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In addition to its antiviral and immunomodulatory properties, the zebrafish β-defensin 2 (zfBD2) is a potent viral DNA vaccine molecular adjuvant

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ABSTRACT

It is well known that β-defensins are key components of the host innate immune response against pathogens and potentially provide a link between innate and adaptive immunity. In zebrafish (*Danio rerio*), a vertebrate model species in numerous biomedical fields, three β-defensin isoforms were recently identified. To our knowledge, however, studies describing antimicrobial or immunomodulatory properties of any of the zebrafish β-defensins isoforms are absent today. Since it is indubitable that deepening the study of zebrafish β-defensins would be of interest in this work we investigated whether or not the zebrafish β-defensin 2 (zfBD2) has the antiviral properties described for their vertebrate counterparts. Our *in vitro* and *in vivo* studies showed that zfBD2 has antiviral activity, immunomodulatory properties and, most importantly, is a potent viral DNA vaccine molecular adjuvant. In addition, a potential relationship between zfBD2 activity and the NF-κB signaling pathway is suggested. Altogether these results show that the zebrafish could be a suitable *in vivo* animal model to study the roles played by β-defensin 2 in viral diseases, vaccinology and even in clinical dermatology. To note that psoriasis can be induced in zebrafish and the over-expression of β-defensin 2 is implicated in the inflammatory response associated with this human skin disorder.

1. Introduction

Pathogen resistance to conventional anti-infective drugs (antibiotics and antivirals) has created a major global health issue. Therefore, there is urgent need to find other non-mainstream therapeutic options (Peters et al., 2010). In this regard, natural anti-infective agents or natural host-defense peptides (HDPs) represent one of the most promising future strategies for combating/preventing infections and microbial drug resistance.

HDPs are gene-encoded components of the immune system that have been selected by evolution as crucial tools of the first line of defense against invading microbes in all living organisms (Hancock and Sahl, 2006; Zasloff, 2002). Overall, these peptides display a potent antimicrobial activity and are rapidly mobilized in order to neutralize pathogens, including viruses, bacteria, protozoa, and fungi (Peters et al., 2010). In addition, the role of HDPs in modulating the innate immune response and boosting infection-resolving immunity, while dampening potentially harmful pro-inflammatory (septic) responses, gives these peptides the potential to become an entirely new therapeutic approach against pathogens (Hancock and Sahl, 2006).

Defensins constitute a family of HDPs with broad range of antimicrobial, antiviral and immunomodulatory activities (Ulm et al., 2012) that have a characteristic β-sheet-rich fold and a framework of six disulfide-linked cysteines (Falco et al., 2008; Ganz, 2005; Lehrer and Ganz, 2002). Among the three subfamilies of defensins (α-, β- and θ-defensins), β-defensins are probably the most interesting because, in contrast to α- and θ-defensins (Owen et al., 2004; Selsted, 2004), they are present in most of the plant and animal species explored so far (Ganz, 2004; Pazgier et al., 2006; Thomma et al., 2002).

The broad spectrum of activities of β-defensins is well-illustrated in studies carried out in both higher and lower vertebrates. Overall, these studies show that in addition to their role as potent antiviral/bacterial agents (Chattopadhyay et al., 2006; Falco et al., 2008, 2009; Garcia et al., 2001; Guo et al., 2012; Harder et al., 1997; Howell et al., 2007; Jiang et al., 2009; Jin et al., 2010; Klotman and Chang, 2006; Liu et al., 2002; Quinones-Mateu et al., 2003; Schroeder et al., 2011; Selsted and Ouellette, 2005; Sun et al., 2005; Valore et al., 1998), β-defensins are key components of the innate response and potentially provide a link between

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innate and adaptive immunity (Bowdish et al., 2006; Funderburg et al., 2007; Ganz, 2002; Oppenheim et al., 2003; Pazgier et al., 2006; Pingel et al., 2008; Yang et al., 2002, 1999, 2010). Moreover, these immunomodulatory properties are consistent with several studies that use β-defensins as vaccine adjuvants (Biragyn et al., 2002; Kohlgraf et al., 2010; Mei et al., 2012; Mutwiri et al., 2007; Tani et al., 2000; Zhang et al., 2010). On the other hand, β-defensins have also relevance in other biomedical fields as dermatology since they are implicated in human skin disorders including psoriasis and atopic dermatitis (Jansen et al., 2009).

In the zebrafish (*Danio rerio*), a vertebrate model species in numerous biomedical fields (Rakers et al., 2013; Rauta et al., 2012), three β-defensin isoforms were recently identified (Zou et al., 2007). To our knowledge, however, studies describing antimicrobial or immunomodulatory properties of any of the zebrafish β-defensins are absent. Taking into account that (i) the successful zebrafish developmental model has expanded and become also a model for the analysis of host-pathogen interactions during infectious disease (Phelps and Neely, 2005), (ii) numerous pathogens have been demonstrated to infect zebrafish, such as rhabdoviruses (Boltana et al., 2013; Encinas et al., 2010; Sanders et al., 2003), and new mechanisms of virulence and host defense have been revealed using this new model (Phelps and Neely, 2005) and (iii) β-defensins are crucial players of the first line of defense against invading microbes, it would be of interest to deepen the study of zebrafish β-defensins.

To that end, in this work we investigated both *in vitro* and *in vivo*, whether or not zebrafish β-defensins have some of the properties described for their vertebrate counterparts. Out of the three isoforms, we chose the zebrafish β-defensin 2 (zfBD2) to carry out this study because a transcriptomic study (Encinas et al., 2010) suggests that, *in vivo*, the induction of zfBD2 may be involved in the early immune response of zebrafish skin and secondary lymphoid-organs to the viral haemorrhagic septicaemia virus (VHSV), a fish rhabdovirus (Gomez-Casado et al., 2011). Our *in vitro* and *in vivo* studies showed that zfBD2 has antiviral activity, immunomodulatory properties and is a potent DNA vaccine adjuvant. In addition, a potential relationship between zfBD2 activity and NF-κB signaling pathway is suggested.

Altogether these results show that the zebrafish could be a suitable *in vivo* animal model to study the roles played by β-defensin 2 in viral infectious diseases, vaccinology and even in clinical dermatology. Psoriasis, for instance, can be induced in zebrafish (Webb et al., 2008) and the overexpression β-defensin 2 is implicated in the inflammatory response associated with this skin disorder.

2. Materials and methods

2.1. DNA constructs

The cDNA encoding the sequences of the zebrafish β-defensin 2 (zfBD2) (GenBank acc. number NM_001081554) and the spring viraemia of carp virus (SVCV) glycoprotein G (gpG_{SVCV}) (Genebank acc. number Z37505) were synthesized by GenScript (CA, USA) and then sub-cloned into the plasmid constructs pAE6 (containing the 5′-regulatory sequences of the carp β-actin gene, (Brocal et al., 2006; Ortega-Villaizan et al., 2009) and pMCV1.4 (containing the CMV promoter, (Chico et al., 2009), respectively, following standard procedures to generate the plasmid constructs pAE6-gpG_{SVCV} and pMCV1.4-zfBD2.

2.2. Cell cultures and virus

The fish cell line ZF4 (zebrafish embryonic fibroblast) (Driever and Rangini, 1993) purchased from the American Type Culture Collection (ATCC number CRL-2050) was used in this work. ZF4 cells were maintained at 28 °C in a 5% CO_2 atmosphere with RPMI-1640 Dutch modified (Gibco, Invitrogen corporation, UK) cell culture medium containing 10% fetal calf serum (FCS) (Sigma, St. Louis, USA), 1 mM Pyruvate (Gibco, Invitrogen Corporation, UK), 2 mM Glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml fungizone.

The isolate 56/70 of SVCV isolated from carp (Stone et al., 2003) was propagated in ZF4 cells at 22 °C. Supernatants from SVCV-infected cell monolayers were clarified by centrifugation at 4000*g* for 30 min and kept in aliquots at −70 °C. Clarified supernatants were used for both *in vitro* and *in vivo* infection assays.

