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How modular protein nanoparticles may expand the ability of subunit anti-viral vaccines: The spring viremia carp virus (SVCV) case

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ABSTRACT

Spring viremia of carp (SVC) remains as a vaccine orphan disease mostly affecting juvenile specimens. Young fish are especially difficult to vaccinate and oral administration of vaccine combined with food would be the election system to minimise stress and the vaccination costs associated to injection. However, administration of prophylactics with food pellets faces off several drawbacks mainly related with vaccine degradation and weak protection correlates of oral vaccines. Here we present a platform based on recombinant proteins (subunit vaccines) manufactured as highly resistant nanostructured materials, and providing excellent levels of protection against SVC virus in a preliminary i.p injection challenge. The G3 domain of SVCV glycoprotein G was overexpressed in *E. coli* together with IFNγ and the modular protein was purified from bacterial aggregates (inclusion bodies) as highly organised nanostructured biomaterial (nanopellets, NP). These SVCV-IFN^{NP} were taken up by zebrafish cells leading to the enhanced expression of different antiviral and IFN markers (e.g *vig1*, *mx*, *lmp2* or *ifngr1 among others*) in zebrafish liver cells (ZFL). To monitor if SVCV^{NP} and SVCV-IFN^{NP} can be taken up by intestinal epithelia and can induce antiviral response we performed experiments with SVCV^{NP} and SVCV-IFN^{NP} in 3 days post fertilization (dpf) zebrafish larvae. Both, SVCV^{NP} and SVCV-IFN^{NP} were taken up and accumulated in the intestine without signs of toxicity. The antiviral response in larvae showed a different induction pattern: SVCV-IFN^{NP} did not induce an antiviral response while SVCV^{NP} showed a good antiviral induction. Interestingly ZF4, an embryonic derived cell line, showed an antiviral response like ZFL cells, although the *lmp2* and *ifngr1* (markers of the IFNγ response) were not overexpressed. Experiments with adult zebrafish indicated an excellent level of protection against a SVCV model infection where SVCV-IFN^{NP} vaccinated fish reached 20% cumulative mortality while control fish reached over 80% cumulative mortality.

1. Introduction

Spring viremia of carp (SVC) is an infectious viral disease common to carp (*C. carpio*) and other cyprinid fish species (including zebrafish (*Danio rerio*), koi (*Cyprinus carpio koi*), silver carp (*Hypophthalmichthys molitrix*), goldfish (*Carassius auratus*) or grass carp (*Ctenopharyngodon idella*) among others, caused by the SVC virus (SVCV). SVCV is a member of the family Rhabdoviridae, with negative-sense ssRNA encoding five proteins—nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA-dependent polymerase (L). The G protein is the most important viral antigenic protein determining the infectivity and serological properties of the virus [1].

Therapeutic or prophylactic approaches to combat SVCV have been ineffective and no commercial vaccine is currently available. The expansion of the geographical and host infection range of SVCV poses a major threat not only to the fish farms and ornamental fish industries,

Abbreviations: IBs, Bacterial inclusion bodies; NPs, Nanopellets; SVCV, Spring Viremia of Carp Virus; iRFP, near-infrared fluorescent protein.

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but also to wild fish populations [2]. SVC outbreaks can reach 90% of mortality in juvenile fish and although there are no approved vaccines for SVC, inactivated, live-attenuated, DNA or other vaccines have been tested in experimental trials [1,3,4]. However, attenuated vaccines pose a slight risk as there were very rare cases where attenuated strains may revert to pathogenic and may cause disease [5], and in the case of DNA vaccines the security concerns are related to the use of DNA vectors. In this context, subunit vaccines overcome these security issues since do not contain exogenous genetic material. Drawbacks of subunit vaccines are: a high susceptibility to degradation of the protein (labile system) and lack of pathogen associated molecular patterns (PAMPS) that the immune system utilizes to recognize pathogens via pattern recognition receptors (PRRs) thus needing adjuvants with co-stimulatory activity to enhance the magnitude of the immune response [5].

In this study, we have designed a vaccination platform based on highly stable protein antigenic protein module (G protein fragment) with in-vaccine included adjuvant properties (IFNγ protein module). In 1957, the IFNs were originally described as molecules that "interfere" with viral replication [6]. Since then, two types of IFNs, (type I and type II) have been characterized in several fish species. Within the fish type II IFNs only two members, IFN-γ and IFN-γrel have been reported that signal through CRFB13 and CRFB17 receptors (IFNGR1-2 and IFNGR1-1 respectively) [7]. IFN-γ and IFN-γrel differs on the number of *N*-glycosylation sites that would provide glycosylated IFN-γ with an extended half-life [8]. Upon activation by the IFN-γ, the IFNGR1 and IFNGR2 recruit Janus Kinase (JAK) 1 and JAK2 to the cytosolic domain respectively, leading to phosphorylation and activation of STAT1 that migrates into the nucleus and binds to the IFN-γ activation sites (GAS) and, initiates the gene expression of IFN-γ dependent genes [9]. In zebrafish also 2 isoforms of IFNγ (IFN-γ and IFNγ-rel) have been reported [8], however treatment of adult zebrafish with IFN-γrel failed to mediate resistance against viral and bacterial infections [10]. We focused our attention on IFNγ because in mammals is a highly versatile molecule with different functions such as regulation of Th1 responses and clearance of intracellular pathogen, regulation of macrophage activation and function [11,12] or promotion of antigen presentation to inhibit viral replication among others [13]. In teleosts the expression of IFNγ is induced by different virus including SVCV, VHSV, RGNNV, IHNV and also by synthetic mimics such as Poly(I:C) [13]. At the same time IFNγ is able to induce expression of antiviral genes and can inhibit virus replication at least *in vitro* [8,9]. IFNγ may also have antibacterial activity by increasing NOS, ROS production and the phagocytic activity of macrophages [8].

On the other hand, toxic effects of IFNγ in zebrafish larvae have been described probably associated to an excessive activation of inflammation [14]. The zebrafish embryo is also able to fight viral infections, such as SVCV, snakehead rhabdovirus and nervous necrosis virus [15,16] and during the early phase of viral infections, pathogens induce *tlr3, tnfa, mxa-c, viperin* [17] whereas bacterial infection led to the induction of pro-inflammatory cytokines such as *tnfa* [17]. All together led us to speculate that inclusion of IFNγ as an in-vaccine adjuvant may potentiate the performance of the novel recombinant vaccine system.

Another important issue previously mentioned is the instability of recombinant proteins when expressed and purified in its native form. To overcome this issue, we use recombinant protein purified from inclusion bodies (IB). These IBs have a specific nanostructured conformation and contain a mixture of aggregate scaffolds and native protein, and are extremely resistant to harsh chemical and biological conditions [18]. These structural features make our vaccination platform also fit very well with oral vaccination approaches, for those situations where oral administration together with food pellets would be the choice in fish vaccinology a non-stressful and inexpensive administration method is highly desirable, and administration of vaccines mixed with feed would be the election for juveniles [19]. In the present study, a proof of concept of vaccination with recombinant nanostructured SVCV G attached to IFN, induced a strong innate immune response in zebrafish that correlated with significant protection against SVCV challenge.

2. Materials and methods

2.1. Design, production, and characterization of nanostructured viral antigenic proteins

2.1.1. Viral strains and plasmids

Recombinant SVCV G protein was designed based on the glycoprotein G sequence (G3 region, AA 254–381, Uniprot acc. no: Q91DS0) reported in Zhu et al., 2019 as the most antigenic [20]. Recombinant SVCV G antigen with the interferon gamma module (SVCV-IFN) was based on the sequence of the G3 region (254–381) linked to the sequence of the IFN gamma 1 from *D. rerio* (27–175) (NCBI GenBank NP_998029.1). Clones were designed using the ORF and pET22b removing the periplasmic location signal and including a C terminal polyHis-tag. Clones were codon optimized for expression in *E. coli*, synthesized by GeneScript and subcloned into pET22b. Plasmids were transformed into *E. coli* BL21 (DE3) (Novagen). The red fluorescent protein (RFP), iRFPHis cloned into pET22b (Genscript), was used as a non-relevant control protein.

