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Vaccination of carp against SVCV with an oral DNA vaccine or an insect cells-based subunit vaccine

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ABSTRACT

We recently reported on a successful vaccine for carp against SVCV based on the intramuscular injection of a DNA plasmid encoding the SVCV glycoprotein (SVCV-G). This shows that the intramuscular (i.m.) route of vaccination is suitable to trigger protective responses against SVCV, and that the SVCV G-protein is a suitable vaccine antigen. Yet, despite the general success of DNA vaccines, especially against fish rhabdoviruses, their practical implementation still faces legislative as well as consumer's acceptance concerns. Furthermore, the i.m. route of plasmid administration is not easily combined with most of the current vaccination regimes largely based on intraperitoneal or immersion vaccination. For this reason, in the current study we evaluated possible alternatives to a DNA-based i.m. injectable vaccine using the SVCV-G protein as the vaccine antigen. To this end, we tested two parallel approaches: the first based on the optimization of an alginate encapsulation method for oral delivery of DNA and protein antigens; the second based on the baculovirus recombinant expression of transmembrane SVCV-G protein in insect cells, administered as whole-cell subunit vaccine through the oral and injection route. In addition, in the case of the oral DNA vaccine, we also investigated the potential benefits of the mucosal adjuvants *Escherichia coli* lymphotoxin subunit B (LTB). Despite the use of various vaccine types, doses, regimes, and administration routes, no protection was observed, contrary to the full protection obtained with our reference i.m. DNA vaccine. The limited protection observed under the various conditions used in this study, the nature of the host, of the pathogen, the type of vaccine and encapsulation method, will therefore be discussed in details to provide an outlook for future vaccination strategies against SVCV.

1. Introduction

Spring Viremia of Carp Virus (SVCV) is a cytopathic virus belonging to the genus *Sprivivirus* of the family *Rhabdoviridae* and is one of the main viruses affecting carp production. Outbreaks occur mainly during Spring, causing an acute systemic infection in several cyprinid species leading to mortality rates of up to 90%, mainly in juvenile fish [2,3]. SVCV virions contain a single, linear, negative-sense, single-stranded RNA molecule, coding for five structural proteins. The glycoprotein (G) is the only one present on the virion surface forming trimeric peplomers that bind to cellular receptors to induce viral endocytosis. For these reasons, the SVCV-G protein is the likely target of protective neutralizing antibodies [3,9,42].

Recently we reported on an experimental DNA vaccine for European common carp (*Cyprinus carpio carpio*) against SVCV able to confer up to 100% protection upon bath challenge with SVCV 2.5 months after vaccination [13]. This protection was achieved by a single i.m. injection of 0.1 μg DNA/g of fish of a pcDNA3 vector encoding the SVCV-G protein. We also showed that besides the local inflammation triggered at the site of injection, the DNA vaccine triggered the production of serum neutralizing antibodies and that SVCV-specific T cells were detectable in the blood of vaccinated fish for at least 3 months after vaccination. Thus, similar to the glycoprotein of other fish rhabdoviruses (i.e. viral haemorrhagic septicaemia virus (VHSV) and infectious haematopoietic necrosis virus (IHNV)) (Reviewed in Ref. [26], the glycoprotein of SVCV is an excellent vaccine antigen, at least when administered through i.m. DNA vaccination.

Although to date i.m or intraperitoneal (i.p.), injection vaccination

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is most effective, oral vaccination would be the ideal delivery method from an animal welfare and handling costs' point of view, (reviewed by Ref. [12]. However, owing to the limited efficacy of the current experimental oral formulations, mass vaccination of fish via the oral route is not common practice. The difficulties in the development of effective oral vaccines are linked to the need to use relatively high vaccine doses, the necessity to protect the antigen against intestinal degradation, as well as the challenges in finding optimal conditions to overcome oral tolerance (Reviewed in Refs. [12,29,30,41]. To date, a strong interest in the use of DNA-based or subunit vaccines also for oral delivery is currently increasing. For example, oral delivery of alginate encapsulated DNA vaccines against infectious haemorrhagic necrosis virus (IHNV) or against infectious pancreatic necrosis virus (IPNV) was shown to confer various degrees of protection in brown trout (*Salmo trutta* L.) and rainbow trout (*Oncorhynchus mykiss*) [4,10], showing the potential of oral DNA vaccination of fish against viruses. Interestingly, high protection against IPNV was achieved not only after oral administration of the vaccine by oral gavage, but also after mixing the alginate-encapsulated DNA vaccine [5], or the chitosan-triphosphate (CS-TPP) nanoparticles containing the DNA vaccine, in feed pellets [1]. Most recently, CLYNAV was the first DNA vaccine to receive a positive recommendation for marketing authorization in the European Union for vaccination of salmon against Salmon Alphavirus 3 (SAV3) [11]. Although this is a major breakthrough in the European legislation, the implementation of DNA vaccines in the daily practice is far from being complete. Alternatives to DNA-based vaccines remain of interest and include inactivated pathogens or subunit vaccines.

While successful oral vaccines against SVCV have not been reported thus far, one study showed the potential of oral vaccination of common carp and koi carp (*Cyprinus carpio koi*) against SVCV and Koi Herpes Virus (KHV), using recombinant *Lactococcus plantarum* (*L. plantarum*) expressing both the SVCV-G protein and the KHV-ORF81 protein [8]. Based on the assessed potential of oral vaccination against SVCV, and on the efficacy of the SVCV-G-based i.m. DNA vaccine [13], in the current study we used two parallel approaches to vaccinate carp against SVCV: one based on the oral administration of the SVCV-G DNA vaccine, and the other based on the use of the SVCV-G protein as subunit vaccine for i.m., i.p. or oral delivery. In the first approach we examined the efficacy of alginate microspheres in assuring intact delivery of protein antigens or DNA plasmid to the carp intestine. Next, we compared various vaccination regimes and antigen doses, either or not in combination with the potent mucosal adjuvant *Escherichia coli* lymphotoxin-beta (LTB) [32]. Furthermore, we analysed local as well as systemic immune responses, based on the expression analysis of immune-relevant genes and the distribution of Igm^+ B cells, mucosal T cells, neutrophilic granulocytes and macrophages in the spleen and intestine of carp vaccinated orally with the SVCV-G DNA plasmid. For the second approach, we generated two recombinant Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) baculoviruses for transmembrane SVCV-G expression in insect cells, based on the proven efficiency of this system to produce membrane-bound and soluble glycoproteins from other rhabdoviruses [14,21]. Indeed, analysis revealed high expression of SVCV-G protein on the membrane of recombinant baculovirus-infected insect cells, which allowed us to use whole-cell preparations as SVCV-G subunit vaccine, using various delivery routes.

Despite the various approaches adopted in this study with regard to vaccine design, dose, vaccination regime, the vaccines did not lead to sufficient protection. These factors, as well as the nature of the pathogen, of the host and of the encapsulation method will be discussed in details with the aim to provide an outlook for future vaccination strategies.

2. Materials and methods

2.1. Animals

European common carp (*Cyrpinus carpio carpio*) R3xR8, originated from cross-breeding of the Hungarian R8 strain and the Polish R3 strain [22], were used in all experiments. In this study we will refer to carp as the European common carp subspecies, unless stated otherwise. Carp were bred in the Aquatic Research Facility Carus of the animal facility at Wageningen University, the Netherlands. Carp eggs were either kept and raised at the local facility or transported to the Institut National de la Recherche Agronomique (INRA, Paris, France) for viral challenge experiments. Carp were raised at 20–23 °C in recirculating UV-treated water and fed pelleted carp food (Skretting, Nutreco) twice daily. All animals were handled in accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). All vaccination and challenge studies were performed at INRA. All animal work at INRA was approved by the Direction of the Veterinary Services of Versailles and COMETHEA (authorization number 78–28, project authorization #2707–2016011318282761), as well as fish facilities (authorization number B78-720). Animal work in Wageningen University was approved by the local animal committee (DEC number 2015098).

2.2. SVCV

The reference SVCV strain VR-1390 (isolate stock of the INRA laboratory [6,15]), was propagated in Epithelioma Papulosum Cyprinid (EPC) cells grown in Glasgow's modified Eagle's medium (GMEM)–25mMHEPES (Eurobio), supplemented with 10% foetal calf serum (FCS; Eurobio), 1% tryptose phosphate broth (Eurobio), 2 mM L-glutamine (PAA), 100 µg/mL penicillin (Biovalley) and 100 µg/mL streptomycin (Biovalley). Virus titers were determined by the method of Reed and Muench [35] and were given as plaque-forming units (pfu).