2.3. Cell transfection assays

Cell transfection assays were performed as previously described (Ortega-Villaizan et al., 2011). Briefly, ZF4 cell monolayers, grown in culture flasks of 75 cm^2, were detached using trypsin (Sigma), washed, resuspended in cell culture medium supplemented with 10% of FCS and dispensed into 96-well cell culture plates. The following day, plasmid DNA incubated with 0.3 μl of FuGene 6 (Roche, Barcelona, Spain) for 30 min in RPMI-1640 was added (1/5 of the total volume of the culture medium in each well) to the wells containing ZF4 cells in culture medium with 10% of FCS. The plates were further incubated at 24 or 28 °C for the times indicated in each experiment. The expression of zfBD2 and gpG_{SVCV} in ZF4-transfected cells was evaluated by quantitative PCR (qPCR) in real time as described below.

2.4. In vitro cell infection assays

ZF4 cells transfected with pMCV1.4.zfBD2 or pMCV1.4 were infected with SVCV (multiplicity of infection, m.o.i., of 3×10^{-1}) in a final volume of 100 μl/well of culture medium supplemented with 2% FCS at 22 °C for 90 min. Infected cell monolayers were then washed, fresh medium added, and plates further incubated until the end of each experiment. SVCV replication in ZF4 cells was evaluated by qPCR using specific primers and probe sequences for the gene encoding the protein N of SVCV (Table 1). Non-transfected ZF4 cells infected with SVCV were included as control.

2.5. In vitro zfmx gene expression

ZF4 cells were transfected with pMCV1.4-zfBD2 or pMCV1.4 as indicated above and then incubated at 28 °C for 72 h. After the incubation period, cell total RNA was extracted and cDNA synthesis carried out as indicated below. The *zfmx* gene transcript levels (both isoforms A and B) were evaluated by RT-qPCR as described below.

2.6. Fish

Adult zebrafish (*D. rerio*) of 2–3 g (~4 cm in length) were obtained from a local fish pet shop and maintained at 28 °C in 30 l tanks equipped with a re-circulating dechlorinated water system. Fish were fed daily with a commercial feed diet. Prior to experiments, fish were acclimatised to laboratory conditions for 2 weeks. All experiments with live animals (zebrafish) were performed using protocols approved by the European Union Council Guidelines (86/609/EU).

2.7. Intramuscular injection of zebrafish with pMCV1.4-zfBD2

To evaluate the expression, protection against SVCV challenge or effect of zfBD2 on the immune system, zebrafish were anaesthetized by immersion in 50 μg/ml buffered tricaine

Table 1
Primer and probe sequences.

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Probe	Gene bank accession No./Ref.
zf βD2	AATGTGCATAATGCCGAAGTACA	ACAACCATGGTGAGCAACAATATATT		NM_001081554.1
mx A-B	GGTCTCTGGGAGTCGAAAAGG	AACTCTTTCCCGAGCTTTGGT		NM 182942-AJ544824
mx C	AAGAGCCCTGCCTAAGGTTGT	ATTTCACACCCAGACGGAACTC		NM_001007284.2
il1 β	TCATCATCGCCCTGAACAGA	CATGTCCAGCACCTCTTTTTCTC		NM 212844
il-10	AGGAACTCAAGCGGGATATGG	TGTTGACTTCAAAGGGATTTTGG		NM_001020785.2
tnf α	AAGCCACTTTTCAGTGCAATCC	AGCGCCGAGGTAAATAGTGTTG		NM 212859
igm	TCGGTTTGTCTCAGTGCAGTTC	TCTGCAGGTGAGAAACCTTTAGC		BC154613.1
t-bet	ACACTGGCACTCACTGGATG	CTCCTTCACCTCCACGATGT		AM 942761/(Mitra et al., 2010)
gata3	ACCGGTGGACATCTCAAAGC	AAGCGGGACAGGTATCAAAGG		BC 162389
ifn-γ	TTGCCAGGCTGTGTTGCTT	TTTATTATCTGACTTGTTCATCATGTCTTG		AB194272.1
mhc2	ACCAGACCGCCGTCAAGA	CCCTCTCTCTGGTTGGAGTAAATG		NM_131590
foxp3	GTGGAAAAGCCGGCACAT	TTCAGTTGCTGCTCTCTCACAGT		FM 881778
cd83	CAGCAGAAGACAGTGGGATATACAG	CGCCTCGCTGGTTCTGAT		BM157226
dc-sign	TGACCCACAGGTGGCTGTT	TTCTGCAGGTGTTAGTCTGCTTTT		NM_001199373.1
nk lysin	CAATGGGAGATGCACAAAGAAC	CACATCCCAGGCAGTTGCT		NM 212741
granzyme	TTGGCTGGTGGGATGGATA	CCATGTATGGTCTTGAATGAGGTTTA		NM 001128550
gpG_{SVCV}	GCTACATCGCATTCCTTTTGC	TCGACCAGATGGAACAAATATGG	ATTGACTCCAACTTAGGAAT-MGB	Z37505.1
N_{SVCV}	GCATTATGCCGCTCCAAGAG	AGCTTGCATTTGAGATCGACATT	CGCAAGACAGCCTGACGAGATCGAC	AJ318079.1
ef1 α	CCACGTCGACTCCGGAAA	CGATTCCACCGCATTTGTAGA		NM 131263

zf βD2, zebrafish beta defensin 2; ***mx A–B***, mx protein (isoforms A and B); *mx C*, mx protein (isoform C); ***il1β***, interleukin-1 beta; ***il-10***, interleukin-10; ***tnf α***, tumor necrosis factor alpha; ***igm***, zebrafish immunoglobulin constant μ chain; ***t-bet***, T-cell-specific t-box transcription factor 21; ***gata3***, trans-acting T-cell-specific transcription factor gata3; ***ifn-γ***, interferon gamma; ***mhc2***, major histocompatibility complex class 2 invariant chain alpha; ***foxp3***, forkhead-box protein P3; ***dc-sign***, C-type lectin domain 4 member L; $\boldsymbol{gpG_{SVCV}}$, SVCV glycoprotein G; $\boldsymbol{N_{SVCV}}$, SVCV nucleocapsid (N) protein; ***ef1α***, cellular elongation factor 1 alpha.

methanesulfonate (MS-222; Sigma) prior to handling and then divided into 3 groups (30 fish each). Then, fish from each group were intramuscularly injected with pMCV1.4-zfBD2 (2 μg of plasmid in 10 μl of phosphate buffered saline, PBS, per fish), pMCV1.4 empty plasmid (2 μg of plasmid in 10 μl of phosphate buffered saline, PBS, per fish) or injected with the same volume of PBS. After DNA injection, fish were transferred to a fresh water tank until they recovered from (1–2 min) and swam normally again. At day 5 post-injection, 5 fish from each group were sacrificed by overexposure to MS-222, and samples of muscle tissue were excised from the site of injection to evaluate the expression levels of a set of immune-related genes by RT-qPCR as indicated below. The genes and their corresponding primers used for *in vivo* gene expression analysis are listed in Table 1. Ten days post-injection, the remaining fish in the tanks (25 fish per tank) were infected with SVCV as indicated below.

2.8. DNA vaccination assays

Zebrafish DNA vaccination assays were carried out as follows. Briefly, fish were anaesthetized by immersion in 50 μg/ml buffered MS-222 prior to handling and then divided into five groups (25 fish each). Groups were intramuscularly immunized with one of the following: 10 μl of PBS (non-immunised or control fish) or 10 μl of PBS containing 10 μg of pAE6-gpG_{SVCV}, 1 μg of pAE6-gpG_{SVCV}, 0.5 μg of pMCV1.4-zfBD2, 1 μg of pAE6-gpG_{SVCV} plus 0.5 μg of pMCV1.4-zfBD2 or 1 μg of pAE6 plus 1 μg of pMCV1.4 empty plasmids. At day 40 post-immunisation fish were challenged with SVCV as indicated below. Two independent DNA immunisation experiments were performed.