2.2. Production of NPs, purification, quantification, and fluorescent labeling

Methods for recombinant protein production and characterization were previously reported [21]. Briefly, some modifications were introduced for these particular constructs: *E. coli* were cultured overnight (O/N) in LB medium (Gibco) with ampicillin (100 μg/ml, Sigma-Aldrich) and recombinant protein expression in *E. coli* was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Panreac) when $OD_{550\ nm}$ reached 0.5–0.8. Growth was carried out for 4,5 h at 37 °C. For NPs purification, the cell cultures were subjected to an enzymatic and mechanical disruption process [18]. Lysozyme (1,5 μg/ml, Roche), protease inhibitor cOmplete EDTA-free Tablets (Roche) and phenyl-methanesulfonyl fluoride (PMSF, Roche) (0.4 mM) were added and incubated at 37 °C for 2 h at 250 rpm. Cultures were then frozen at −80 °C overnight (O/N). After thawing, Triton X-100 (Roche) was added (0.2% v/v) and stirred for 1 h at room temperature (RT). Then, DNase I (1 μg/ml) and $MgSO_4$ (1 M) were added, and the cultures were incubated for 1 h at 37 °C with shaking. NPs were harvested by centrifugation at 15,000×*g*, 15 min at 4 °C and resuspended in lysis buffer (50 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA) at 1/10 the original culture volume. Finally, several freeze thaw cycles were performed to remove any remaining viable bacteria. Pellets of purified NPs, named $SVCV^{NP}$ and $SVCV\text{-}IFN^{NP}$ were stored at -−80 °C until use. Protein was semi-quantified by western blot using an anti-His-tag antibody (Genscript A00186-100), and the protein concentration was calculated using Image Lab 6.0.1 software (Bio-Rad). For flow cytometry or confocal microscopy, NPs were conjugated with Atto-488 NHS ester (Sigma-Aldrich) according to the manufacturer's instructions. Labeling efficiency was determined with the Nanodrop ND-1000 (Thermo Scientific).

2.3. Characterization of viral recombinant protein NPs

Electron Microscopy was used to determine the external morphology and physical dimensions of the NPs. Samples were prepared by resuspending NPs at 100 μg/ml in mQ water, pipetting 20 μl onto silicon chips, and air-dried O/N. NPs were exposed to different *in vitro* conditions. For pH, NPs were incubated under gentle stirring at pH 2 and 10 for 3 h together with a control sample. For temperature, NPs were incubated for 5 min at 100 °C. After incubation all NPs were centrifuged at 10.000×*g* for 5 min at 4 °C and resuspended at 10 and 50 μg/ml in phosphate buffered saline (PBS, Sigma-Aldrich) and finally 20 μl were deposited onto silicon chips, and air-dried O/N. Electron microscopy

images were taken with a FESEM (Zeiss Merlin) and analyzed using Fiji package (National Institute of Health, USA). At least 60 particles were measured, and size distribution histograms were generated using Past 4.04 software (University of Oslo).

2.4. Cell culture and virus

Zebrafish ZFL cells (CRL-2643, ATCC) were cultured according to Thwaite et al. [21] at 28 °C and 5% CO_2 in DMEM + GlutaMAX (Gibco), 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 0.01 mg/ml insulin (Sigma-Aldrich), 50 ng/ml epidermal growth factor (Sigma-Aldrich), 2% (v/v) antibiotic/antimycotic (Gibco), and 0.5% (v/v) trout serum which had been filtered (0.20 μm filter Corning) and heat inactivated for 30 min at 56 °C.

Zebrafish embryonic fibroblast (ZF4 cells) were cultivated at 28 °C and 5% CO_2 in RPMI medium (Gibco) supplemented with 10% FBS (Cultek), 1 mM pyruvate (Gibco), 2 mM L-glutamine (Gibco), 50 μg/ml gentamicin (Gibco) and 2 μg/ml amphotericin B (Gibco).

Fathead minnow (*Pimephales promelas*) EPC cells were maintained at 28 °C and 5% CO_2 in medium RPMI-1640 supplemented with 10% FBS, 1 mM pyruvate, 2 mM L-glutamine, 50 μg/ml gentamicin and 2 μg/ml amphotericin B.

SVCV (strain 56/70) originally isolated from carp (Fijan, 1971) was propagated in EPC cells at 28 °C until a cytopathic effect was observed. Then, the cell culture medium was harvested, centrifuged at 4000×*g* for 30 min, and virus aliquots were stored at −80 °C.

2.5. Uptake of nanostructured viral antigens by ZFL cells

To test cellular uptake, fluorescently labeled NPs were added to ZFL cells cultures at 70–80% confluence after 2–3 h incubation in minimal media (0–0.5% FBS) at the doses and times indicated below. For dose–response assays, $SVCV^{NP}$ and $SVCV\text{-}IFN^{NP}$ were added at 2.5, 5, 10, 20 and 40 μg/m L and cultures were then incubated O/N (12–14 h). For time-course assays, SVCV NPs were added at 5 and 10 μg/ml and cultures were simultaneously incubated for 12 and 24 h. For comparative assays between $SVCV^{NP}$ and $SVCV\text{-}IFN^{NP}$ were added at 5 and 10 μg/ml and cultures were then incubated O/N (12–14 h). All experiments were performed in duplicate. Post treatment, ZFL cells were washed in PBS and incubated at 28 °C and 5% CO_2 with 1 mg/ml Trypsin (Gibco) for 15 min. This strong trypsinization step aimed to remove NPs attached to the cell surface [22]. Then, two volumes of complete medium were added, and cells were pelleted by centrifugation at 300×*g* for 5 min at RT. Pellets were resuspended in PBS for flow cytometry (FACSCalibur BD), and 10,000 events were counted. Data was analyzed using FlowJo 10.4 (Leland Stanford University) and plotted with Prism 8.01 (GraphPad Prism). To confirm the fluorescent NPs were inside the cells, we performed confocal microscopy (Zeiss LSM 700). ZFL cells were seeded on Ibidi plates (μ-Dish 35 mm) and incubated at 28 °C and 5% CO_2. The next day cells at approximately 50–60% confluence were placed in minimal media. $SVCV^{NP}$ and $SVCV\text{-}IFN^{NP}$ at 20 μg/ml were added 2 h later and cells were incubated for 16 h at 28 °C. The cells were stained with Hoechst (nuclei) and CellMask Deep Red (membrane) (Life Technologies). Images were analyzed using Imaris software v8.2.1 (Bitplane) and ImageJ (National Institute of Health, USA).

2.6. RNA extraction and Q-PCR

Total RNA of ZFL cells was extracted using TriReagent (Sigma-Aldrich) following the manufacturer's instructions and RNA yield and quality was determined on Nanodrop ND-1000 (Thermo Fisher Scientific) and integrity assessed on the Agilent 2100 Bioanalyzer using RNA 6000 Nano Lab-Chip kit (Agilent Technologies). Then, cDNA was synthesized from 1 μg high quality total RNA using iScript cDNA systhesis kit (Bio-Rad). Quantitative real-time PCR (qPCR) was performed at 60 °C annealing temperature in a CFX384 touch real-time PCR detection system (Bio-Rad) using iTaq Universal SYBR Green Supermix kit (Bio-Rad). Each PCR mixture consisted of 5 μl SYBR green supermix, 0.4 μM specific primers, 2 μl diluted cDNA and 2.6 μl water (Sigma-Aldrich) in a final volume of 10 μl. The *ef1-a* gene was used as a reference gene. The dilution factor for all the genes tested was 1:10 (*vig1*, *mx*, *ccl4, lmp2, ifngr1, il1b, tnfa, il10, prf19b, gzma* and *ef1*-α) (Supplementary Table 1). Amplification efficiencies for new primers *lmp2* and *ifngr1* were calculated (Supplementary Table 1). All the samples (n = 6 per treatment) were run in triplicate, and data were analyzed for individual replicates using the Livak method [23].

For total RNA isolation of ZF4 cells, the E.Z.N.A.® Total RNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA) was used according to the manufacturer's instructions. Then, RNA was quantified with a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and M-MLV reverse transcriptase (Invitrogen, Thermo Fisher Scientific) was used for cDNA synthesis from RNA, as previously described [24]. To evaluate the gene expression in ZF4 cells, RT-qPCR was carried out in 20 μl reactions containing 24 ng of cDNA, 10 μl of SYBR green PCR master mix (Thermo Fischer Scientific), and a 900 nM final concentration of each primer using the QUANTSTUDIO 3 System (Applied Biosystems, Thermo Fisher Scientific Inc.). The *ef1-a* gene was used as a reference gene.