2.3. Insect cells

Spodoptera frugiperda 21 (Sf21) cells were used for the construction of the recombinant baculoviruses and initial validation of the constructs; *S. frugiperda* 9 (Sf9) cells were used for the preparation of the SVCV-G subunit vaccine for *in vivo* vaccination experiments.

Sf21 cells were cultured in Grace's insect medium (Gibco) supplemented with 10% foetal calf serum (FCS) (Gibco) and 10 µg/mL Gentamycin at 27 °C. Sf9 cells were cultured in Sf-900 II SFM (Thermo Fisher) supplemented with 5% FCS and 10 µg/mL Gentamycin. For infection of both cell lines, medium without addition of Gentamycin was used.

2.4. Construction of recombinant AcMnPV baculoviruses expressing SVCV-G

Three recombinant AcMnPV baculoviruses were constructed: one encoding the SVCV-G protein under the control of the polyhedrin (PH) promotor and the reporter gene green fluorescent protein (GFP) under the control of the p10 promotor (bAc-GFP-SVCV-G); the second encoding the SVCV-G protein alone under the PH promotor (bAc-SVCV-G); and the third encoding the GFP protein alone under the p10 promotor (Fig. 1). The SVCV-G coding sequence was obtained by PstI-BamHI (NEB) digestion of the pcDNA3-SVCV-G vector [13,43], followed by ligation in the PstI-BamHI restriction sites of the pFastBac Dual-GFP/Polyhedrin vector (Invitrogen), thereby replacing the polyhedrin gene. The pFastBac Dual vectors were then used to transform competent DH10Bac cells (Thermo Fisher) for subsequent bacmid isolation.

For construction of bAc-SVCV-G, the SVCV G gene was amplified

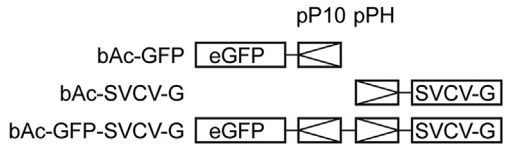

Fig. 1. Schematic representation of the construct design of the three recombinant bacmids encoding GFP under the p10 promotor (pP10), SVCV-G under the polyhedrin promotor (PH) or both.

using primers containing a PstI restriction site and Gateway AttB1 or AttB2 sites (AttB1-SVCV-G_FW GGGGACAAGTTTGTACAAAAAAGCAG GCTTAGAATTCACCATGTCTATCATCAGCTACATC and AttB2-SVCV-G_RV GGGGACCATTTGTACAAGAAAGCTGGGTACTGCAGTCAAACTA AAGACCGCATTT). The amplicon was inserted into a pDONR207 (Thermo Fisher) and a pDEST8 vector (Thermo Fisher) subsequently, using Gateway cloning. Finally, the product was used to transform competent DH10Bac cells for subsequent bacmid isolation.

2.5. Insect cell transfection, infection and SVCV-G protein expression

For generation of baculovirus stocks, Sf21 cells were transfected with the recombinant bacmids using FectoFly (Polyplus Westburg) in non-supplemented Grace's insect medium. Virus was collected at 4–5 days post-transfection and was used to infect a T75 flask of Sf21 to generate the virus master stock for further studies. Virus titres were determined using an end-point dilution assay and calculated according to the formula of Reed and Muench [35].

For subsequent infection and recombinant protein expression, Sf21 or Sf9 cells were infected with the obtained viruses with an multiplicity of infection (MOI) of 4. Cells were harvested for subsequent immunohistochemical analysis or for use as vaccine antigens in vaccination trials. Expression of the SVCV G protein on the cell membrane of infected Sf21 cells was verified by immunofluorescence analysis. Cell were harvested by centrifugation and fixed in 4% PFA for 15 min at 4 °C, followed by staining with the mouse monoclonal antibody anti-SVCV-G (clone 13C10c) [13] diluted 1:150 and with a goat-anti-mouse RPE (1:200, BioLegend). Pictures were made using a Zeiss Observer Z1m inverted microscope (Zeiss).

2.6. Encapsulation of DNA plasmid and proteins in alginate microspheres

The pcDNA3-SVCV-G vaccine was prepared as described previously [13,43] using the G protein sequence of the CAPM V 539 strain Jaroslavicky 97 (Accession number: KU934300). The pcDNA3 empty plasmid and PBS were used as negative controls. Alginate encapsulation was performed according to the method used for oral DNA vaccination of brown trout and rainbow trout against IPNV [10] with slight modifications. Briefly, 3% alginate (alginic acid sodium salt from brown algae, Sigma) in distilled water was mixed in a 1:1 (v/v) ratio with the DNA vaccine (at various concentrations), with or without 5 μg LTB/vaccine dose, in PBS. LTB kindly provided by Dr. John D. Clemens [32]. The mixture was added dropwise to an Erlenmeyer with paraffin oil or cod liver oil containing 1% v/v Span-80 surfactant (Sigma). The obtained emulsion was mixed for 10 min at 1000 rpm. Paraffin oil was used in the first challenge experiment including the histological and gene expression analysis, cod liver oil was used in all follow-up *in vivo* experiments. Microspheres were generated by addition of 0.15M $CaCl_2$ (equal volume to 3% alginate solution) and stirring for 2 h at 1000 rpm. With this method, alginate particles between 1.6 and 9 μm were obtained. Microspheres were then pelleted by centrifugation for 10 min at 1000 × *g* and the oil supernatant was removed. Alginate preparations were immediately used for oral vaccination or stored at 4 °C.

For analysis of the efficacy of delivery of alginate-encapsulated protein antigens to the intestine of carp, Sf21 cells were infected with bAc-GFP using an MOI of 4 and harvested 72 h post-infection. Cells were pelleted, washed once with PBS and disrupted by passing 10 times through a 22-gauge needle on ice, after which the lysate was centrifuged at 21.000 × *g* to pellet the nuclei and collect the cytosolic fraction containing the recombinant GFP. The obtained lysate was encapsulated in alginates as described above, to obtain an alginate-antigen solution equivalent to 2×10^7 cell/mL alginate suspension. Non-encapsulated lysates were used as control. Alginate preparations were immediately used for oral administration and subsequent immunofluorescence analysis.

2.7. Immunofluorescence

Carp of six months were anesthetized using 0.3 g/L Tricaine Methane Sulfonate (TMS, Crescent Research Chemicals) and received 100 μL of encapsulated insect cells lysate containing recombinant GFP by oral gavage with a 200 μL pipet. Carp were euthanized 24 h later using 0.6 g/L TMS and bled through the caudal vein. The first, second and third part of the intestine were isolated and cleaned, mounted in CryoCompound mounting medium (Klinipath) and snap-frozen in liquid nitrogen. Five μm cryosections were air-dried, fixed in 4% paraformaldehyde in PBS for 30 min at room temperature, rinsed twice with PBS and embedded in Vectashield with DAPI nuclear staining (Vectorlabs). GFP uptake was visualized using a M205 FA fluorescence stereomicroscope (Leica).

2.8. Immunohistochemistry

The efficacy of encapsulation and oral delivery of the DNA vaccine was examined by immunohistochemical analysis of G protein expression in the intestine of orally vaccinated carp. For this purpose, carp (3-month-old) received 2 × 10 μg of alginate-encapsulated pcDNA3 or pcDNA3-SVCV-G by oral gavage, with a 48 h interval. After 14 days, carp were sacrificed using an overdose of TMS (0.6 g/L) and bled through the caudal vein before collection of the second segment of the intestine. Cryosections (5 μm) from the intestine were stained with an antibody against the G protein as described before [13]. Briefly, slides were stained with anti-SVCV-G clone 13C10c diluted 1:150 followed by incubation with alkaline phosphatase (AP) conjugated goat-anti-mouse (Dako, 1:200). Development was performed using AP substrate (4.5 μL/mL nitro-blue-tetrazolium (NBT; Roche Applied Science) and 3.5 μL/mL 5′-bromo-4′-chloro-3'indolyl phosphatase (BCIP; Roche Applied Science)) in AP buffer (0.1M Tris-Cl, 0.1M NaCl, 0.05M $MgCl_2$) until sufficient staining was observed.

2.9. Vaccination and challenge trials

Carp of 3 months (2–4 g, $n = 20$ per group) were vaccinated at 20 °C with the alginate-encapsulated DNA vaccine (10 μL/fish) by oral gavage using a 10 μL pipet. Controls included i.m. injections of the pcDNA3 empty plasmid or the pcDNA3-SVCV-G vaccine (0.1 μg DNA/g of fish, 10 μL/fish) and alginate-encapsulated PBS. All orally delivered vaccines were encapsulated in alginate microspheres as described in paragraph 2.3. In the first experiment, carp were orally vaccinated either once with 3 μg DNA/g of fish, or three times with 1 μg DNA/g fish with a 72 h interval. In the second experiment, carp received 20 μg or 100 μg encapsulated DNA, with or without the addition of 5 μg *E. coli* lymphotoxin-beta (LTB) [32]; kindly provided by Dr. John D. Clemens).