2.9. Challenge with SVCV

The fish were first acclimated at 22 °C for two days prior to challenge with SVCV. Then, zebrafish were challenged by bath immersion. For this, fish were introduced in 2 l of water containing 10^7 PFU/ml for 90 min at 22 °C. The tanks were then filled with fresh dechlorinated water and the flow through the filters was restored. Mortality was recorded daily for 21 days and the cumulative percentages of mortality (CPM) were calculated using the formula: CPM = (amount of dead fish/initial amount of fish in the tank) × 100.

2.10. Virus recovery from fish injected with pMCV1.4-zfBD2 and then infected with SVCV

Ten days after intramuscular injection with pMCV1.4-zfBD2 or pMCV1.4, fish were infected with SVCV as indicated above to determine whether zfBD2 conferred some protection against SVCV. At 4 days post infection, three fish with SVCV-infection symptoms from each of the groups were sacrificed by overexposure to MS-222 and tissue samples from fins, spleen, gills and head kidney removed. Tissue samples were then mixed, disaggregated using a sterile nylon cell strainer (BD Falcon, MA, USA) and subsequently passed through 0.2 μm sterile filters to remove bacterial contamination. Tissue homogenates were suspended in 3 ml of RPMI-1640 2% FBS cell culture medium and then used to infect ZF4 cell monolayers. Virus titers present in tissue homogenates were determined by plaque assays. Briefly, different dilutions of the tissue homogenates (from 0.1 to 0.0001) were added to ZF4 cell monolayers, grown in 24-well plates, at 22 °C for 90 min. Then, the culture media were removed and infected cell monolayers covered with a solution of RPMI-1640 cell culture medium with 2% FCS and a 2% aqueous solution of methyl cellulose (Sigma). Cell plates were incubated at 22 °C for 5 days and then the media with methyl cellulose was removed. Finally, wells were stained with crystal violet-formalin to count plaques. Virus titers were expressed as plaque forming units (pfu) per ml.

2.11. RNA isolation and RT-qPCR assays

The E.Z.N.A HP Total RNA kit (Omega bio-tek, Norcross, GA, USA) and E.Z.N.A HP Tissue RNA kit (Omega bio-tek) for the *in vitro* and the *in vivo* experiments, respectively, were used for total RNA extraction in accordance with the manufacturer's instructions; isolated RNAs were stored at −80 °C until used. One microgram of RNA, as estimated by a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.), was used to obtain the cDNA using the Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) as previously described

in (Falco et al., 2008). Quantitative PCR was performed using the ABI PRISM 7300 System (Applied Biosystems, NJ) as previously described (Chico et al., 2010). Primers and probes used in both *in vitro* and *in vivo* expression assays are listed in Table 1. Reactions were performed in a total volume of 20 µl volume comprising 2 µl of cDNA reaction mixture, 900 nM each primer, 10 µl of TaqMan universal PCR master mix (Applied Biosystems) and 200 nM of probe or SYBR green. The cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

In cell transfection assays transcript abundances of zfBD2 and SVCV N protein genes were analyzed by 2^{-Ct} method (Martinez-Alonso et al., 2011) where ΔCt is determined by subtracting the Ct value of the endogenous gene, the cellular elongation factor 1 α (EF1α), from the target gene Ct. Results of SVCV infection were expressed as percentage of infectivity and calculated by the formula (SVCV infectivity in pMCV1.4-zfBD2 transfected cells/SVCV infectivity in non-transfected cells) × 100. In the case of *in vivo* gene expression and *in vitro zfmx* induction assays results were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak, 2001), also using as an endogenous control for quantification the EF1α gene. Control fish (fish injected with PBS) served as calibrator and fold of increases were calculated relative to gene expression levels of these fish. Non-template controls (NTCs) and controls without reverse transcriptase (RT) were included for each gene in all RT-qPCR assays.

2.12. Immunofluorescence assays

To study the presence and localization of the subunit p65 of the NF-κB factor in response to zfBD2 expression, ZF4 cell monolayers, grown in 96-well plates at 28 °C, were transfected with either pMCV1.4.zfBD2 or pMCV1.4. (75 µg/ml) plasmids as indicated above. One and 48 h after transfection, cell monolayers were fixed for 15 min with a 4% paraformaldehyde solution (in phosphate buffered saline, PBS) and then 15 min in cold methanol. For p65 detection, fixed ZF4 cell monolayers were incubated overnight with a polyclonal antibody antihuman RelA/p65 (Thermo Fisher Scientific Inc.) diluted 200-fold in PBS with 0.3% Triton ×100 (Merck, Darmstadt, Germany) at 4 °C. Cells were then washed with PBS and incubated for 2 h with FITC-labeled goat anti-rabbit immunoglobulin G antibody (Sigma) diluted 300-fold in PBS with 0.3% Triton ×100. Finally, the cells were washed 3 times with PBS. To visualize the nuclei of the cells, the monolayers were then counterstained with 4′-6-diamidino-2-phenylindole (DAPI) for 10 min and washed again with PBS. Stained cells were viewed and photographed with an inverted

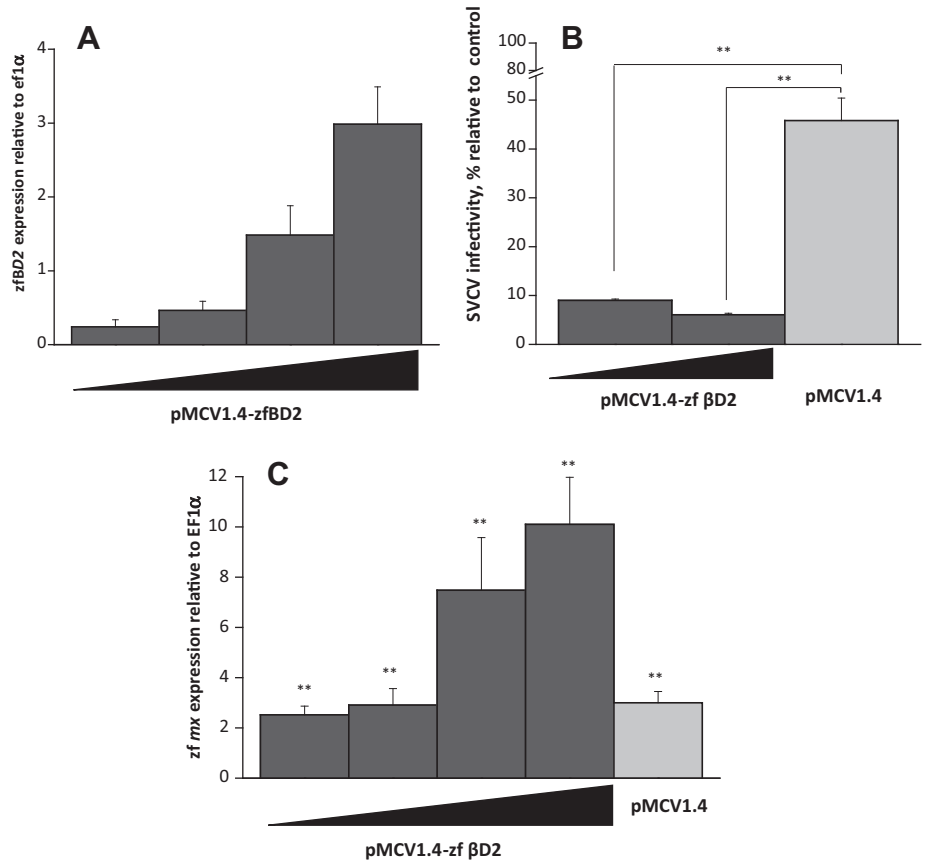

Fig. 1. zfBD2 expression (A), SVCV infectivity (B) and fold changes in the zf*mx*A-B genes expression in response to the expression of zfBD2 (C). (A) ZF4 cells were transfected with different concentrations of the pMCV1.4-zfBD2 (0.10, 0.25, 0.50 and 0.75 µg/ml). After 72 h of incubation at 28 °C total RNA was extracted and the expression of transcripts of zfBD2 was estimated by RT-qPCR. Fold increases were calculated relative to the expression levels of the *ef1α* endogenous gene. Results represent the mean ± SD of two independent experiments each performed in duplicate. (B) ZF4 cells previously transfected with pMCV1.4-zfBD2 or pMCV1.4 empty plasmids were infected with SVCV (m.o.i. 3×10^{-1}) at day 3 post-transfection. 24 h after infection, SVCV infectivity was evaluated by RT-qPCR. Results are expressed as percentages and represent the mean ± SD of two independent experiments each performed in duplicate (**$p < 0.01$ vs. pMCV1.4 empty plasmid transfected cells). Dark grey bars, ZF4 cells transfected with 0.50 and 0.75 µg/ml of pMCV1.4-zfBD2; light grey bar, ZF4 cells transfected with 0.75 µg/ml of pMCV1.4 (C) ZF4 cells were transfected with pMCV1.4-zfBD2 using the DNA concentrations indicated in A. 72 h post-transfection total RNA was extracted and the expression of *zfmx*A-B transcripts was estimated by RT-qPCR. Fold increases were calculated relative to the expression levels for non-transfected ZF4 cells. Results represent the mean ± SD of two independent experiments each performed in duplicate (**$p < 0.01$ vs. non-transfected ZF4 cells).