2.7. Zebrafish husbandry

Wild type zebrafish were kept in a re-circulating aquarium with water temperature at 28 ± 1 °C. The lighting conditions were 14:10 h (light:dark) and adult fish were fed twice a day at a rate of 2% bodyweight. Ammonia, nitrite, nitrate and pH levels were measured once a week and were kept below toxic levels and pH at 7.5 ± 0.5. For in-tank breeding, two females and four males were transferred in groups to a breeding tank in the late afternoon. The next morning after 3 h of the onset of light embryos were collected and cultured in embryo medium (E3 medium) in a Petri dish (Deltalab). Fertilized eggs were separated from unfertilized eggs using a plastic pipette (Deltalab). All experiments involving zebrafish were performed following International Guiding Principles for Research Involving Animals (EU 2010/63) and the Ethics Committees of the Universitat Autònoma de Barcelona (UAB, CEEH) and Generalitat Valenciana (2019/VSC/PEA/0203)

2.8. In vivo uptake in zebrafish larvae

Groups of five larvae per treatment were distributed on 6-well plates (Thermo Fisher) in duplicate and immersed in 1 ml of E3 medium with PBS, fluorescent $SVCV^{NP}$ and $SVCV\text{-}IFN^{NP}$ (10 μg/ml), GFP (10 μg/ml) and LPS (10 μg/ml). Biodistribution after 24 and 48 h zebrafish larvae was evaluated using a fluorescence stereomicroscope (Nikon SMZ800) coupled with a camera (Nikon DS-Fi2). In parallel groups of n = 5 larvae were treated as indicated, washed and homogenized in TriReagent for gene expression analysis as indicated in RNA extraction and Q-PCR section.

2.9. SVCV infection challenge

Zebrafish, average size 35 mm and 0.4 g, were purchased from a local pet store. Fish were acclimatized in 20 L tanks at 21 °C for 14 days before the vaccination trial. Zebrafish were divided into five groups with n = 12 animals each: the non-infected control, the SVCV-infected control, the $iRFP^{NP}$ control group, $SVCV^{NP}$ group, and $SVCV\text{-}IFN^{NP}$ group. Each individual was anesthetized with tricaine (tricaine methanesulfonate, Sigma-Aldrich) (40 mg/l) and intraperitoneally (i.p.) injected with 30 μl of PBS containing 5 μg of each antigen using 23G syringe-needle (Terumo Europe N.V.). At 30 days post-vaccination (dpv), fish were infected by bath-immersion for 2 h in aquarium water containing SVCV (1.4×10^7 SVCV infectious particles). Individuals were maintained at 21 °C over the course of the challenge, and the mortality rate of each group

was recorded daily.

2.10. Statistics

Graphs and analyses were performed with Prism 8.01 software (GraphPad Prism). Comparisons of means for each experimental group versus control and between treatments (NPs) were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons test or unpair non parametric Mann-Whitney test. Data are shown as mean ± standard deviation (SD) and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of modular nanostructured protein antigens $SVCV^{NP}$ and SVCV-IFN^{NP}

We produced two different recombinant viral proteins in *E. coli* as bacterial IBs (i.e., NPs) with moderate to good yields: $SVCV^{NP}$ 0.3 mg/l and SVCV-IFN^{NP} 3.6 mg/l (Supplementary Fig. 1). Expression of recombinant proteins is an empiric process with several experimental constraints that affect the production yield. $SVCV^{NP}$ and SVCV-IFN^{NP} production yields ranged from moderate to good compared with other NPs such as $VHSV^{NP}$ and VHSV-IFN^{NP} where we obtained 11.7 and 14.2 mg/l, respectively (unpublished data).

The size and morphology of both NPs were evaluated by FESEM (Fig. 1). SVCV-IFN^{NP} is generally oviform (elliptical shaped) while $SVCV^{NP}$ is rounder and smaller. We have observed similar morphologies in other IBs produced in *E. coli* using the same strain BL21(DE3) and in M15(pREP4) [21]. The size range is shown in Fig. 1 with average width and length being 420 ± 67.5 nm and 693 ± 154 nm for $SVCV^{NP}$ and 624 ± 104.9 nm and 982 ± 216.9 nm for SVCV-IFN^{NP}, respectively. The morphological features of the nanostructured immunologically irrelevant control protein $iRFP^{NP}$ were previously reported in Torrealba et al. [25]. Morphological and structural integrity of NPs were evaluated under different environmental conditions of temperature and pH to assess the resistance of the NPs under industrial extrusion procedures

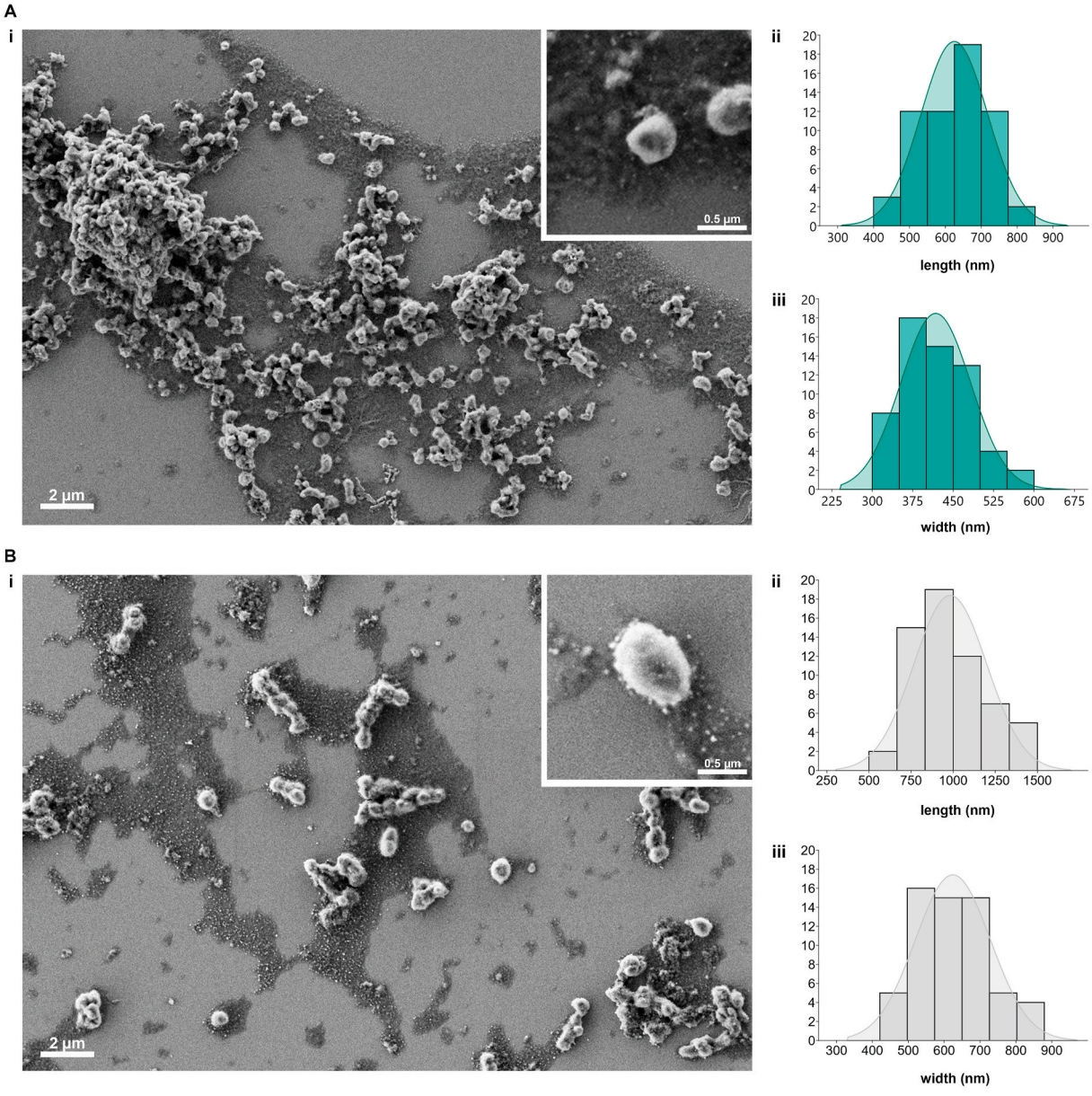

Fig. 1. Characterization of nanostructured $SVCV^{NP}$ and SVCV-IFN^{NP} proteins. Field Emission Scanning Electron Microscopy images (FESEM) (i) of $SVCV^{NP}$ (A) and SVCV-IFN^{NP} (B). Size distribution histograms (n = 60) of length (ii) and width (iii) measurements of $SVCV^{NP}$ and SVCV-IFN^{NP}.

and gastrointestinal pH conditions. SVCV^{NP} (Fig. 2A) and SVCV-IFN^{NP} (Fig. 2B) were subjected to high temperature (100 °C) and extreme pH conditions (pH 2 and 10) and we did not observe significant changes in size or morphology.