In the third vaccination trial, the SVCV-G subunit vaccine was used for injection and oral vaccination. Sf9 cells were infected with bAc-SVCV-G, or with bAc-GFP as control, at an MOI of 4. After 3 days, cells were collected and the number of SVCV-G^+ or GFP^+ cells was estimated by counting the number of syncytia or the number of GFP^+ cells. Cells were harvested by centrifugation at 300 × *g*, washed in PBS, fixed in 4% PFA for 10 min at 4° and washed twice with PBS again. Carp (3-month-old, 2–4 g, $n = 20$ per group) were vaccinated with PFA-fixed whole-Sf9 cells expressing the recombinant SVCV-G. For injection

vaccination, the equivalent of 100, 1.000 or 10.000 non-encapsulated SVCV-G$^+$ cells were resuspended in 10 μL PBS and administered through i.m. or i.p. injection. For oral vaccination, the equivalent of 1.000, 10.000 or 100.000 SVCV-G$^+$ cells were encapsulated in a final volume of 10 μL alginate microspheres and administered through oral gavage. As controls, GFP-expressing cells were used: 10.000 non-encapsulated GFP$^+$ cells for i.m. and i.p. injection, or 100.000 encapsulated GFP$^+$ cells for oral gavage. As a positive control, i.m. injection of the pcDNA3-SVCV-G vaccine (0.1 μg DNA/g of fish) was used. Prior to vaccination, carp were anesthetized using 0.3 g/L TMS. All vaccinations were performed at 20 °C and before bath challenge with SVCV, 2.5 months after vaccination, the water temperature was gradually lowered to 15 °C at a rate of 1–2 °C per day. Challenge was performed by bath, using a viral exposure time of 48 h and a viral load of 8×10^6 pfu/mL, as previously optimized [13]. Mortality was recorded over a period of 3–4 weeks.

2.10. RNA isolation and cDNA synthesis

Carp (3-month-old, $n = 3$/treatment) received two times 10 μg of alginate-encapsulated pcDNA3 or pcDNA3-SVCV-G with an 48 h interval and were sacrificed 5 days later to collect the intestine (all three segments separately), spleen and gills. Spleen and gills were stored in RNA later, the intestinal segments were snap-frozen immediately. All organs were stored at −80 °C until further processing. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions including on-column DNase treatment using the RNase-free DNase set (Qiagen). RNA concentrations were measured using a Nanodrop-1000, the integrity was verified on a 1% agarose gel and RNA was stored at −80 °C until further use. Prior to cDNA synthesis of 1 μg total RNA, a second DNase treatment was performed using DNAse I, Amplification Grade (Invitrogen). Reverse transcription of the RNA was performed using random primers (300 ng) and Superscript™ III (200U) First Strand Synthesis Systems for RT-PCR (Invitrogen). cDNA samples were further diluted 25 times in nuclease-free water and stored at −20 °C.

2.11. Gene expression analysis

Real-time quantitative PCR (RT-qPCR) was performed using a Rotor-Gene™ 6000 (Qiagen). Fluorescence data were analysed using Rotor-Gene Q series software version 2.3.1. Briefly, 5 μL of 25 times diluted cDNA was mixed with 2 μL of forward and reverse primers (2.1 μM of each primer) and 7 μL of 2× ABsolute qPCR SYBR Green Mix (Thermo Scientific) as detection chemistry. The list of primers can be found in Table 1. The take-off value for each sample and the average reaction efficiencies (E) for each primer set were obtained upon comparative quantitation analysis from the Rotor-Gene software [16]. The relative expression ratio (R) of each sample was calculated based on the take-off deviation of sample versus each of the unhandled control and normalized relative to the *s11* protein of the *40s* subunit as reference gene.

2.12. Flow cytometry

Carp received 10 μg of encapsulated pcDNA3 or pcDNA3-SVCV-G by oral gavage, or were left unhandled. After 3, 5, 7 and 14 days the intestine (all segments together) and spleen ($n = 3$/treatment/timepoint)were isolated for flow cytometric analysis of cell populations as described before [18,23]. Briefly, single-cell suspensions were loaded onto a 1.02–1.083 Percoll (GE Healthcare) density gradient and centrifuged for 15 min at 800 × *g*. The obtained total leukocyte populations were stained with specific antibodies against carp Igm (WCI12, 1:100 [39]), putative mucosal T cells (WCL38, 1:100 [38]), macrophages (WCL15, 1:50 [44]) or neutrophilic granulocytes (TCLBE8, 1:50 [17,31]) and goat-anti-mouse PE (1:200, BioLegend). Cells were analysed on a FACS CantoA (BD Biosciences) and data were analysed using FlowJo V10 (BD Biosciences).

2.13. Statistics

For gene expression data, statistical analysis was performed using SPSS Software 22 (IBM). Relative expression ratios R were transformed (LN(R)) and significant differences ($p < 0.05$) were determined by Student's T tests.

3. Results

3.1. Delivery of alginate-encapsulated pcDNA3-SVCV-G results in G protein expression in the intestine

To investigate whether alginate encapsulation of our pcDNA3-SVCV-G vaccine would allow for delivery of intact DNA to the second intestinal segment, we analysed the local expression of the SVCV-G protein after oral DNA delivery.

Carp received 2 × 10 μg of alginate-encapsulated pcDNA3-SVCV-G by oral gavage. The second intestinal segment was isolated 14 days later and was processed for immunohistochemical detection of the SVCV-G protein. While no G protein staining was observed in the intestinal epithelium of carp receiving the empty pcDNA3 plasmid (Fig. 2A), a clear signal of the G protein could be observed in the pcDNA3-SVCV-G group (Fig. 2B). The G protein was clearly expressed

Table 1
Primers used for Real-Time quantitative PCR analysis.

Primer	FW primer 5′-3′	RV primer 3′- 5′	Acc. No.
Housekeeping genes			
40s	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
Cytokines			
cxcb1	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	AB082985
il1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGAGGCTGTCA	AJ245635
ifnγ1	TGCACTTGTCAGTCTCTGCT	TGTACTTGTCCCTCAGTATTT	AM261214
ifnγ2a/2b	CGATCAAGGAAGATGACCCAGTC	GTTGCTTCTCTGTAGACACGCTTC	AM168523
Interferon stimulated genes			
mx1	ACAATTTGCGGTCTTTGAGA	CCCTGCCATTTCTCTTCG	cypCar_00015892
vip2	CTGTCGGACACATCAGC	TCAATGGGCAAGACGAAA	cypCar_00024055
pkr3	CACGGTGTTTGAAAAGAGC	GACTGGGTCTCAGCATTC	cypCar_00039221
isg15.2	AGTGTTCGTCAAGAATGAGG	CCTCGCAGACGGAAAAC	cypCar_00039111
Adaptive immune genes			
igm	CACAAGGCGGGAAATGAAGA	GGAGGCACTATATCAACAGCA	AB004105
igt1	AAAGTGAAGGATGAAAGTGT	TGGTAACAGTGGGCTTATT	AB598367
igt2	GATTCTACTGGGT8CTTCAC	GACATCACTCAACTC8TTCT	AB598368
zap70	GGAACAAGCCATCATTAGCC	GTCGTCTCTCACCCTCCTG	Scaf 2523 & 63374

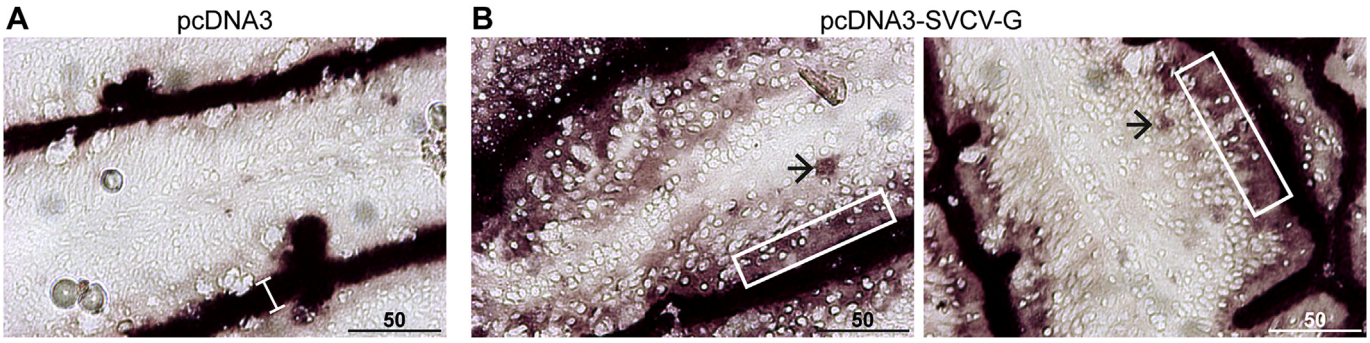

Fig. 2. Immunohistochemical analysis of SVCV-G protein expression after oral administration of alginate-encapsulated pcDNA3-SVCV-G vaccine. Carp (3-month-old) received 2 × 10 μg of DNA plasmid (pcDNA3 or pcDNA3-SVCV-G) with a 48 h interval. The intestine was isolated 14 days later and stained with an antibody specific for the SVCV-G protein. **A)** Intestinal villi of the second gut segment from fish that received pcDNA3 control plasmid. White brackets indicate the regions of a-specific background staining due to endogenous phosphatase activity. **B)** Intestinal villi of two different regions of the second gut segment from fish that received the pcDNA3-SVCV-G vaccine plasmid. White boxes indicate strong specific staining of the SVCV-G protein in the epithelial layer; arrows point to putative G protein-positive cells present throughout the lamina propria, most likely indicating G protein-expressing leukocytes. Scale bars indicate μm.