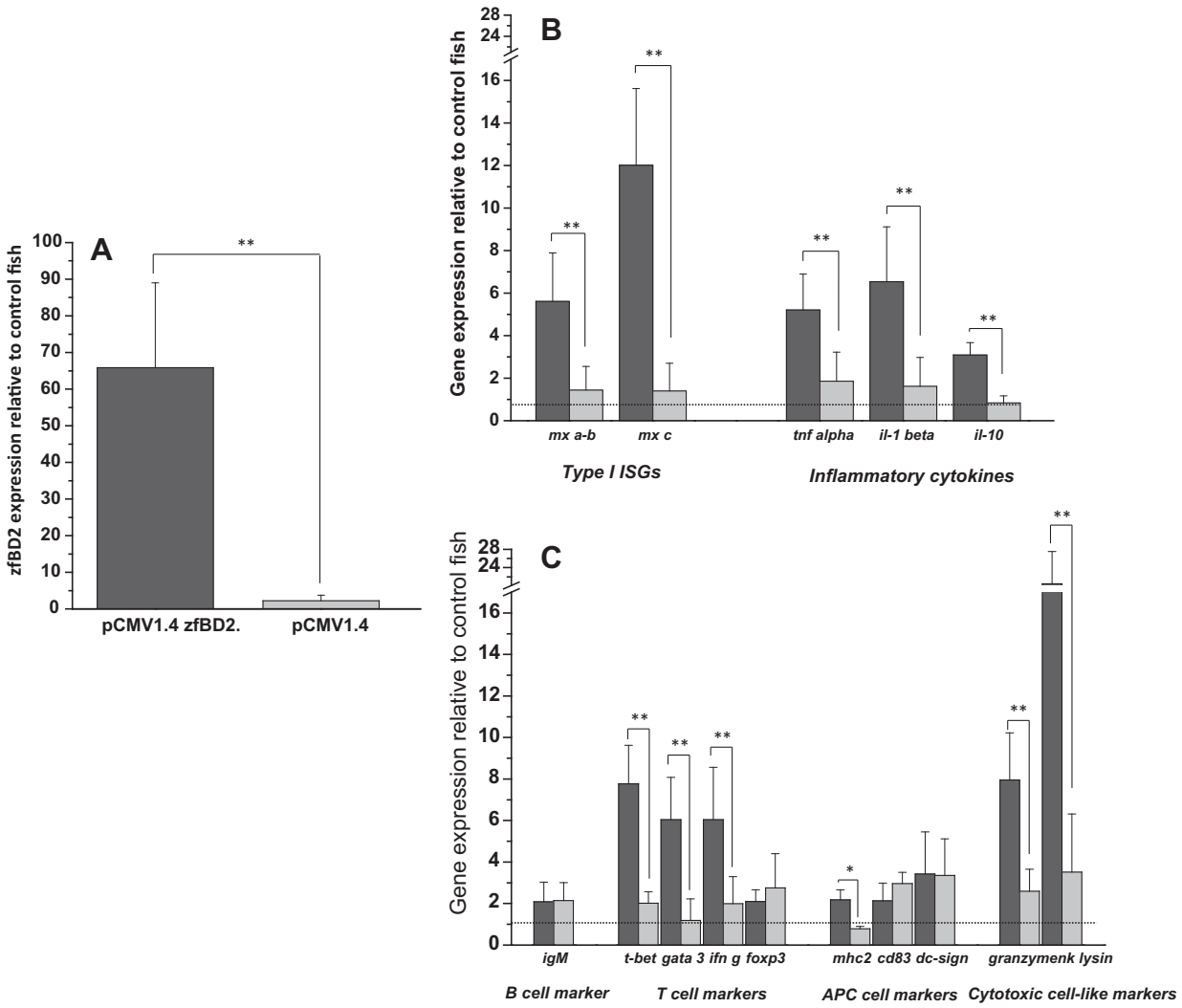

Fig. 2. zfBD2 expression in skeletal muscle of zebrafish (A) and RT-qPCR analysis of the immune-related gene expression pattern induced by zfBD2 expression (B and C). Zebrafish were injected intramuscularly with 2 μg pMCV1.4-zfBD2 or 2 μg pMCV1.4. After 5 days, the injected animals were sacrificed by anaesthetic overdose and muscle tissue at the injection site excised and processed. (A) Expression of zfBD2 transcripts in zebrafish muscle tissue estimated by RT-qPCR. (B and C) Gene expression pattern of immune-relevant genes induced by the intramuscular injection of the plasmids. In all cases, fold increases were calculated relative to the expression levels of the same genes in mock-injected fish (PBS-injected) and results represent the mean ± SD of five fish per group from two different experiments. Dark grey bars, fish injected with pMCV1.4-zfBD2; light grey bar, ZF4 fish injected with pMCV1.4 ($^{*}p < 0.05$ and $^{**}p < 0.01$ vs. pMCV1.4 empty plasmid injected fish).

fluorescence microscope (Nikon Eclipse TE2000-U; Nikon Instruments, Inc., NY, USA) provided with a digital camera (Nikon DS-1QM).

2.13. Statistical analysis

Statistical analysis was carried out using the Graph Pad Prism 5 software. All data are shown as means ± SD. Differences between groups were evaluated using ANOVA followed by a post hoc test (Tukey's). Statistical significance was defined by *p*-values $p < 0.05$ and <0.01. Mortality data were analyzed using the nonparametric Mann–Whitney *U* test $p < 0.05$.

3. Results

3.1. Resistance of ZF4 cells expressing zfBD-2 to SVCV infection

To study the antiviral properties of the zebrafish β-defensin 2 (zfBD2), the zfBD2 cDNA sequence was cloned into the pMCV1.4 (Chico et al., 2009) downstream of the human cytomegalovirus (CMV) promoter, thus generating the pMCV1.4-zfBD2.

First, the *in vitro* expression of zfBD2 was evaluated by RT-qPCR. For that, ZF4 cells were transiently transfected with different amounts of the pMCV1.4-zfBD2 (from 0.1 to 0.75 μg/ml) and the zfBD2 expression was determined at 72 h post-transfection. RT-qPCR analysis revealed that pMCV1.4 vector efficiently expressed zfBD2 mRNA in a dose-dependent manner in ZF4 cells (Fig. 1A) showing the maximal expression at a pMCV1.4-zfBD2 concentration of 0.75 μg/ml.

Having confirmed the successful expression of zfBD2, their potential antiviral properties were studied using as model a virus, the SVCV, that infects zebrafish *in vivo* (Boltana et al., 2013; Sanders et al., 2003). ZF4 cells transiently expressing the zfBD2 were infected with SVCV and 24 h later SVCV infectivity evaluated. The results showed an 89% and 94% reduced SVCV infectivity in ZF4 cells transfected with 0.50 and 0.75 μg/ml of pMCV1.4-zfBD2, respectively (Fig. 1B). To evaluate whether or not the reduction of SVCV infectivity was due to a specific effect of zfBD2 expression, cells transfected with 0.75 μg/ml of pMCV1.4 were also infected. As

expected, the cells transfected with the empty plasmid showed partial resistance to SVCV infection but propagated the virus much more efficiently than the cells transfected with the plasmid encoding the zfBD2 (Fig. 1B).