3.2. Uptake of modular nanostructured antigens by ZFL cells

Modular SVCV NPs were taken up by ZFL cells. In dose–response experiments, uptake of SVCV^{NP} was found to be slightly more efficient than SVCV-IFN^{NP}, achieving ~100% fluorescent positive cells at 10 μg/ml versus ~90% fluorescent positive cells at 10 μg/ml, respectively (Fig. 3A and C). In both cases, the mean fluorescence intensity (MFI) increased with dose until 5–10 μg/ml (Fig. 3A and C) and at higher doses dropped significatively. This decrease was not related with toxicity or apoptosis since cell survival was 100% across doses and time (data not shown). Maybe, the MFI dropped indicating cells were not able to take up more NP (100% of cells were fluorescent) and NP had started to be metabolized. For time course experiments, a 10 μg/ml dose was chosen. In the time course experiments, at 12 and 24 h ZFL cells treated either with SVCV^{NP} or SVCV-IFN^{NP} reached the same percentage of

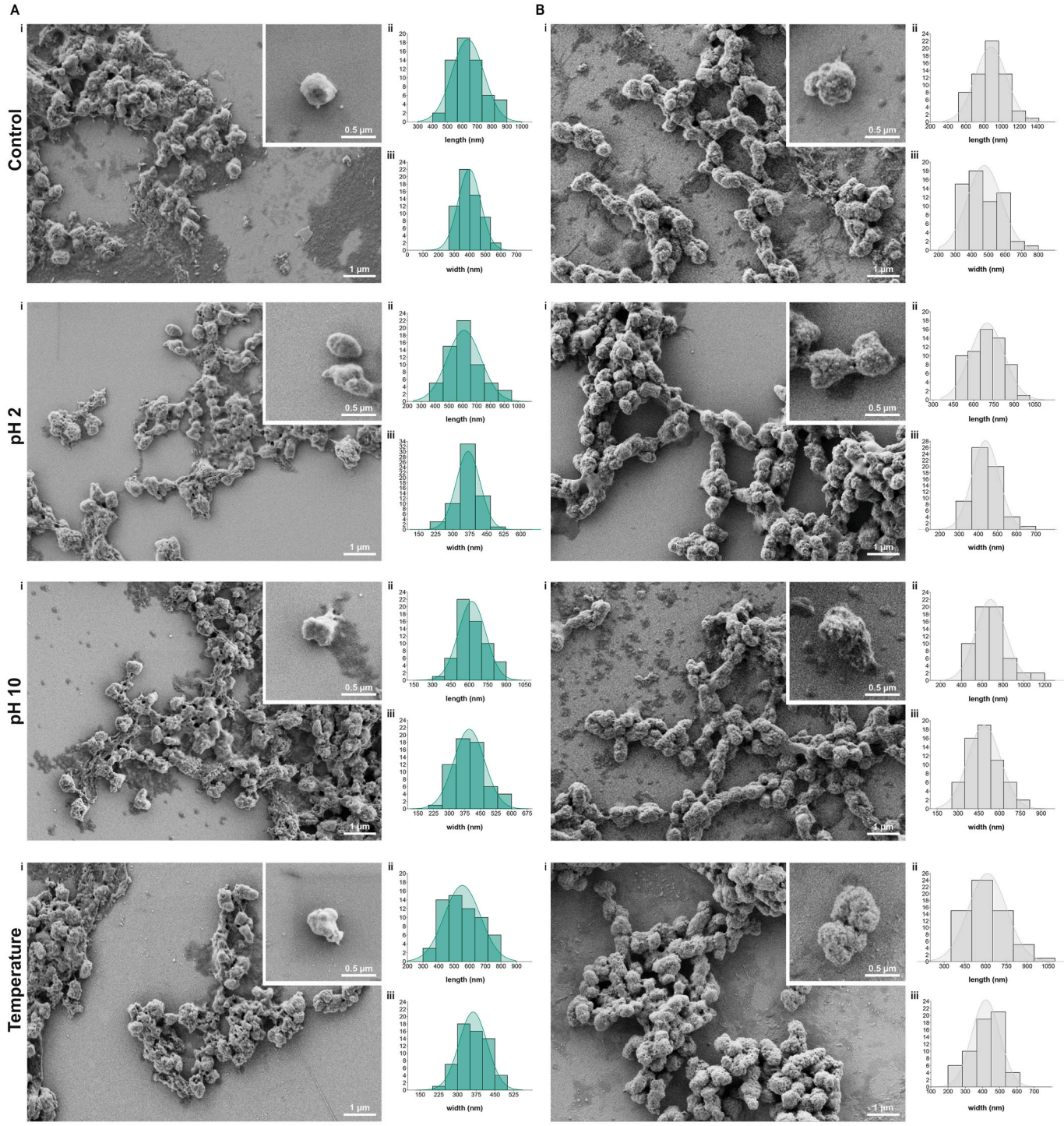

Fig. 2. Characterization of SVCV^{NP} and SVCV-IFN^{NP} under different conditions pH and Temperature. FESEM images (i) of SVCV^{NP} (A) and SVCV-IFN^{NP} (B) submitted to pH 2, pH 10 and high temperature (5 min at 100 °C). Size distribution histograms (n = 60) of length (ii) and width (iii) measurements of SVCV^{NP} and SVCV-IFN^{NP}.