along the epithelium, as indicated by the strong purple staining. Furthermore, it was found in concentrated areas throughout the lamina propria, possibly indicating SVCV-G-expressing leukocytes (Fig. 2B). Overall, we show that encapsulation of the pcDNA3-SVCV-G vaccine in alginate microspheres effectively protects the DNA against intestinal degradation thereby ensuring delivery of intact DNA and subsequent protein expression in the second intestinal segment.

3.2. Oral administration of low doses of pcDNA3-SVCV-G does not confer protection against SVCV, nor does it induce a strong local or systemic immune response

After confirming the delivery of the intact DNA plasmid to the intestine of carp, characterized by a strong local expression of the SVCV-G protein, we next examined the protection induced by oral delivery of relatively low doses of the pcDNA3-SVCV-G DNA vaccine. Carp were vaccinated orally with 1 dose of 3 μg DNA/g of fish or with 1 μg DNA/g of fish 3 times with a 72 h interval. All orally delivered vaccines were encapsulated in alginate microspheres. The naked DNA vaccine injected intramuscularly was used as positive control and all vaccinations where performed at 20 °C. At 2.5 months post-vaccination (mpv) carp were challenged at 15 °C by bath using 8×10^6 pfu/mL for 48 h and mortality was recorded over a period of 3 weeks. As expected, i.m. vaccination with pcDNA3-SVCV-G induced a 95% survival (RPS of 92) (Fig. 3). In contrast, no protection was induced by oral delivery of the same DNA vaccine at any of the given regimes. The group receiving a single dose of pcDNA3-SVCV-G had a survival of 20% and had similar

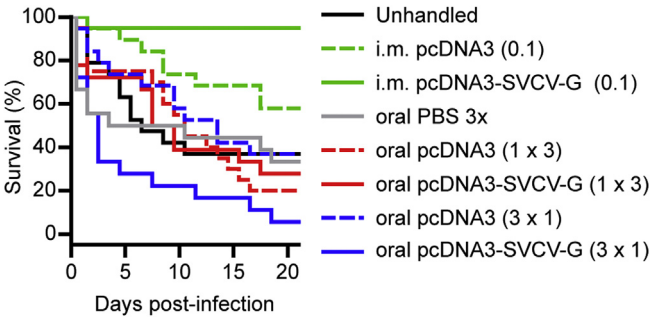

Fig. 3. Efficacy of orally administered alginate-encapsulated pcDNA3-SVCV-G vaccine. Carp (3-month-old, 2–4 g, $n = 20$/group) were kept at 20 °C and were orally vaccinated with 1 × 3 μg DNA/g of fish or 3 × 1 μg alginate-encapsulated DNA/g of fish with a 72 h interval. I.m. injection of 0.1 μg DNA/g of fish was used as a positive control and oral gavage of alginate-encapsulated PBS or pcDNA3 plasmid was included as negative controls. Carp were challenged at 15 °C by bath for 48 h, using 8×10^6 pfu/mL at 2.5 mpv. Mortality was recorded for 3 weeks.

kinetics as all control groups that received either a low or a high dose of pcDNA3 plasmid, with the highest incidence around 8–11 days. Surprisingly, the group receiving three times a low dose of pcDNA3-SVCV-G resulted in only 5% survival after 21 days, with most of the mortalities occurring already within the first 3 days after challenge.

After observing the lack of protection induced by oral delivery of the pcDNA3-SVCV-G, we next set out to investigate the local response to higher doses of the orally delivered DNA vaccine through gene expression analysis. The panel of genes was selected based on previous analysis of the local response triggered by the same plasmid upon i.m. DNA vaccination, which was previously shown to induce full protection against SVCV [13]. In parallel, we also investigated whether oral vaccination of carp would lead to changes in the relative distribution of leukocytes in the intestine and spleen.

For analysis of local gene expression, carp received 2 × 10 μg of alginate-encapsulated plasmid (pcDNA3 or pcDNA3-SVCV-G) with a 48 h interval. Intestine (all three segments separate), spleen and gills were isolated 5 days later and used for subsequent gene expression analysis. In the intestine, although a significant upregulation was observed only for *cxcb1,* in the first segment, in the group receiving the vaccine plasmid (Fig. 4A), a similar but lower response was observed in the second and third segment. *Ifnγ1, ifnγ2ab, mx1, pkr3* and *isg15.2* were all elevated in the intestine but their upregulation was not significantly different from the one observed in the group receiving the empty plasmid. Similarly, the panel of B cell- and T cell-related genes (*igm, igt1, igt2, zap70*) did not show a vaccine-specific response. In parallel, we analysed the expression of selected genes also in spleen (Fig. 4B) and gills (Fig. 4C) of control and vaccinated carp. While most of the genes showed some degree of response, this was often observed in both, the control and vaccinated group.

For the flow cytometric analysis of leukocyte distribution in the intestine and spleen of vaccinated carp, organs were isolated at various time points after oral administration of 10 μg of either pcDNA3 (control) or pcDNA3-SVCV-G (vaccine) and leukocytes were stained with specific antibodies against Igm^+ B cells, putative mucosal T cells, macrophages and neutrophilic granulocytes. In the intestine, no apparent changes in the relative percentage of Igm^+ B cells, mucosal T cells and neutrophilic granulocytes could be observed in the control or vaccine group when compared to the unhandled group (Fig. 5A). The relative percentage of macrophages however, was elevated in both the control and vaccinated group, but such increase was not significantly different between the two groups, suggesting that macrophage recruitment is most likely due to the administration of alginate microspheres rather than of the plasmid. In the spleen, moderate changes in the percentage of Igm^+ B cells, mucosal T cells and macrophages was observed in both groups (Fig. 5B), again suggesting that leukocyte recruitment might be triggered by the alginate administration and not