3.2. Transfection of ZF4 cells with zfBD2 induces expression of the zfmx gene

Since it is has been reported that antiviral properties of fish β-defensins can be related to the type I IFN system induction (Falco et al., 2008, 2009), possible changes in the expression levels of the *mx* gene (both A and B *mx* gene isoforms) were analyzed by qPCR in ZF4 cells transfected with pMCV1.4-zfBD2. The interferon stimulated gene (ISG) *mx* was chosen as a marker for IFN-mediated response because Mx proteins have proven to be very specific and sensitive markers for type I IFN induction (Simon et al., 1991; von Wussow et al., 1990). As expected, *zfmx* gene expression was up-regulated in zfBD2-ZF4 transfected cells (Fig. 1C). The level of expression of *zfmx* transcripts was proportional to the amount of pMCV1.4-zfBD2 transfected showing the maximal induction (12-fold) at DNA concentrations $\geqslant$0.75 μg/ml (Fig. 1C). In response to pMCV1.4 (empty plasmid) cell transfection also up-regulated the *zfmx* gene expression (3-fold) (Fig. 1C). On the other hand, expression levels of *zfmx* gene (Fig. 1C) in ZF4-transfected cells correlated with the reduction of SVCV infectivity (Fig. 1B).

3.3. Immuno-related gene expression pattern induced by the in vivo expression of zfBD2

First, since the *in vivo* conditions governing the expression of the proteins encoded by the plasmids might be different from those *in vitro* (Xiang et al., 1995), the expression of the zfBD2 in the muscle of zebrafish injected with pMCV1.4-zfBD2 was assessed by RT-qPCR. Fish injected with pMCV1.4-zfBD2 overexpressed zfBD2 transcripts by 65-fold over control fish (PBS injected fish); this overexpression was not observed in fish injected with the pMCV1.4 empty plasmid (Fig. 2A) suggesting that plasmid DNA does not induce the *in vivo* expression of β-defensins, at least in zebrafish.

Next, the immune response induced by the *in vivo* expression of zfBD2 was evaluated through RT-qPCR at day 3 post-injection. To analyze this response a representative set of immune-related genes was chosen (Table 1). These included *mx* gene isoforms A-B and C (ISGs), *tnfα*, *il1β* and *il10* (inflammatory cytokines), *igm* (the B-cell membrane form of IgM), *tbet* and *gata3* (T-cell transcription factors implicated in T helper (Th) cell polarization towards either Th1 or Th2, respectively), *ifnγ* (a characteristic cytokine of a Th1-mediated immune response), *foxp3* (a master regulator in the differentiation of T regulatory cells), *nklys* and *granzyme* (antimicrobial peptides secreted by immune cytotoxic cells such as cytotoxic T cells and NK cells), *mhc2* (major histocompatibility complex class 2), *cd83* (a marker of mature dendritic cells, DCs) and *dc-sign* (an important protein that associates with antigen presenting cells, APCs, and participates in the adaptive response (Lin et al., 2009)).

Overall, at the primary site of zfBD2 delivery (skeletal muscle), the expression of all genes was significantly induced by day 3 after injection in the fish injected with the pMCV1.4-zfBD2 plasmid compared to control fish. Moreover, some of these genes (*tnfα*, *igm*, *tbet*, *foxp3*, *cd83*, *dc-sign* and *granzyme*) were also significantly induced in response to the empty plasmid injection compared to control fish. However, in this case the induction levels were always much lower than those in pMCV1.4-zfBD2-injected fish (Fig. 2B and C). Therefore, since the aim of this study was to determine whether or not the changes in the gene expression pattern were due to zfBD2 expression and not to the plasmid DNA, the degree of statistical significance of the differences observed between pMCV1.4-zfBD2 and pMCV1.4-injected fish was evaluated.

The expressions of all isoforms (A–B and C) of the *mx* gene were higher in fish overexpressing zfBD2 (pMCV1.4-zfBD2-injected fish) than in the fish injected with the empty plasmid (~3-fold for the isoform A–B and ~6-fold for the isoform C) (Fig. 2B). Strikingly, the transcripts of *mx* isoform C were more abundant (2-fold) than those of the *mx* A–B in the fish overexpressing zfBD2. Since all *mx* gene isoforms showed similar expression levels in the fish injected with the empty plasmid this results suggest a specific induction of the *mx* gene isoform C by the zfBD2. Additionally, overexpression of zfBD2 had a potentiating effect on the transcript expression levels of cytokines associated with inflammatory processes such *as tnfα*, *il1β* and *il10* compared to both control and empty plasmid-injected fish (Fig. 2B). In fact, in the fish injected with pMCV1.4-zfBD2 *tnfα* and *il1β* expression levels were 2- to 3-fold higher than

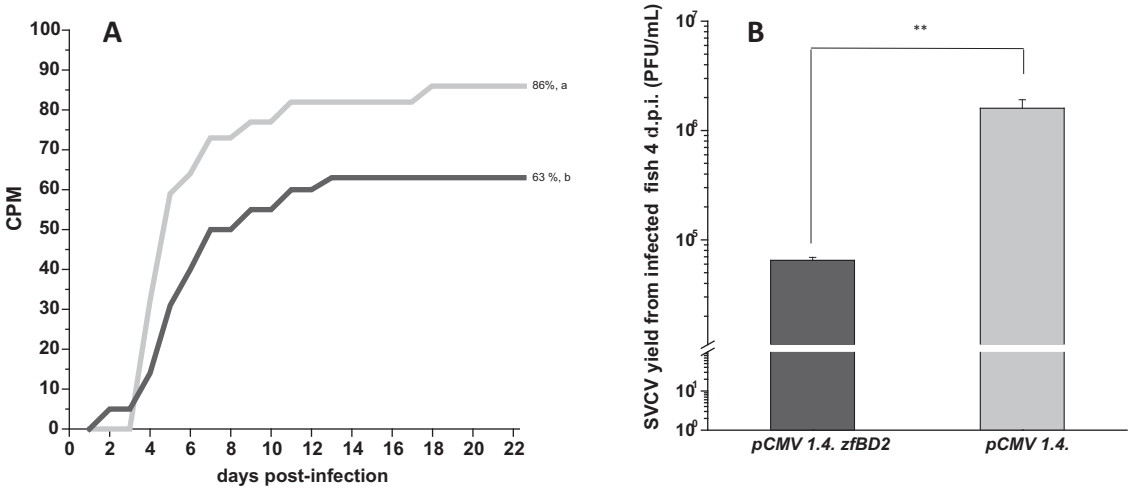

Fig. 3. Cumulative percent mortality after SVCV challenge (A) and virus yields recovered from infected fish (B). Adult zebrafish were intramuscularly injected with 2 μg of pMCV1.4-zfBD2 or pMCV1.4 plasmids and then exposed by bath immersion to a lethal dose of SVCV (107 PFU/ml) at 10 days post-immunisation. (A) CPM, Cumulative percent mortality. Mortality was monitored daily for 21 days after challenge. The graph is representative of the results obtained in two independent experiments. Dark grey line, fish injected with pMCV1.4-zfBD2; light grey line, fish injected with pMCV1.4. Different letters denote statistically significant differences between the groups according to Mann–Whitney *U* test, $p < 0.05$. Data followed by the same letter are not significantly different. (B) Virus yields recovered from tissue homogenates of zebrafish ($n = 3$) injected with pMCV1.4-zfBD2 (dark grey bars) or pMCV1.4 (light grey bars) at 4 days after challenge with SVCV. Results represent the mean ± SD of two independent experiments each performed in triplicate.