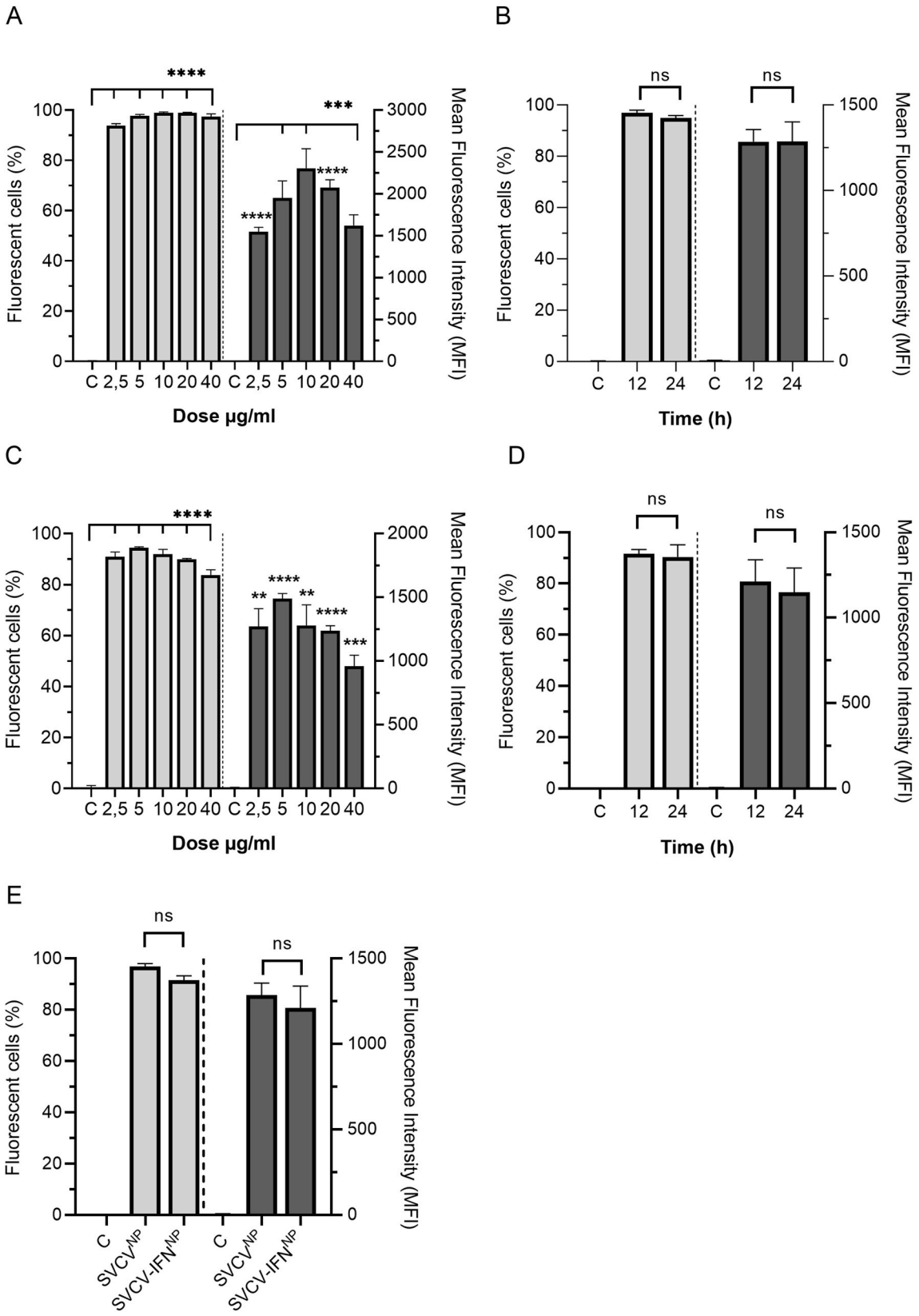

Fig. 3. Uptake of SVCV^{NP} and SVCV-IFN^{NP} nanopellets by ZFL cells. (A) SVCV^{NP} dose response; (B) SVCV^{NP} time course; (C) SVCV-IFN^{NP} dose response; (D) SVCV-IFN^{NP} time course and (E) Comparative uptake of SVCV^{NP} and SVCV-IFN^{NP} at 10 µg/ml for 12 h. For dose response assays 2,5–40 µg/ml of fluorescently labeled SVCV^{NP} and SVCV-IFN^{NP} were added to ZFL cells and incubated for 12 h and the uptake evaluated by cytometry. For time course 10 µg/ml of fluorescently labeled SVCV^{NP} and SVCV-IFN^{NP} were added to ZFL cells and incubated for 12 and 24 h. Differences between means were analyzed by a one-way ANOVA with Dunnett's multiple comparisons test, treatments versus control. Significance levels ns: no significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

fluorescence and the same MFI (Fig. 3B and D). No differences in ZFL uptake at 5 or 10 μg/ml at 12 h incubation were observed between SVCV^{NP} and SVCV-IFN^{NP} when both NPs were assayed in parallel (Fig. 3E). In addition, these results (Fig. 3E) were highly consistent with the previous ones when the NPs were assayed separately (Fig. 3A–D).

The confocal microscopy images of SVCV^{NP} and SVCV-IFN^{NP} treated ZFL cells showed that both NPs were internalized and accumulated inside the cytosol. The 3D images demonstrated the complete internalization of both SVCV^{NP} and SVCV-IFN^{NP} in ZFL cells (Fig. 4). Confocal microscopy assays were performed at 20 μg/ml of NPs at 24 h and the cytometry uptake data showed that 100% and 90% of the cells were fluorescent at 20 μg/ml. However, the Mean Fluorescent Intensity (MFI) in SVCV-IFN^{NP} treated cells (Fig. 3C) was lower than in SVCV^{NP} treated cells (Fig. 3A). Apparently, confocal microscopy images did not show any difference between SVCV^{NP} and SVCV-IFN^{NP} treated cells in terms of size or qualitative intensity of the fluorescent agglomerates (Fig. 4).

3.3. Gene expression analysis in ZFL stimulated with NPs

To see whether the antigenic NPs could induce an antiviral response in line with that provoked by viral infection, ZFL cells were stimulated with the viral NPs overnight (16 h) at 10 μg/ml. We selected the time and dose from the uptake data shown in section 3.2: those conditions which the highest percentage of fluorescent positive cells (SVCV^{NP} ~90% and SVCV-IFN^{NP} ~100%), with a significant intensity of fluorescence (MFI) and no differences among time. We also used Poly(I:C) (10 μg/ml), as a viral dsRNA mimic and LPS as a bacterial mimic. iRFP^{NP} (10 μg/ml) was the control of a NP made with immunologically irrelevant protein but with all the potential *E. coli* contaminants washed down during NPs preparation. The expression of genes, markers of the immune response to viral infection was tested by qPCR (Fig. 5). In ZFL cells a typical strong antiviral response after Poly(I:C) treatment was previously reported [26]. iRFP^{NP} protein without a relevant immune role does not induce the expression of antiviral genes while SVCV^{NP} and SVCV-IFN^{NP} were able to stimulate ZFL cells by increasing the gene expression of *vig1*, *mx*, *lmp2*, *irf1*, *ccl4* or *ifngr1*. Importantly, the inclusion of the IFNγ protein module enhanced the ability of SVCV^{NP} to stimulate an antiviral response showing that IFNγ could act as a potent adjuvant when included within the same NP (Fig. 5). Induction of the expression of IFNγ receptor (*ifngr*) and low molecular mass polypeptide (*lmp*)-2 were observed after SVCV-IFN^{NP} indicating a parallel induction of INFγ signalling pathways presumably after IFNγ binds to its receptor. Pro-inflammatory (*tnfa*) and anti-inflammatory cytokines (*il10*) also were up-regulated in ZFL but no significant differences between SVCV^{NP} and SVCV-IFN^{NP} were observed.

3.4. Uptake of SVCV^{NP} and SVCV-IFN^{NP} by zebrafish larvae

Hatched zebrafish larvae (approximately at 72 h post fertilization (hpf)) were incubated with 10 and 20 μg/ml of fluorescent SVCV^{NP} and SVCV-IFN^{NP} in E3 medium. After 24 and 48 h living larvae were anesthetised and imaged using a stereoscopic microscope (Fig. 6A). Uptake of SVCV^{NP} at 24 h was almost undetectable (n = 6 larvae) while we observed high levels of fluorescence at 48 h in around 100% of larvae mainly showing accumulation in the gastrointestinal tract and pancreas (Fig. 6A). Importantly, SVCV^{NP} and SVCV-IFN^{NP} treated larvae died after 24 h incubation at the highest dose, 20 μg/ml. However, at 10 μg/ml the whole set of larvae survived after immersion in the SVCV^{NP} and SVCV-IFN^{NP} solution.

We also tested the antiviral response in zebrafish larvae after SVCV^{NP} and SVCV-IFN^{NP} treatments. To try to understand the *in vivo* response to SVCV^{NP} and SVCV-IFN^{NP} modular proteins, we selected a panel of genes potentially representative of differential immune responses: direct virus response, macrophage response, Th1 or cytotoxic T cell responses. However, as is shown in Fig. 6B almost all the selected genes were stimulated *in vivo* by SVCV^{NP} but not by SVCV-IFN^{NP}. To better explore why SVCV-IFN^{NP} treatments failed to induce the IFNγ response in larvae, we used the zebrafish embryonic cell line ZF4 to assess whether this pattern was common in cells from same origin and developmental stage. ZF4 cells were tested for gene expression after treatment with SVCV^{NP}, SVCV-IFN^{NP}, Poly(I:C) (10 μg/ml), LPS (10 μg/ml), and iRFP^{NP}. As is shown in Fig. 6C embryonic derived cells ZF4 treated with SVCV^{NP} and SVCV-IFN^{NP} stimulated a canonical antiviral response at similar levels of those observed in ZFL cells (Fig. 5) although not showing difference in