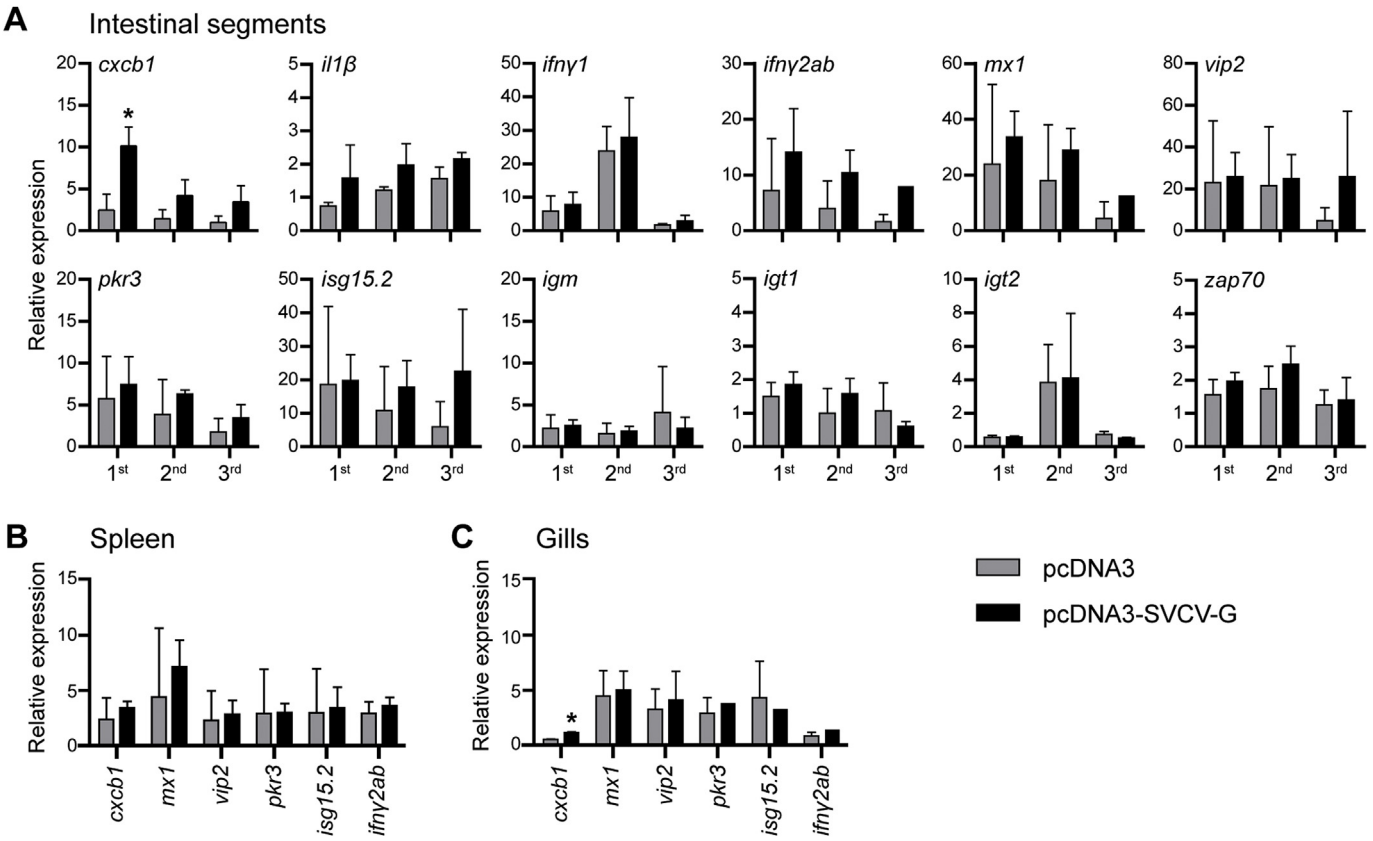

Fig. 4. Gene expression analysis of immune-related genes after oral administration of pcDNA3-SVCV-G. Carp (3-months-old) received 2 × 10 μg of plasmid (either pcDNA3 or pcDNA3-SVCV-G) with an interval of 48 h and organs were isolated 5 days later. Gene expression analysis in the three intestinal segments **(A)**, spleen **(B)**, and gills **(C)** was normalized against the housekeeping gene *s11* of the ribosomal subunit 40S and expressed relative to the unhandled control. Significant differences ($p < 0.05$, indicated with an asterisk *) were analysed by a Student's T-test. Bars indicate average and SD of $n = 3$ fish per time point.

specifically by the DNA plasmid.

In conclusion, analysis of the local response to the orally delivered DNA vaccine revealed no clear specific response to the oral vaccine and most changes could be ascribed to the administration of alginate microspheres. This moderate response might in part explain the lack of protection observed.

3.3. High doses of orally delivered pcDNA3-SVCV-G, with or without the mucosal adjuvant LTB, do not confer protection against SVCV

After observing a higher and faster mortality in the group receiving repeated administration of a low dose of the DNA vaccine (Fig. 2), in the follow-up experiment we used single high doses of encapsulated DNA vaccine, in the presence or absence of a mucosal adjuvant. Carp received 20 μg or 100 μg of DNA (pcDNA3 or pcDNA3-SVCV-G), with or without the addition of 5 μg LTB. Carp were challenged as described above and mortality was recorded over a period of 4 weeks. The i.m. pcDNA3-SVCV-G vaccinated group showed full protection against the bath challenge (Fig. 6). Strikingly, in none of the orally vaccinated groups, independently of the presence of the LTB adjuvant, protection could be observed. As already observed in Fig. 3, the group orally vaccinated with a high plasmid dose, this time also in combination with LTB adjuvant, showed a faster kinetic of mortality.

In conclusion, we found that under the tested vaccination regimes, oral administration of alginate-encapsulated pcDNA3-SVCV-G vaccine, even in the presence of a potent mucosal adjuvant as LTB, does not confer protection against a bath challenge with SVCV.

3.4. Validation of recombinant baculovirus for the production of a SVCV-G subunit vaccine

In an attempt to generate a vaccine that would allow for a DNA-independent administration of the vaccine antigen, we designed an insect cell-based subunit vaccine. To this end, recombinant AcMNPV baculoviruses encoding the transmembrane form of the SVCV-G protein together with GFP (bAc-GFP-SVCV-G), the SVCV-G protein alone (bAc-SVCV-G) or the GFP protein alone (bAc-GFP) were constructed and were used to infect Sf21 or Sf9 cells. The expression of the SVCV-G protein on the cell surface was analysed by examining cell morphology and by using a specific monoclonal antibody against the SVCV-G protein [13]. All cells infected with the recombinant baculoviruses encoding for the SVCV-G protein showed clear syncytia formation 3 days after infection, whereas no syncytia could be observed in cells infected with bAc-GFP (Fig. 7A upper panel). Upon extracellular staining with the anti-SVCV-G antibody, expression of the SVCV-G protein on the Sf21 cell membrane could be confirmed (Fig. 7A, middle panel), also coinciding with GFP expression in the bAc-GFP-SVCV-G-infected group (Fig. 7A, lower panel) and with the syncytia in the bAc-GFP-SVCV-G and bAc-SVCV-G group (Fig. 7A, middle and lower panel). A similar picture was observed in infected Sf9 cells (data not shown). Together, the formation of syncytia and the use of the specific antibody against the SVCV-G protein allowed us to confirm that the SVCV-G protein is expressed on the membrane of insect cells infected with the recombinant baculoviruses.

After having assessed that alginate encapsulation allows for delivery of intact DNA plasmid to the intestine, we used the same encapsulation method to assess whether encapsulation was required for efficient delivery of protein antigens to the intestine of carp. To this end, the