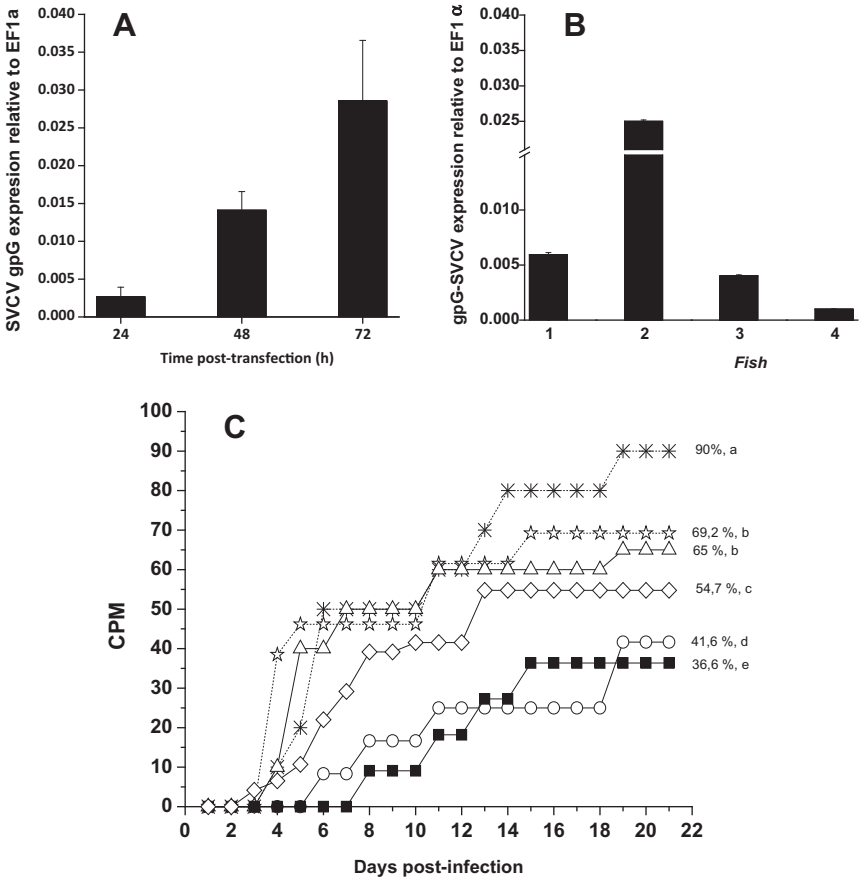

Fig. 4. In vitro (A), *in vivo* (B) expression of the surface glycoprotein G (gpG) of SVCV and DNA vaccine adjuvanting effects of zfBD2 (C). (A) Time course of the transcript expression of gpGsvcv in ZF4 cells after transfection at 24 °C with 0.75 μg/mL of pAE6-gpGsvcv. Results represent the mean ± SD of two independent experiments each performed in duplicate. (B) gpGsvcv expression in zebrafish muscle after intramuscular injection with 1 μg of pAE6-gpGsvcv. Results represent the mean ± SD of two independent experiments each performed in duplicate. (C) Cumulative percent mortality after challenge with SVCV (~107 PFU/ml) of fish immunised by intramuscular injection with 10 μg of pAE6-gpGsvcv (open circles), 1 μg of pAE6-gpGsvcv (open diamond), 0.5 μg of pMCV1.4-zfBD2 (triangles), 1 μg of pAE6-gpG$_{svcv}$ + 0.5 μg of pMCV1.4-zfBD2 (black squares), 1 μg of pAE6 + 1 μg of pMCV1.4 empty plasmids (asterisks). Non-immunised or PBS-injected control group (open stars). Challenge was carried out at 40 days post-immunisation and mortality monitored daily for 21 days. The graph is representative of the results obtained in two independent experiments. Different letters denote statistically significant differences between the groups according to Mann–Whitney *U* test, $p < 0.05$. Data followed by the same letter are not significantly different.

in the fish injected with the empty plasmid. Interestingly, up-regulation of the *il10* gene expression was only observed in the fish overexpressing the zfBD2 suggesting that plasmid DNA has no effect of the on *il10* gene transcription (Fig. 2B).

On the other hand, the transcripts of major histocompatibility class II (*mhc2*) in adult zebrafish were significantly more abundant in pMCV1.4-zfBD2-injected fish compared to control fish (2-fold) whereas in fish injected with the empty plasmid expression of this gene was not induced (Fig. 2C). In contrast, expression levels of *dc-sign* (or *cd209*) and *cd83*, all up-regulated compared to control fish, shows no differences between fish groups (pMCV1.4-zfBD2 or pMCV1.4-injected fish) (Fig. 2C). Therefore, it is likely that no specific recruitment of mature APCs after zfBD2 expression at the primary site of plasmid delivery occurs.

In contrast to what is suggested in relation with APC cells, a significant increase in the recruitment of cytotoxic cells, according to the expression levels of cytotoxic T and NK cell peptides *granzyme* (Chowdhury and Lieberman, 2008) and *NK lysin* (Andersson et al., 1995), in response to the expression of zfBD2 was detected in the muscle of fish injected with pMCV1.4-zfBD2 (Fig. 2C) compared to both control and pMCV1.4-injected fish (Fig. 2C). In particular, this difference was very significant in the expression of *NK lysin* since the transcript expression levels of this antimicrobial peptide (AMP) were 25 and 6-fold higher in pMCV1.4-zfBD2-injected fish than in muscle samples of control and pMCV1.4-injected fish, respectively.

Regarding the presence of B-cells, no significant differences were found between pMCV1.4-zfBD2 and pMCV1.4-injected fish since the expression levels of the membrane form of *Igm* were similar in both groups of fish (Fig. 2C). Finally, to characterize the potential migrating CD4$^+$ (or T helper, Th) cell population, the expression levels of *t-bet* and *gata3*, two specific transcription factors (TFs) of the Th1 and Th2 cells subsets, respectively (Kanhere et al., 2012), were measured. Both TFs increased their expression in pMCV1.4-zfBD2-injected fish (7- and 6-fold, respectively, compared to control fish) (Fig. 2C) as opposed to fish injected with empty plasmid where no up-regulation of these genes was observed. This might indicate that zfBD2 is directly implicated in the recruitment of Th cells. The differences between the expression levels of t-*bet* and *gata3* and the up-regulation of the hallmark cytokine of Th1 cells, IFNγ, in the muscle of fish injected with the pMCV1.4-zfBD2 suggests a Th1-biased immune cell response induced by zfBD2. Interestingly, *foxp3*, a TF involved in the differentiation of T regulatory (Treg) cells and autoimmunity was not differentially expressed in either pMCV1.4 or pMCV1.4-zfBD2-immunised fish (Fig 2C).

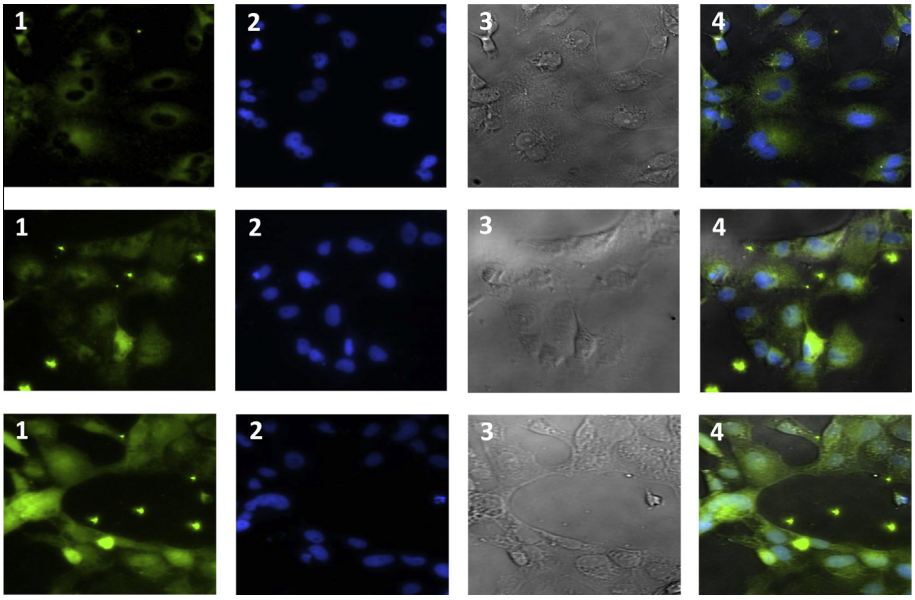

Fig. 5. Localisation of the subunit p65 of the transcription factor NF-κB in ZF4 cells at 1 (Fig. 5) and 48 (Fig. 6) hours post-transfection with 0.75 μg/mL of pMCV1.4-zfB2 (lower panel) or pMCV1.4 empty plasmid (middle panel) and in non-transfected cells (upper panel). Transfected cells were incubated overnight with a specific polyclonal antibody anti-p65. Subsequently, cells were incubated with a FITC-labelled secondary antibody to stain p65 antibody-bound units (green) and viewed by means of fluorescence microscopy. Nuclei of the cells were stained with DAPI (blue). 1, p65 of the transcription factor NF-κB; 2, cell nuclei, 3 bright field and 4 merged images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