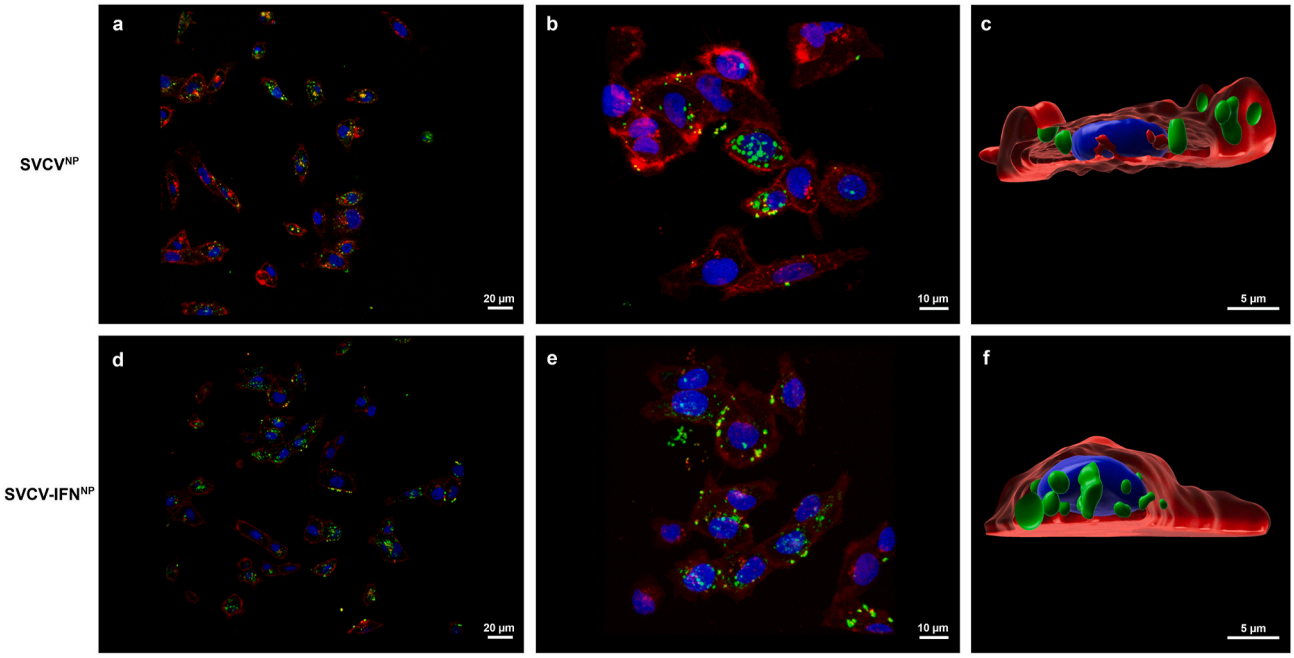

Fig. 4. Confocal microscopy and digitalized image (z-stacks) of ZFL cells with SVCV^{NP} and SVCV-IFN^{NP}. NPs are green, cell membrane red, and nuclei blue. SVCV^{NP}; (a, b) confocal microscopy and (c) digitalized image (z-stacks), and SVCV-IFN^{NP} treated ZFL cells; (d, e) confocal microscopy and (f) digitalized image (z-stacks). Cells were incubated for 16 h with SVCV^{NP} and SVCV-IFN^{NP} at 20 μg/ml.

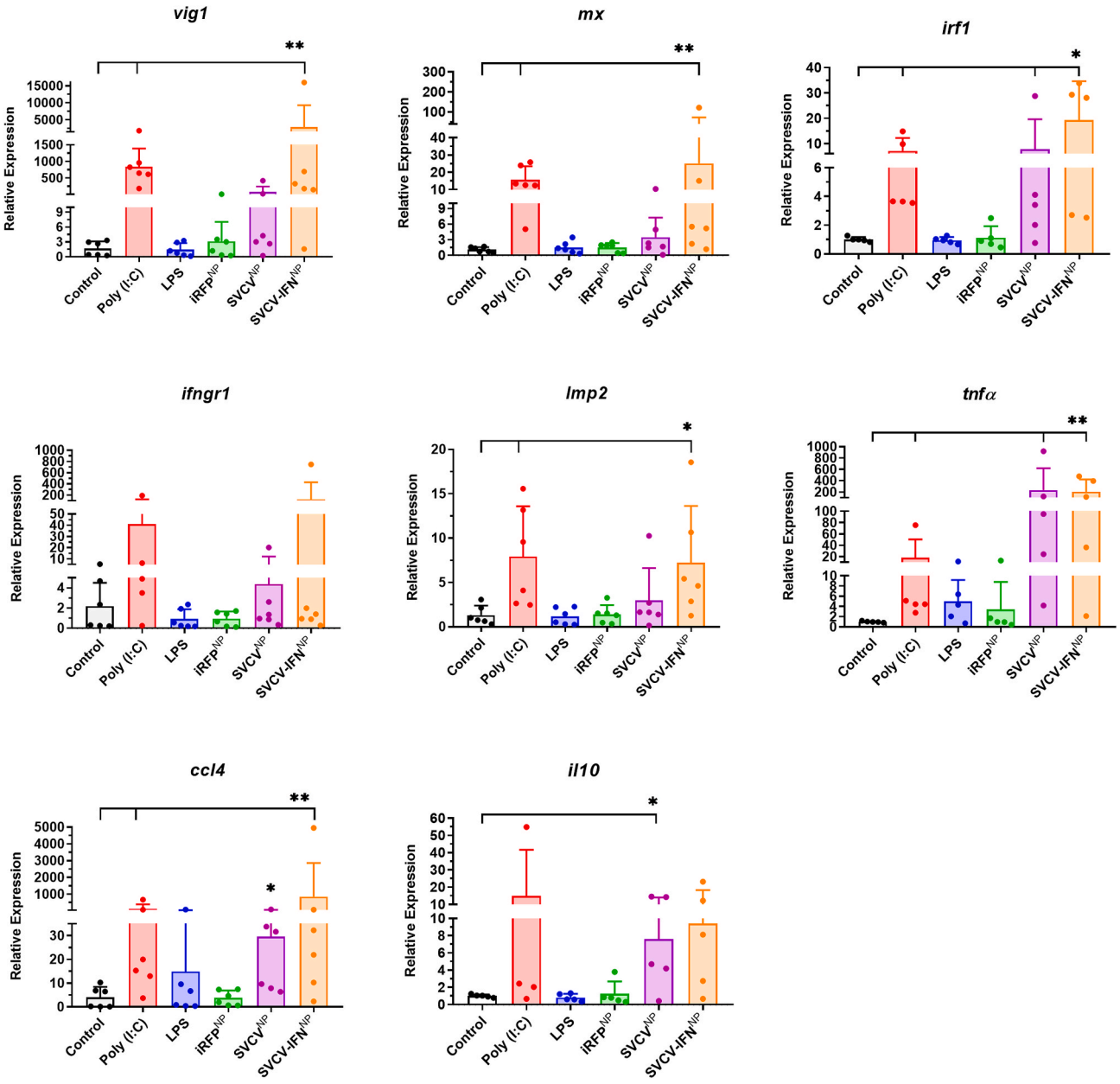

Fig. 5. Gene expression analysis of ZFL treated with SVCV^{NP} and SVCV-IFN^{NP}. (A) ZFL cells were incubated as follow: unstimulated control cells (black), 10 μg/ml of PolyI:C and LPS (red and blue respectively), 10 μg/ml of SVCV^{NP} (purple), 10 μg/ml of SVCV-IFN^{NP}(orange) and 10 μg/ml of iRFP^{NP} as immunogenically irrelevant control (green). Data are mean ± SD (n = 6). Statistical differences between treatments and controls were analyzed by unpair non parametric Mann-Whitney test. Significance levels $*p < 0.05$; $**p < 0.01$.

stimulation levels between SVCV^{NP} and SVCV-IFN^{NP}. However, ZF4 failed to up-regulate *lmp-2* and *ifngr*.

3.5. Protective effect of the vaccines against SVCV infection in zebrafish

A preliminary test of NPs toxicity on adult animals was run after i.p injection of 5 and 10 μg of SVCV^{NP} and SVCV-IFN^{NP} and no signs of macrotoxicity were observed (data not shown). A preliminary survival rate assay was performed on SVCV-infected zebrafish after different nanoparticle vaccine treatments. Unvaccinated zebrafish had 83.3% cumulative percentage mortality after 16 days of infection with SVCV. Mortality rates in the iRFP^{NP}, SVCV^{NP} and SVCV-IFN^{NP} were 41.7%, 50.0% and 20.0%, respectively (Fig. 7B). The main onset of mortality occurred between 4 and 8 days post-infection (dpi), although this was delayed in the SVCV-IFN^{NP} group where first mortalities were recorded at 7 dpi. Dead fish showed clinical signs of SVCV (Fig. 7A) and presence of virus in the diseased fish was confirmed by RT-qPCR (data not shown).