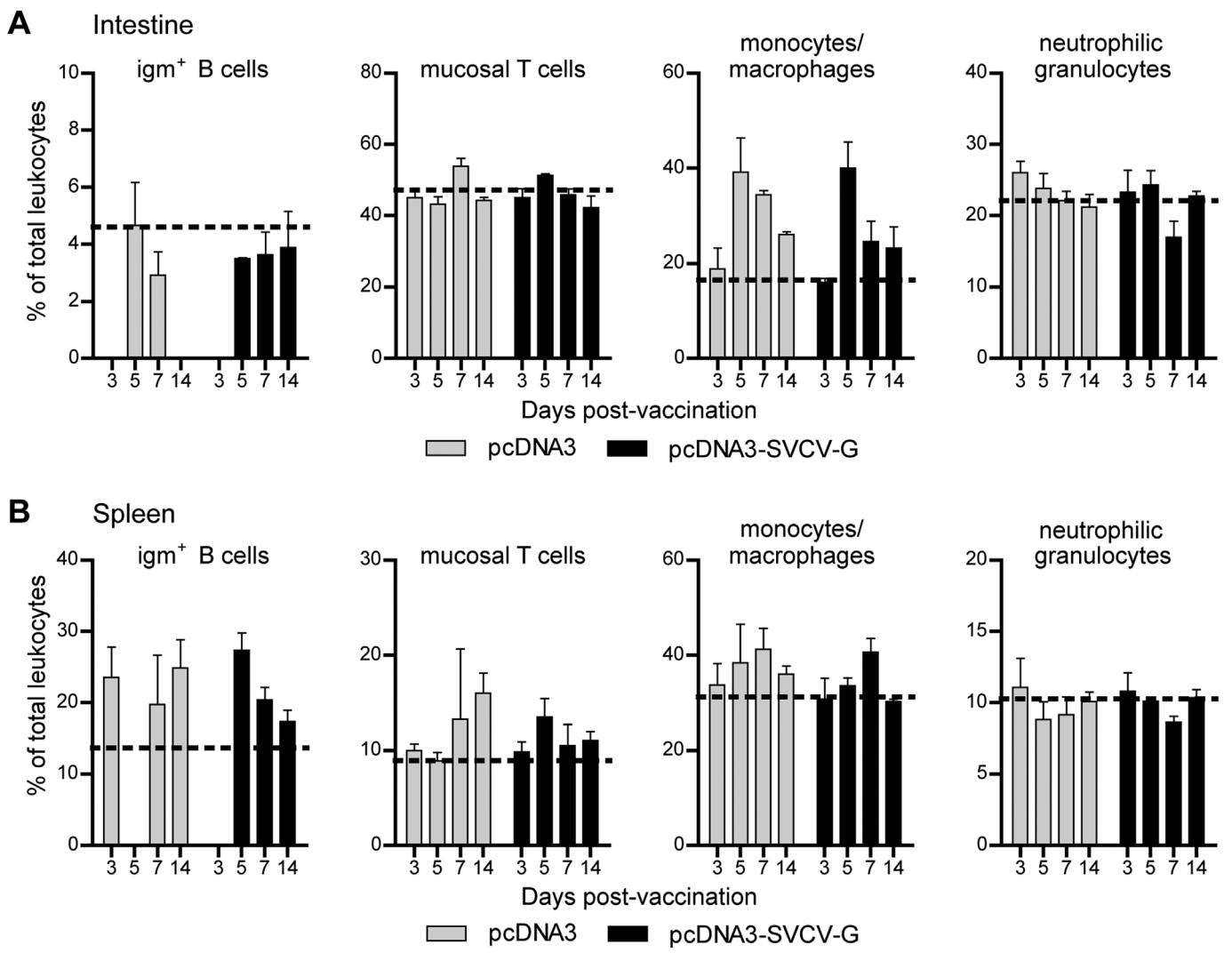

Fig. 5. Leukocytes distribution in intestine and spleen after oral delivery of alginate encapsulated pcDNA3-SVCV-G. Carp (3-month-old) received 10 μg of plasmid (either pcDNA3 or pcDNA3-SVCV-G), or were left unhandled; 3, 5, 7 and 14 days later the intestine **(A)** and the spleen **(B)** were isolated for flow cytometric analysis. Cells were stained with monoclonal antibodies against Igm$^+$ B cells, mucosal T cells, monocytes/macrophages and neutrophilic granulocytes. The dotted line at each graph indicates the average % of the indicated cell type in unhandled fish. Bars indicate average and SD of $n = 3$ fish per time point.

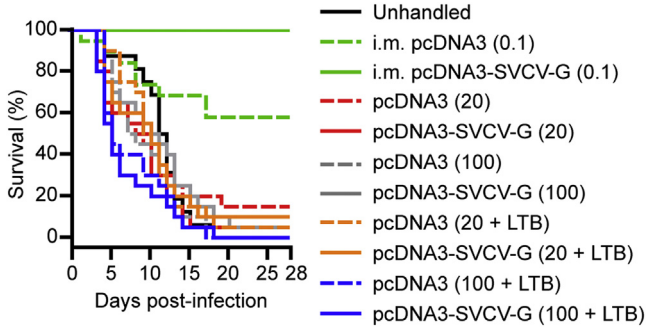

Fig. 6. Efficacy of orally administered alginate-encapsulated pcDNA3-SVCV-G vaccine. Carp (3-month-old, 2–4 g, $n = 20$/group) were kept at 20 °C and orally vaccinated with 20 μg or 100 μg alginate-encapsulated DNA plasmid/fish, with or without the addition of 5 μg LTB. I.m. injection of 0.1 μg DNA/g of fish was used as a control. Carp were challenged at 15 °C by bath for 48 h, using 8×10^6 pfu/mL at 2.5 mpv. Mortality was recorded for 4 weeks.

cytosolic fraction of Sf21 cells infected with bAc-GFP was encapsulated in alginate microspheres and administered to carp by oral gavage; unhandled fish or fish that received non-encapsulated antigens were used as control (Fig. 7B upper panel). After 24 h, the intestine was isolated and examined for the presence of GFP. A strong GFP signal was detected in all intestinal segments of carp that received encapsulated antigens (Fig. 7B lower panel), whereas GFP was detected at lower level, only in the last intestinal segment, in carp that received non-encapsulated antigens (Fig. 7B, middle panel). Altogether, this confirms that encapsulation of proteins greatly enhances protein delivery to all intestinal segments of carp and might thus be suitable for the formulation of subunit vaccines for oral delivery.

3.5. Insect cells-based SVCV-G subunit vaccine does not confer protection against SVCV

After verifying the expression of the SVCV-G protein on bAc-SVCV-G-infected insect cells, whole-cells were used as source of the subunit vaccine and administered to carp using different administration routes. Carp received PFA-fixed whole Sf9 cells expressing the recombinant SVCV-G protein by oral gavage of alginate-encapsulated cells (1.000, 10.000 or 100.000 SVCV-G$^+$ cells/fish) or by i.m. or i.p. injection of non-encapsulated cells (100, 1.000 or 10.000 SVCV-G$^+$ cells/fish). Cells expressing recombinant GFP were used as control using the maximum dose used of the SVCV-G-expressing cells and the administration route of the corresponding vaccinated group. In addition, a

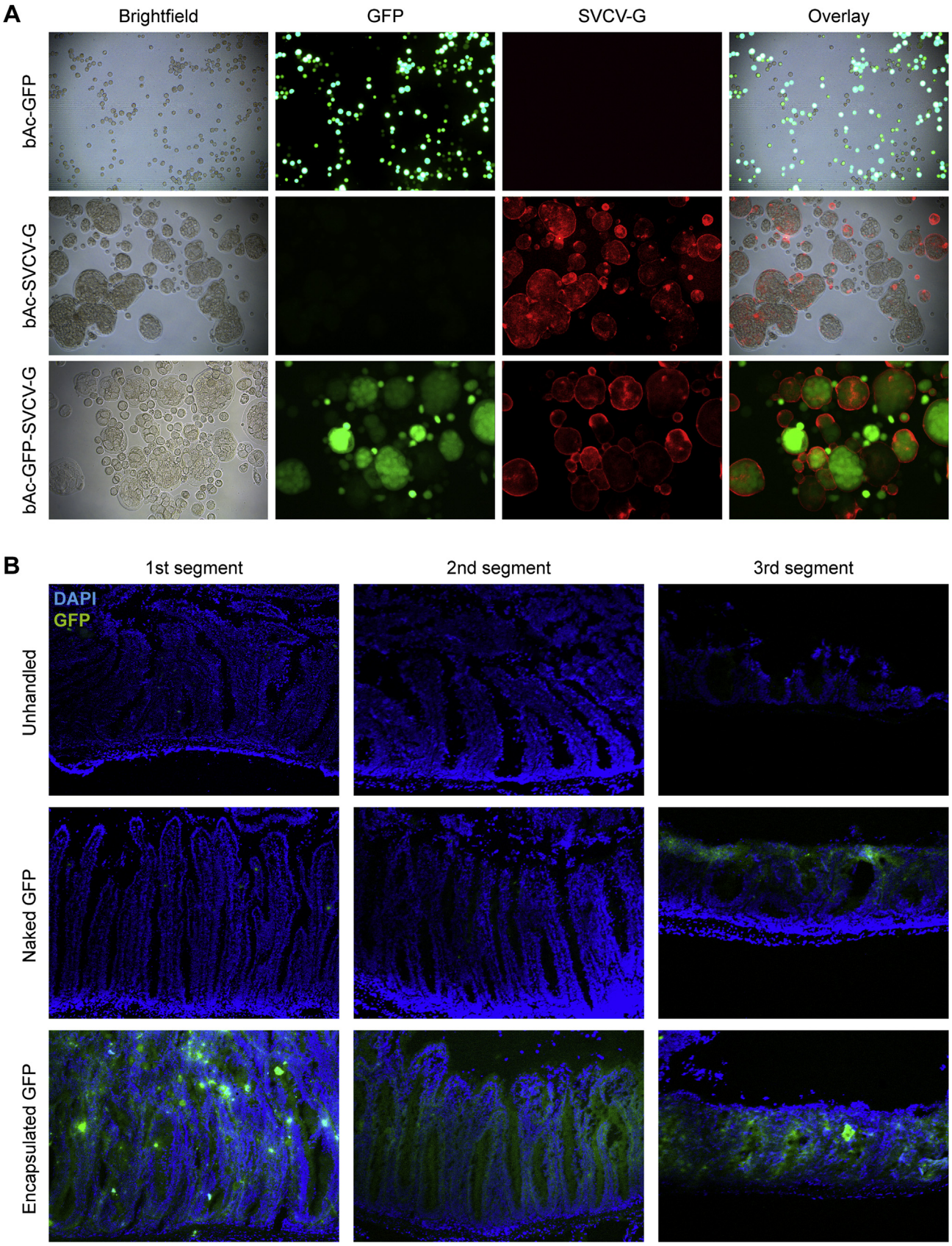

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Fig. 7. Validation of an insect-cell based SVCV-G expression system and encapsulation efficacy of protein antigens. A) Sf21 cells were infected with the indicated recombinant baculoviruses (bAc) at an MOI of 4 and after 48 h GFP and SVCV-G protein expression was visualized. Cells were fixed with PFA and stained with a monoclonal antibody against the SVCV-G protein (1:150) and goat-anti-mouse RPE (1:200). Images were acquired with a Zeiss Observer Z1m inverted microscope and all images were obtained using a 20 times magnification. Note the formation of syncytia only in the cells infected with the baculovirus encoding the SVCV-G protein. For clarity, the brightfield channel is not shown in the overlay picture in the bottom tight panel. **B)** Sf21 cells were infected with the bAc-GFP baculovirus at an MOI of 4 for 72 h. Cells were harvested and the cytosolic fraction containing the GFP was encapsulated in alginate microspheres. Carp (6-month-old) received 100 μL of encapsulated cell lysate (equivalent to 150.000 GFP^+ cells). After 24 h, the intestine was isolated and the presence of GFP signal was examined in cryosections. A counterstaining with DAPI was included to visualize the cell nuclei. Images were acquired using a M205 FA fluorescence stereomicroscope (Leica).