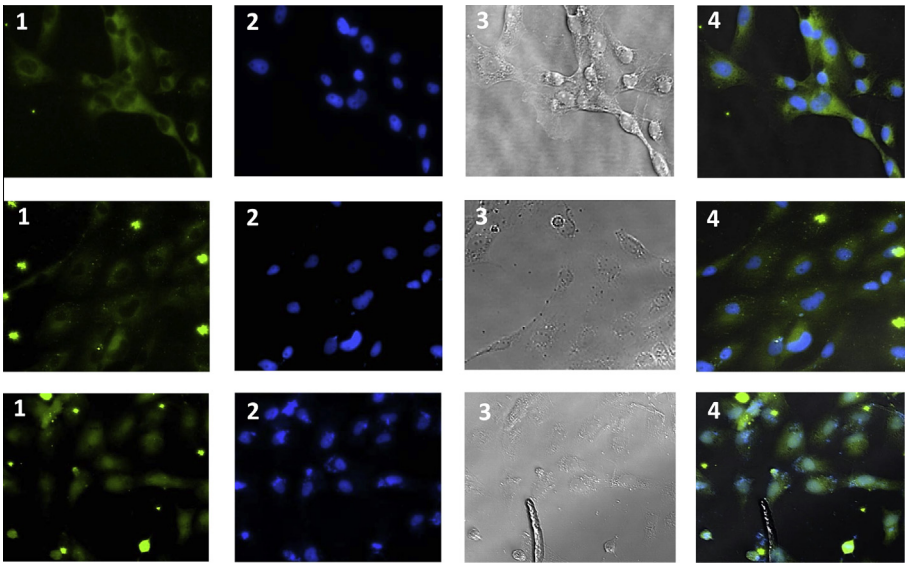

Fig. 6. Localisation of the subunit p65 of the transcription factor NF-κB in ZF4 cells at 1 (Fig. 5) and 48 (Fig. 6) hours post-transfection with 0.75 μg/mL of pMCV1.4-zfB2 (lower panel) or pMCV1.4 empty plasmid (middle panel) and in non-transfected cells (upper panel). Transfected cells were incubated overnight with a specific polyclonal antibody anti-p65. Subsequently, cells were incubated with a FITC-labelled secondary antibody to stain p65 antibody-bound units (green) and viewed by means of fluorescence microscopy. Nuclei of the cells were stained with DAPI (blue). 1, p65 of the transcription factor NF-κB; 2, cell nuclei, 3 bright field and 4 merged images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Resistance of zebrafish expressing zfBD2 to SVCV infection

Since, (i) the immune-related gene expression pattern induced by the *in vivo* overexpression of *zfBD2* (type I IFN system induction, increased expression of pro-inflammatory cytokines and genes of the major histocompatibility class II, Th cell recruitment, etc.) partially resembles a typical antiviral response and (ii) *zfBD2* showed antiviral activity *in vitro*, the potential antiviral activity of zfBD2 against SVCV infection was evaluated *in vivo*. To that end, adult zebrafish were intramuscularly injected with 2 μg of pMCV1.4-zfBD2 or pMCV1.4 and after 10 days infected with a lethal dose of SVCV (~10^7 PFU/ml). As expected, the resistance to SVCV infection was higher (24%) in the fish injected with pMCV1.4-zfBD2 than in the fish injected with the empty plasmid (Fig. 3A). In addition, the viral yield recovered at 4 days post infection was over 20-fold lower in fish over-expressing zfBD2 (Fig. 3B) indicating a direct correlation among zfBD2 expression, virus replication rates and fish survival rates.

3.5. zfBD2 as DNA vaccination adjuvant

These results as well as previous studies (Mei et al., 2012; Tani et al., 2000; Zhang et al., 2010) suggest that one of the potential uses of the zfBD2 could be as an adjuvant in DNA vaccination. To assess this possibility we designed an immunization/infection experiment where fish were immunized with a DNA vaccine based on the SVCV surface glycoprotein G (pAE6-gpG_{SVCV}), a strategy that has been proven less effective than similar vaccines against other fish rhabdoviruses such as VHSV or IHNV (Emmenegger and Kurath, 2008; Kanellos et al., 2006), in the presence or absence of the plasmid encoding the zfBD2 (pMCV1.4-zfBD2).

Before vaccination/challenger assays, the gpG_{SVCV} was cloned into a vector under the control of 5′-regulatory sequences of the beta-actin carp gene, pAE6, and the expression of gpG_{SVCV} expression assessed both *in vitro* (ZF4 cell monolayers) and *in vivo* (zebrafish skeletal muscle). RT-qPCR analysis revealed that pAE6 vector efficiently drives the expression of gpG_{SVCV} in ZF4 cells showing the maximum expression at 72 h post-transfection (Fig. 4A). Likewise, gpG_{SVCV} transcripts could be detected in the muscle of fish injected with pAE6-gpG_{SVCV}. Since gpG_{SVCV} transcript expression levels were highly variable, Fig. 4B shows the expression of each individual fish.

Having established the successful expression of gpG_{SVCV}, fish were immunized 1 μg of the plasmid encoding the gpG_{SVCV} (pAE6-gpG_{SVCV}) and 0.5 μg of the plasmid encoding zfBD2 (pMCV1.4-zfBD2). As controls, fish were injected with PBS, 1 μg of pAE6 plus 1 μg of pMCV1.4 (empty plasmids) or 1 μg of pAE6-gpG_{SVCV} alone (DNA vaccine). As well, fish were injected with 10 μg of the plasmid encoding the gpG_{SVCV} gene (pAE6-gpG_{SVCV}) because it has been demonstrated that 10 μg of a plasmid DNA encoding the gpG of SVCV provided protection (50–80%) in vaccinated fish against challenge at low, moderate, and high virus doses of an homologous virus (Emmenegger and Kurath, 2008).

Immunised and non-immunised fish were challenged by bath-immersion at 40 days post-immunisation with 10^7 PFU SVCV/ml. Fig. 4C shows the cumulative percentage of mortality (CPM) from groups immunized intramuscularly with each of the plasmid vaccine constructs. In agreement with previous reports (Emmenegger and Kurath, 2008), significant protection (∼60%, Fig. 4C open circles) was observed in fish immunised with 10 μg of the pAE6-gpG_{SVCV} plasmid. As expected 1 μg of the vaccine alone (pAE6-gpG_{SVCV}) conferred less protection (45%, Fig. 4C open diamonds) than at the highest dose (10 μg). Nonetheless, when 1 μg of the DNA vaccine was combined with 0.5 μg of pMCV1.4-zfBD2 the protection level achieved was even higher than that observed with 10 μg of vaccine (65%, Fig. 4C black squares). This confirms the hypothesis that proposes that zfBD2 should be considered as an adjuvant candidate in DNA vaccination studies. As expected, fish that received either PBS (Fig. 4C, asterisks) or the empty plasmid combination (Fig. 4C, open stars) had higher rates of mortality showing CPM values of ∼90% and 70% respectively (Fig. 4C). Regarding the fish injected only with the plasmid encoding the zfBD2, protection rates of ∼65% were found (Fig. 4C, open triangles).

On the other hand, every dead fish presented extensive haemorrhages in the abdominal cavity, a typical symptom of SVCV infection (data not shown).