4. Discussion

Finding cost effective, safe and efficient therapeutic and prophylactic methods for fish viral diseases is still a challenge. The main issue when using subunit vaccines is the short lifetime of soluble proteins. This is particularly important for oral administration methods. To overcome degradability, encapsulation using different biocompatible materials have been investigated. Among others, liposomes, poly-lactic-*co*-glycolic acid (PLGA) or carbon nanotubes (CNTs) have shown to be efficient for therapeutics and prophylactics delivery (see Ref. [27] for a review). However, the cost of encapsulation is still an issue in fish health, particularly for those species with moderate market value and high production such as common carp (FAO, 2020). These high levels of production of common carp (C. *carpio*) and grass carp (*C. idella*) (a total

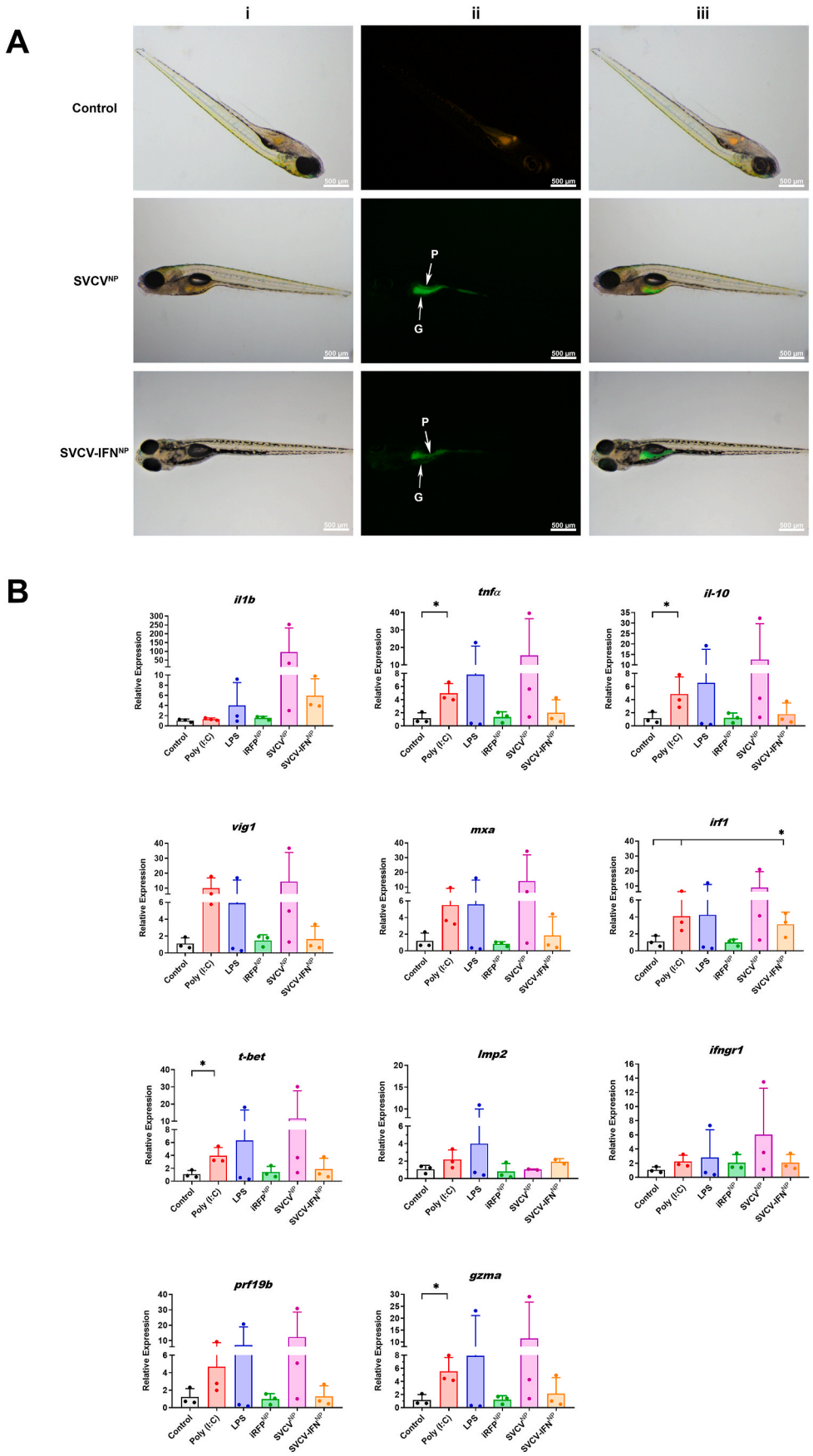

Fig. 6. SVCV^{NP} and SVCV-IFN^{NP} biodistribution *in vivo* in zebrafish larvae. (A) Uptake in larvae. Zebrafish larvae (3 dpf) were immersed with 10 μg/ml of fluorescent SVCV^{NP} and SVCV-IFN^{NP} for 48 h. E3 medium with PBS were used as control. (i) Transmitted image, (ii) fluorescent image and (iii) merged image. (B) Gene expression in larvae. Groups of 5 larvae were inmersed for 24 h as follow: unstimulated control cells (black), 50 μg/ml of PolyI:C (red), 25 μg/ml of LPS (blue), 10 μg/

ml of SVCV^{NP} (purple), 10 μg/ml of SVCV-IFN^{NP} (orange) and 10 μg/ml of iRFP^{NP} as immunogenically irrelevant control (green). Data are mean ± SD (n = 3). No differences were detected between treatments and control (unpair non parametric Mann-Whitney test). (C) ZF4 cells were incubated as follow: unstimulated control cells (black), 10 μg/ml of Poly(I:C) and LPS (red and blue respectively), 10 μg/ml of SVCV^{NP} (purple), 10 μg/ml of SVCV-IFN^{NP} and 10 μg/ml of iRFP^{NP} as immunogenically irrelevant control (green). Data are mean ± SD (n = 6). Statistical differences between treatments and controls were analyzed by unpair non parametric Mann-Whitney test. Significance levels *$p < 0.05$; **$p < 0.01$.

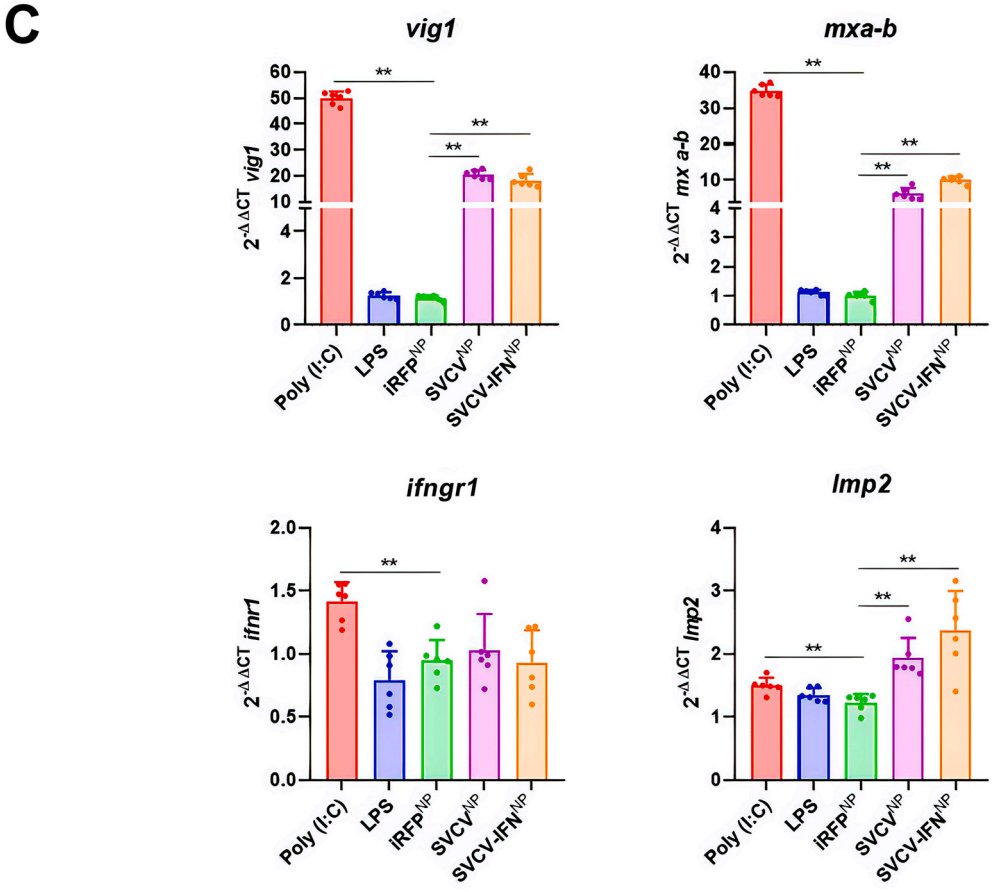

Fig. 6. (*continued*).