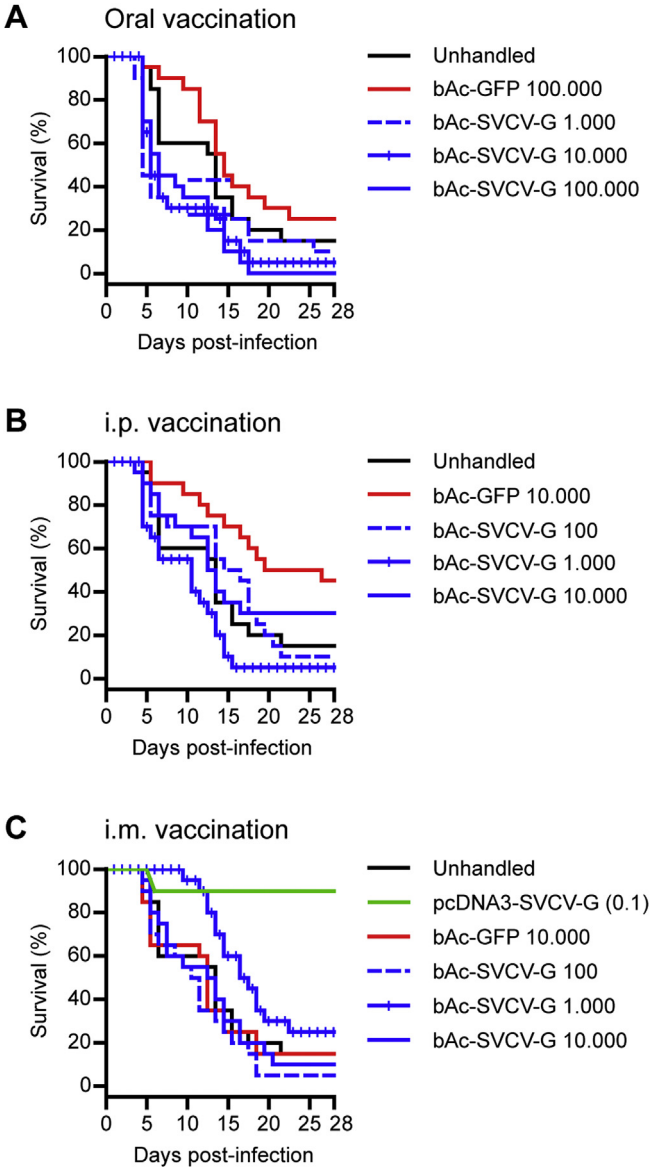

Fig. 8. Efficacy of injection and oral vaccination with a recombinant SVCV-G-based subunit vaccine.

group vaccinated by i.m. injection with the pcDNA3-SVCV-G vaccine (0.1 μg DNA/g of fish) was used as a positive control.

Carp were challenge 2.5 months later. In the orally vaccinated groups, 25% of carp survived in the control group, whereas only 10% or 5% survived in the group vaccinated with 1.000 or 10.000 cells, respectively, and none of the fish survived in the group receiving the highest dose (Fig. 8A). In the i.p. injected groups, all vaccinated fish showed a survival ranging between 5% and 30%, which was lower than the corresponding injected control (Fig. 8B). In the i.m. injected groups, the highest survival (25%) and a delayed onset of mortality was observed in the group i.m. injected with the medium dose of 1000 SVCV-G^+ cells/fish (Fig. 8C). The level of protection however, was much lower than the one observed in the corresponding positive control group i.m. injected with the pcDNA3-SVCV-G plasmid in which 90% survival was observed. In general, compared to the groups vaccinated through the i.m. or i.p. route, the group vaccinated orally showed the fastest kinetics of mortality, with the highest incidence at 5 days after challenge, and the highest mortality in the group receiving the highest vaccine dose. In conclusion, the use of an insect cell-based subunit vaccine against SVCV, under the conditions, doses and vaccination regimes used in our study, was not able to induce significant protection against a bath challenge with SVCV.

Despite the lack of protection observed under the various conditions used, the fact that we used different vaccine types, administration routes, and vaccine doses, in the presence or not of an adjuvant, provides us with valuable information to discuss in details the design of future vaccination strategies.

Carp (3-month-old, $n = 20$/group) were kept at 20 °C and were vaccinated with SVCV-G^+ PFA-fixed whole Sf9 cells using various dose and administration routes. For clarity, the vaccinated groups and respective controls are shown in separate graphs, whereas the unhandled group is shown in each graph as a reference. **(A)** Carp received 1.000, 10.000 or 100.000 alginate-encapsulated SVCV-G^+ cells, or 100.000 GFP^+ cells through oral gavage. **(B)** Carp received 100, 1.000 or 10.000 SVCV-G^+ cells, or 10.000 GFP^+ Sf9 cells through i.p. injection or **(C)** through i.m. injection. The group vaccinated i.m. with pcDNA3-SVCV-G (0.1 μg/g of fish) served as positive control.

4. Discussion

In this study we tested two approaches for vaccination of carp against SVCV. The first approach focused on the oral delivery of alginate-encapsulated SVCV-G plasmid DNA; the second approach consisted of a baculovirus-based recombinant expression of the transmembrane SVCV-G protein in insect cells, which were subsequently used as a whole-cell subunit vaccine for oral or injection delivery. We did not only test different vaccine types but also various doses, regimes, administration routes and, in the case of the DNA-based oral vaccine, the use of a strong mucosal adjuvant (*E. coli* lymphotoxin-beta, LTB). Protection under all tested conditions proved insufficient, certainly when compared with the full protection obtained with the reference pcDNA3-SVCV-G vaccine when injected i.m. We discuss how our results may have been influenced by the nature of the pathogen, host species and encapsulation method used in our study.

In our first approach, we used an encapsulation method based on alginate microspheres. Immunohistochemical analysis revealed high levels of SVCV-G protein expression in the intestinal epithelium after oral administration of encapsulated pcDNA3-SVCV-G plasmid, confirming that the plasmid was delivered intact, was taken up by epithelial cells, finally resulting in SVCV-G protein translation.

In a first vaccination trial we tested relatively low DNA vaccine doses, administered orally either once (3 μg DNA/g of fish) or three times (1 μg DNA/g of fish with a 72 h interval). Under these conditions, no protection was obtained and a steeper and quicker mortality was observed in the group vaccinated three times with the lowest dose of the vaccine. As further discussed later, this might be indicative of tolerance induction. Despite the ascertained SVCV-G protein expression, oral delivery of the encapsulated DNA vaccine did not induce strong local and systemic immune responses (antiviral genes expression, recruitment of neutrophilic granulocytes, macrophages, Igm^+ B cells, and

putative mucosal T cells) as those typically observed upon i.m. injection of the same DNA vaccine [13].

In our second trial, we used a single but higher vaccine dose and orally administered either 20 μg or 100 μg of alginate-encapsulated plasmid per fish, with or without further addition of the mucosal adjuvant LTB. Despite the use of a higher dose and addition of an adjuvant, no protection was observed. Again, a slightly steeper mortality curve in the group vaccinated with the highest dose of plasmid in the presence of LTB was observed. Bacterial toxins and their derivatives, although among the strongest mucosal adjuvants, are often most effective when administered nasally (not orally) and coupled to antigens [20,36,40], which may partly explain our observation. In addition, in our study, the absence of a clear effect of LTB on vaccine efficacy, could be explained by mistiming: possibly immune stimulation by LTB could have occurred prior to sensing of the SVCV-G protein by immune cells since SVCV-G protein expression first required uptake of the plasmid, transcription, and finally translation. Future studies focusing on oral delivery of DNA plasmid could deliver LTB as a plasmid thereby synchronizing its expression with the one of the vaccine antigens.