3.6. zfBD2 activates the nuclear factor κB (NF-κB) signaling pathway

Other vertebrate β-defensins have been shown to activate the NF-κB through TLR-mediated signaling pathways (Yang et al., 2010) and do so even by binding TLRs (Biragyn et al., 2002). Thus, we investigated whether or not the zfBD2 could induce the translocation of NF-κB to the cell nucleus. Immunofluoresce assays in ZF4 cells showed that at one hour post-transfection with either pMCV1.4-zfBD2 or pMCV1.4 empty plasmid, NF-κB translocated to the nuclei (Fig. 5 middle and lower panels) as opposed to non-transfected cells where NF-κB remained in the cytoplasm (Fig. 5 upper panel). This effect is likely to be caused by the CpG bacterial DNA contained in both plasmid constructs (Kawai and Akira, 2007). At 48 h post-transfection however, NF-κB was translocated to the nuclei in the cells transfected with pMCV1.4-zfBD2 plasmid (Fig. 6 lower panel); in contrast, NF-κB was not translocated in either pMCV1.4-tranfected cells or non-transfected control cells (Fig. 6 upper and middle panels) which indicates that the translocation of this transcription factor at 48 h is specifically due to zfBD2 expression.

4. Discussion

In the present work we described for the first time several biological traits of a zebrafish β-defensin isoform, the β-defensin 2 (zfBD2). To that end, the zfBD2 cDNA sequence obtained from the zfBD2 mRNA (Zou et al., 2007) was cloned into a DNA backbone, the pMCV1.4 plasmid, containing the CMV promoter sequence with demonstrated ability to drive the expression of different transgenes in fish cells both *in vitro* and *in vivo* (Chico et al., 2009; Tafalla et al., 2007) including the rainbow trout β-defensin 1.

Using this plasmid construct (pMCV1.4-zfBD2) *in vitro* transfection–infection assays in a zebrafish-derived cell line (ZF4) showed that zfBD2 drastically inhibited the infectivity of SVCV, a fish rhabdovirus. Likewise, it was previously described that trout β-defensin 1 abolished the infectivity of VHSV, another fish rhabdovirus (Falco et al., 2008, 2009). Fish rhabdoviruses seem, therefore, to be highly susceptible to β-defensins actions; this opens the possibility of developing new strategies/agents based on β-defensins/β-defensins-like peptides to treat fish rhabdoviral infections. Similarly, antiviral activity of vertebrate β-defensins against other virus including HIV-1 (Quinones-Mateu et al., 2003; Sun et al., 2005), adenovirus (Klotman and Chang, 2006), influenza virus (Gong et al., 2010; Jiang et al., 2009; Leikina et al., 2005), parainfluenza virus 3 (PIV-3) (Grubor et al., 2004), respiratory syncytial virus (RSV) (Meyerholz et al., 2007), vaccinia virus (VV) (Howell et al., 2007) herpes simplex virus (Hazrati et al., 2006), Chandipura virus (Chattopadhyay et al., 2006) and iridovirus (Jin et al., 2010), has been reported.

On the other hand, up-regulation of the interferon stimulated (IS) *zfmx* gene was found in ZF4 cells overexpressing zfBD2 suggesting some interrelationship between the induction of the type I IFN system and zfBD2 antiviral activity as it was previously reported for other fish β-defensins (Falco et al., 2008; Guo et al., 2012). Consequently, the modulation of the innate antimicrobial immunity rather than a direct effect on virions might be underlying the antiviral actions of zfBD2 in transfected ZF4 cells. In this case, zfBD2 should also show immune enhancement capabilities *in vivo*. To verify this hypothesis adult zebrafish were injected with pCMV1.4-zfBD2 and the immune response induced by the overexpression of zfBD2 at the injection site was evaluated at early times post-injection. In addition, to confirm that the immune responses were specifically induced by zfBD2 a group of fish was injected with the empty plasmid, pCMV1.4. Briefly, gene expression analysis showed that, *in vivo,* zfBD2 (i) triggered the activation of the type I IFN-system as indicated by the up-regulation of the 3 different isoforms of the IS *mx* gene, (ii) induced the transcription of genes related to the inflammatory response such as *tnfα* and *il1β*, (iii) increased the presence of *mhc2* transcripts and therefore the potential presentation of antigens by MHC class II molecules, (iv) enhanced the immune-related cytotoxic cell responses as indicated

by the increased presence of transcripts of the AMPs granzyme and mainly NK lysine, (v) mediated the recruitment of Th cells at the injection site and (vi) elicited a Th1-biased immune response as indicated by the differences between the expression levels of t-*bet* and *gata3* and the up-regulation of IFNγ, the hallmark cytokine of Th1 cell responses. Taken together, these properties, also shown by other β-defensins including the human β-defensin 2 (Pazgier et al., 2006; Tani et al., 2000; Yang et al., 2002; Yeung et al., 2011) manifest the strong regulatory-like properties of the zfBD2 upon the host antimicrobial immunity as well as its functions as cell-derived signals that promote the adaptive immune response.

At this point the question was whether or not zebrafish over-expressing zfBD2 were more resistant to a viral infection. As expected, immunization with pMCV1.4-zfBD2 reduced fish mortality after SVCV lethal challenge by 24% compared to fish injected with the empty plasmid. Moreover, this reduced mortality rates correlated with the lower viral yields recovered from different organs of infected fish suggesting that the overexpression of zfBD2 impedes, to a certain extent, the entry and/or replication and/or spread of SVCV. Prior to this study, inhibition of influenza virus replication and viral protein synthesis by defensin cell-mediated mechanisms has also been described (Salvatore et al., 2007).

Finally, we evaluated the potential of zfBD2 to enhance the efficacy of a DNA vaccine based on the glycoprotein G of SVCV (gpG_{SVCV}), the only surface antigen of this virus (Huang et al., 2012). We chose to use vaccination against SVCV in this small proof of concept study because although DNA vaccines containing the gpG_{SVCV} gene can partially protect fish (50–80%) against SVCV, at least doses of 10 μg of DNA per fish are needed (Emmenegger and Kurath, 2008). In contrast, administration of 1 μg of DNA encoding the sequences of the glycoprotein G of the fish rhadoviruses VHSV or IHNV is enough to coffer protection levels ⩾90% against virus lethal challenge (Alonso and Leong, 2013; Corbeil et al., 2000; Chico et al., 2009; Lorenzen et al., 2002; McLauchlan et al., 2003). Here we demonstrate that the protection rates upon SVCV lethal challenge in zebrafish immunized with 1 μg of DNA vaccine encoding the gpG_{SVCV} gene in combination with 0.5 μg of zfBD2 encoding plasmid were higher than those conferred by the immunization with 10 μg of DNA vaccine encoding gpG_{SVCV} gene alone. Thus, these results clearly indicate that zfBD2, through its adjuvant-like effects, improves the gpG_{SVCV} DNA vaccine efficacy. For other defensins, adjuvanting effects in DNA immunization assays have been reported in cancer studies (Mei et al., 2012) and DNA vaccination studies against viruses (Zhang et al., 2010).

In other species of vertebrates, β-defensins are ligands of the cellular receptors of immune cells such as CCR6 (Yang et al., 1999) or TLR4 (Biragyn et al., 2002). TLR activation triggers a signaling pathway that can lead to the translocation of the TF NF-κB (Kawai and Akira, 2007), a master TF of immune processes (Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009) and crucial for the activation of T cells (Kawai and Akira, 2007). In mice, the avian β-defensin 13 (AvBD13) activates NF-κB, probably after binding TLR4 (Zhang et al., 2010). These results strongly suggest that responses elicited by defensins or other HDPs in vertebrates are orchestrated initially by TFs, such as NF-κB. Taking advantage of the existence of primary antibodies to detect p65, a protein subunit of NF-κB, in zebrafish cells (Kanther et al., 2011) we set out to investigate the zfBD2 effect on this TF. The immunofluorescence assays showed that zfBD2 has the ability to induce NF-κB activation as indicated by the presence of this TF in the nuclei of zfBD2-expressing cells. This is the first time this effect of a β-defensin upon NF-κB is reported in teleost fish, shedding light on how HDPs may regulate immunity in this group of animals. In summary, zebrafish could be a suitable *in vivo* animal model to study the roles played by β-defensins in an age in which most pathogens have developed resistance to commonly used antimicrobial agents and improved viral vaccines need to be developed.

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