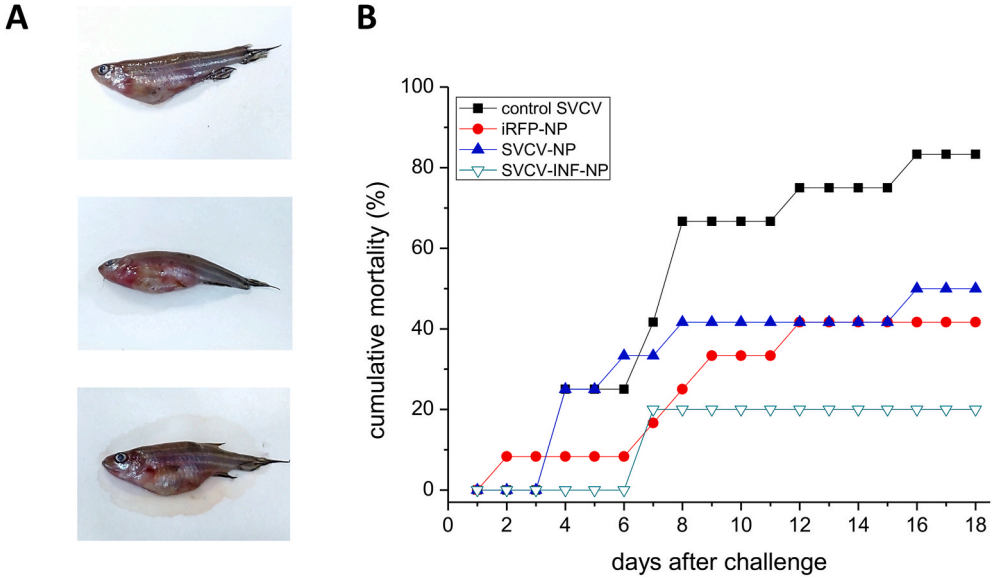

Fig. 7. SVCV^{NP} and SVCV-IFN^{NP} administration protects from SVC virus *in vivo* infection. (A) Representative fish images of SVC virus clinical signs. (B) Cumulative mortality rate of zebrafish after SVCV challenge. Fish were vaccinated with 5 μg/fish of different antigens (iRFP^{NP}, SVCV^{NP}, SVCV-IFN^{NP}) by intraperitoneal injection. Thirty days post-vaccination fish were challenged with SVCV (4×10^4 TCID50/ml). Number of dead fish in each group was counted daily.

of 9.89 million tonnes/year) makes even more relevant to develop vaccines against SVC virus.

Here we present a biocompatible, cheap and modular platform that allow the production of recombinant proteins in a highly stable conformation, suitable for injection and for feed inclusion and oral administration. SVCV^{NP} and SVCV-IFN^{NP} exposed to high temperatures and extreme pH did not show changes in shape or size. Previous works demonstrated that nanostructured TNFα^{NP} also remains structurally and functionally intact when subjected to extreme temperature or pH [18,21]. Moreover, high salinity has no impact either on structure or function of NPs (data not shown). Although IFNs can be used for treatment of different human diseases its stability and plasma half-life remained the main obstacles that hindered the utilization of recombinant proteins as therapeutics [28]. IFNγ failed to show the expected bioactivity *in vivo* due to degradation, and also to the tendency to aggregate irreversibly under mild denaturing conditions [29].

In the present study, nanostructured IFNγ combined with the SVCV antigenic module remained stable and functional even under the harshest conditions of pH and temperature. This modular approach is highly versatile and could include other modules such as ligands for specific molecules or receptors, that would provide a plethora of extra functions to the recombinant antigenic protein. The production yield of these modular proteins is good although is highly dependent on the protein and the SVCV^{NP} and SVCV-IFN^{NP} showed moderate expression levels compared to other viral antigens such as VHSV^{NP} or VNNV^{NP} [21].

At the functional level SVCV^{NP} and SVCV-IFN^{NP} were efficiently uptaken by ZFL cells and elicited a strong antiviral response. The IFNγ module within the SVCV-IFN^{NP} conferred the NPs a stronger ability to induce an antiviral response probably by stimulating the signalling pathways downstream of the IFN receptor, and leading for example to upregulation of *lmp-2*. Sieger et al. described in zebrafish larvae that except for some *irg* family members (immune-related GTPases) the only genes that responded to IFNγ were *ifn-phi1* and *lmp-2* [17]. *Lmp-2* is involved in degradation of intracellular proteins into antigenic peptides and is a good marker of IFNγ activation. It is well described that IFNγ genes in the zebrafish are inducible by Poly(I:C) in cultured cells [30]. The SVCV^{NP} and SVCV-IFN^{NP} were even more efficient than the Poly(I:C) stimulating the antiviral response in ZFL cells, indicating excellent immunostimulant properties compared with adjuvants such as Montanide, saponin or peptides [4,31–33].

In ZF4 cells the picture is slightly different and although SVCV^{NP} and SVCV-IFN^{NP} stimulated a similar antiviral response and within the range of the Poly(I:C), the SVCV-IFN^{NP} failed to induce the IFNγ response observed in ZFL cells. Lopez-Muñoz et al., had previously tested the ability of HEK293-expressed zebrafish recombinant IFNγ stimulating the expression of anti-viral genes such as *mx* in ZF4, and preventing SVCV infection [10]. Worth to mention that recombinant protein production using a bacterial system or a mammalian heterologous system like HEK293 are substantially different in terms of structure and bioactivity.

The zebrafish larvae showed high gastrointestinal and pancreas uptake of both SVCV^{NP} and SVCV-IFN^{NP} but again zebrafish larvae failed to respond to SVCV-IFN^{NP} while could mount a good virus specific response and a proinflammatory response. Similar results in 4 dpf larvae infected with SVCV were reported by Lopez-Muñoz et al. [34]. Not less important, zebrafish larvae exposed to SVCV^{NP} and SVCV-IFN^{NP} died after incubation (24 h) at higher dose (20 μg/ml). However, at dose of 10 μg/ml – double of the dose used in the zebrafish challenge - all set of larvae survived in the SVCV^{NP} and SVCV-IFN^{NP} solution. Toxicity of IFNγ has been previously described by Lopez-Muñoz et al. in microinjected zebrafish embryos overexpressing IFNγ [14]. Although it is difficult to compare bath immersion administration of IFNγ with microinjection administration to zebrafish embryos.

Importantly, zebrafish larvae and ZF4 cells are from the same origin (embryo) and the antiviral responses against the recombinant antigen alone were consistent among them (*vig1* and *mx* up regulation). The adaptive immune system of the zebrafish develops at about 4 weeks of age [35]. This means that during the first weeks of development the embryo/larvae depend solely on innate immune system and could be that within this period the different antiviral response mechanisms mature at a different pace and only adult systems show the full repertoire of antiviral mechanisms. Dios et al. characterized the progress of antiviral system maturation in zebrafish between 2 and 29 dpf using IFNγ-1 expression and they describe how IFNγ-1 started to be detectable at 14 dpf without viral stimuli, and at 8 dpf with viral stimuli. In contrast, IFN type I genes were overexpressed after viral stimuli at high levels at 2 dpf [36].

Adult zebrafish vaccinated with SVCV-IFN^{NP} and infected with SVC virus showed excellent levels of survival compared with non-vaccinated and control fish. Previous attempts at protecting carp from SVCV using a DNA vaccine engineered with a plasmid that drives the expression of the SVCV-G protein provided different degree of protection against SVC virus [37,38], although some of these DNA vaccines achieved excellent protection levels even at low doses [39]. These experimental vaccines involve the use of exogenous genetic material with the corresponding biosecurity concerns. Other successful approaches that do not involve exogenous genetic material, engineered *Lactobacilus* to express SVCV-G protein on the bacterial external surface [3,40] obtaining good levels of protection against SVCV infection. Overall, our findings demonstrate the capacity of self-adjuvanted modular NP vaccines to confer efficient protection against viral challenge in a bio secure and cheap context.

Future work should be done to adapt this promising vaccination platform to oral administration and to incorporate NPs into aquafeeds to be tested as oral vaccines against SVC virus infection in aquaculture relevant species (e.g carp), paying special attention to survival and correlates of protection and gut mucosal immune response.

Ethics statement

All methods were performed in accordance with the Spanish and European regulations (RD53/2013 and EU Directive 2010/63/EU) for the protection of animals used for research experimentation and other scientific purposes. Zebrafish procedures were conducted under Generalitat Valenciana permit 2019/VSC/PEA/0203.

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CRediT authorship contribution statement

Mauricio Rojas: Formal analysis, Data curation. **Patricia Aceituno:** Formal analysis, Data curation. **Maria E. Salvador:** Formal analysis, Data curation. **Marlid Garcia-Ordoñez:** Formal analysis, Data curation. **Maria del Mar Ortega-Villaizan:** Formal analysis, Supervision. **Luis Perez:** Formal analysis, Supervision. **Nerea Roher:** Formal analysis, Conceptualization, Supervision, Writing - original draft.

Declaration of competing interest

The authors declare that the research was performed without any conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.10.067.

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