With regard to the host species, it is of interest to compare our study to previous studies in trout in which oral DNA vaccination against IPNV and IHNV was reported with various degrees of success. The encapsulation method, plasmid dose, regime and age of vaccination used in our first trial were comparable to those previously reported for oral DNA vaccination of trout against IHNV and IPNV. However, while a single oral administration of 10 μg of alginate-encapsulated plasmid induced full protection against IPNV in 3-month-old brown trout and rainbow trout of 1–1.5 g [10], the same plasmid dose and vaccination regime was not successful in protecting rainbow trout against IHNV [4]. In the latter study, a significant degree of protection (RPS = 56) was observed only in trout receiving a very high dose (100 μg) of encapsulated DNA plasmid. Although significant, protection was lower than the one achieved after i.m. injection of 5 μg of the same plasmid (RPS = 76) [4]. The clear difference with respect to the plasmid dose required to achieve protection, suggests that the nature of the pathogen (IPNV versus IHNV) might play a crucial role in achieving protection after oral DNA vaccination. While IPNV is a mucosal pathogen, mainly targeting intestine and pancreas, SVCV is a systemic pathogen similarly to IHNV. SVCV mainly targets kidney and spleen and a strong systemic response might therefore be more important than a strong local mucosal immune response. Although oral vaccination is known to trigger both a local and a systemic response [28,30,37], the systemic response induced by our oral DNA vaccine may not have been strong enough to induce protection. This could maybe also explain the difference in success between oral DNA vaccination against IPNV and against IHNV, since IHNV is a rhabdovirus requiring a stronger systemic response than the mucosal pathogen IPNV [4]. In addition, the time of challenge post-vaccination, 30 days (450° days approximately) in the trout studies versus 2.5 months (1500° days approximately) in our study, is a well-known determining factor when evaluating protection. It could be informative to further investigate the duration of protection induced by the oral DNA vaccines against IPNV and IHNV.

In our second approach, we generated an SVCV-G-based subunit vaccine and tested its efficacy after both, injection (i.m and i.p.) and oral vaccination. To this end, formalin-fixed whole Sf9 cells expressing the recombinant transmembrane SVCV-G protein where used to vaccinate carp through i.p., i.m., and oral delivery. Only the cells used for oral delivery were encapsulated in alginate microspheres, whereas the cells used for injection vaccination were whole-cell suspensions. Despite the use of various doses and routes of administration, none of the tested regimes induced significant protection in carp when challenged with SVCV 2.5 months after vaccination. We hypothesised that the insect cells would act both as vaccine vehicle by displaying the recombinant SVCV-G protein on the cell surface in its native conformation, and as adjuvant by expressing non-self-proteins. To illustrate the potency of our approach, i.m. injection of only few thousands cells expressing the rabies virus G protein was shown to be sufficient to achieve 100% protection in mice [34]. Furthermore, a strong protection was achieved also in raccoons vaccinated orally with lysate of insect cells infected with a baculovirus encoding the rabies G protein [19]. Yet, we did not observe a similar protection in our carp study. In the mouse study, animals received two i.m. injections with 1-week interval and protection was assessed 14 days post-immunization. Only mice receiving 2×1000 cells, but not lower number of cells, showed full protection. Comparison of the weight of these mice (approximately 20 g at 6 weeks) with the weight of the fish used in our study (2–4 g at 3 months), indicates that the relative antigen dose (i.e. absolute number of SVCV-G^+ insect cells) was higher in our study, and thus dose may not explain the difference in protection observed in carp injected with whole Sf9 insect cells expressing the recombinant transmembrane SVCV-G protein.

While injection by i.m. route of the pcDNA3-SVCV-G vaccine, already at a low dose of 0.1 μg/g of fish, was shown to confer full protection against challenge with SVCV [13], i.m injection of the SVCV-G^+ insect cell-based recombinant vaccine induced 20% protection at best. This suggests that crucial protective mechanisms that are triggered by the injection of DNA plasmid, are not (sufficiently) triggered by the injection of the whole-cell-based subunit vaccine. These could include inflammatory responses seen in the muscle upon i.m. injection of DNA plasmid and ascribed to vaccine-induced local damage and recognition of molecular patterns on the plasmid backbone (*i.e.* CpG motifs) [13,24,27]. Possibly, also local antigen expression by host cells, normally induced by the DNA vaccine, assuring peptide presentation on MHC-I molecules and likely activation of the cytotoxic T cell compartment, may not have been triggered by the subunit vaccine. Yet, the co-presence of insect cells as non-self-antigens, and the damage inflicted by the injection should have mimicked at least some of the conditions normally induced by i.m. injection of DNA plasmid. For example, the presence of the native G protein on insect cells should have activated specific humoral responses. One possible explanation could be that the insect cells are (too) rapidly eliminated by the host: it is not possible to accurately estimate the total amount and persistence of G protein locally in the muscle after injection of DNA plasmids since several copies of plasmid DNA can be incorporated in each cell and transcribed at various rates. However, what we and others observed, was that G^+ myocytes could be detected up to 14–21 days after plasmid injection although myocytes were in the process of being eliminated by host cells [13,25]. If indeed insect cells are (too) rapidly eliminated, this would reduce the duration of antigen persistence *in vivo* and negatively affect the required antigen presentation and subsequent induction of a protective immune response. Given the importance of the three-dimensional conformation of the SVCV-G protein and the possible impact of detergents used to lyse cells, we decided to administer the SVCV-G-expressing insect cells as (encapsulated) whole cells instead of (non-encapsulated) lysates as in the raccoon study [19]. Possibly, the presence of an encapsulation matrix and/or the lower availability of damage-associated molecular patterns (DAMPs) might partly explain the observed differences in vaccine efficacy between the two studies.

Altogether, when considering the host species and the oral vaccination approach we took, although alginates were very successful for oral vaccination of brown trout and rainbow trout against IPNV and IHNV [5,10], alginate might not be a universal encapsulation matrix for all fish species. Salmonids are carnivorous species and plant-based alginates are not part of their standard diet. In contrast, carp are omnivorous species and might tolerate plant-based alginate much better, minimizing the co-stimulatory effect seen in salmonids. Therefore, encapsulation matrixes that are not plant-based, like the copolymer poly (lactic-co-glycolic acid) (PLGA) or chitosan, might prove more effective in carp. Finally, the ability of alginates to protect or release their cargo in the intestinal lumen can be highly affected by the pH of the intestinal environment [45], which is different between fish species. Thus, the maximum release should be assessed and optimized for each species.

Furthermore, with respect to the adjuvant used, while linking of GFP to LTB was previously found to enhance GFP uptake and specific humoral responses in carp [7], LTB did not increase protection in our case when administered as protein antigen together with the DNA vaccine mix. Possibly, other adjuvants or immunostimulants may be more suitable for oral vaccination of carp, and may include saponins, flagellin, β-glucans, and molecular adjuvants including cytokines [33,41]. Some of these could be administered as plasmids along with DNA vaccines, or directly as proteins or polysaccharides along with subunit vaccines, in both cases assuring similar kinetics of uptake and expression of the adjuvant and vaccine antigen.

With respect to vaccine efficacy, in all our trials a faster kinetic of mortality was generally observed in the groups orally vaccinated using alginate-encapsulated DNA or proteins. Although the induction and mechanisms of tolerance in fish are still elusive, some reports hint at the possible induction of tolerance in carp when low doses of antigens are delivered over a longer period of time [45,37]. In our study, we administered antigens orally once, two or three times with a short time interval. Whether the lack of protection was due to insufficient antigen dose or to the induction of oral tolerance, possibly due to the regimes used, is not known and was not the focus of the current study. Perhaps, using our SVCV-G DNA vaccine as model, it will be possible in the future to more systematically compare which protective mechanisms are triggered upon injection vaccination, and which are triggered (or are absent) upon (unsuccessful) oral vaccination using the same vaccine. The paucity of tools to thoroughly investigate mucosal responses, in particular B and T cell responses at mucosal surfaces, greatly hampers such in-depth type of analysis. There is no doubt however, that more insights into the mechanisms behind induction of oral tolerance in fish will greatly advance future oral vaccine design.

To conclude, while injection vaccination regimes can provide a situation where the exact vaccine dose and exact location of the antigen uptake are known, oral vaccination regimes appear more difficult to optimize and standardize. This is mostly caused by the large surface area of the intestine, the possibility of antigen breakdown in the gastrointestinal tract and/or the highly tolerogenic environment. Furthermore, an improved understanding of the mechanisms important for optimal functioning of mucosal adjuvants would help to steer the subtle balance between tolerance and activation of protective responses. It may be clear that in order to formulate an effective vaccine, parameters such as vaccine dose, vaccination regime, antigen formulation, encapsulation, and choice of adjuvants, all need to be tailored to the fish species and targeted to the specific pathogen of interest.

Conflicts of interest

The authors declare to have no conflict of interests.

Acknowledgments

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