xL induced more potent specific and IFN-γ secreting $CD8^+$ and $CD4^+$ T cell responses than those induced by the pFMDV plasmid ($p<0.05$). Our results open new expectations for the use of the pFMDV/Bcl-xL plasmid as a vaccine, alone or in combination with adjuvanted peptide, in FMDV hosts.

Acknowledgements This project is partly funded by The Scientific and Technological Research Council of Turkey (110O809), Ege University, Science and Technology Center (2011BIL020) and a complementary action between Spain and Turkey (PCI2005-A7-0092). The authors acknowledge EP Martin, Ph.D. and JM Marques, Ph.D. for their technical help. The specific MAbs against FMDV were kindly provided by Emilliana Brocchi, Ph.D., from Istituto Zooprofilattico Sperimentale Della Lombardia, Italy.

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