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Intra-muscular and oral vaccination using a Koi Herpesvirus ORF25 DNA vaccine does not confer protection in common carp (*Cyprinus carpio L.*)

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ABSTRACT

Koi Herpes Virus (KHV or Cyprinid Herpesvirus 3, CyHV-3) is among the most threatening pathogens affecting common carp production as well as the highly valuable ornamental koi carp. To date, no effective commercial vaccine is available for worldwide use. A previous study reported that three intramuscular injections with an ORF25-based DNA vaccine, led to the generation of neutralizing antibodies and conferred significant protection against an intraperitoneal challenge with KHV. In the present study, we set out to optimize an ORF25-based DNA vaccination protocol that required fewer injections and would confer protection upon a challenge that better resembled the natural route of infection. To this end, ORF25 was cloned in pcDNA3 either as a soluble protein or as a full-length transmembrane GFP-fusion protein. We tested our ORF25-based DNA vaccines in multiple vaccination trials using different doses, vaccination routes (i.m. injection and oral gavage) and challenge methods (bath and cohabitation). Furthermore, we analysed local and systemic responses to the i.m. injected DNA vaccine through histological and RT-qPCR analysis. We observed a strong protection when fish received three injections of either of the two DNA vaccines. However, this protection was observed only after bath challenge and not after cohabitation challenge. Furthermore, protection was insufficient when fish received one injection only, or received the plasmid orally. The importance of choosing a challenge model that best reflects the natural route of infection and the possibility to include additional antigens in future DNA vaccination strategies against KHV will be discussed.

1. Introduction

Common carp (*Cyprinus carpio carpio* L.) is among the five most cultured species worldwide but its sharp increase in production, along with the global intensification of aquaculture, have led to the increasing incidence of infectious diseases outbreaks (FAO, 2010- [1]; [2]. Koi Herpes Virus (KHV), also known as Cyprinid Herpesvirus 3 (CyHV-3), is one of the pathogens causing high losses in common carp aquaculture [3,4]. Furthermore, KHV affects ornamental koi carp (*Cyprinus carpio koi*) and wild carp populations, resulting not only in high economical losses but also in environmental damage [3,5,6]. KHV is part of the *Alloherpesviridae* family and has a linear double stranded DNA genome of ~295 kB, encoding 156 functional Open Reading Frames (ORFs) [7]. While effective inactivated, live attenuated or live recombinant experimental vaccines against KHV have been reported [8–16], no commercial vaccine is yet available for worldwide use due to legislative restrictions concerning associated risks of reactivation [17] or reversion to virulence. The only available live attenuated immersion vaccine is commercialized and used exclusively in Israel (KV3, KoVax Ltd, Israel).

In contrast to live virus vaccines, subunit or DNA vaccines would provide suitable alternatives owing to their safety profile. The large size of the KHV genome and the complexity of the virion require identification and characterization of ORFs before they can be selected as vaccine candidates. While 40–43 functional proteins has been identified in various KHV isolates [7,18,19], the immunogenicity of only a few has been characterized [8,21–24]. Recently, two studies showed the potential of using the membrane proteins ORF25 and ORF81 as candidates for intra-muscular (i.m.) DNA vaccination of carp against KHV [25,26]. In these studies, three consecutive i.m. injections of 1, 10 or 50 μg of pcDNA3 encoding the soluble form of the surface glycoproteins ORF25 or ORF81, triggered the formation of neutralizing antibodies, and induced up to 87.5% survival when carp were challenged with KHV by intra-peritoneal (i.p.) injection. While this is promising, fewer injections or vaccination routes other than injection would be preferred

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Table 1
Primers used in this study.

	FW primer 5′-3′	RV primer 3′- 5′	Acc. Nr.
Primers used for amplification and cloning of KHV ORF25			
tmORF25	ATATAC**GGTACC**ATGGCGGGTTGTGGGGTT	ATATAT**CTCGAG**GGGCCTCCGGGAAACCTGGGC	
solORF25	ATATAC**GGTACC**ATGGCGGGTTGTGGGGTT	ATATAT**CTCGAG**TTAAGCGTAGTCTGGGACGTCGTATGGGTAGGTGGCGTTGAGGTCCT	
Primers used for RT-qPCR			
Housekeeping genes			
40s	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	AB012087
Cytokines			
cxca	CTGGGATTCCTGACCATTGGT	GTTGGCTCTCTGTTTCAATGCA	AJ421443
cxcb1	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	AB082985
il1β	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGAGGCTGTCA	AJ245635
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

Restriction sites (*KpnI* in the FW and *XhoI* in the RV primer) are in bold; the Kozak sequence is underlined in the common FW primers. The HA tag is underlined in the RV primer used for the soluble ORF25 construct.

from a practical point of view. Furthermore, a challenge method better resembling the natural route of infection might be more reliable to assess vaccine efficacy. The potential of oral vaccination against KHV was assessed using liposomes containing formalin-inactivated KHV [16,27] and using recombinant *Lactococcus plantarum* (*L. plantarum*) encoding the KHV ORF81 [28]. In both studies, protection around 70–75% was obtained, indicating that oral vaccination against KHV is possible but needs further refinement. More recently, during the course of this study, a report described the generation of an ORF25-deleted KHV virus and its efficacy as vaccine strain. Despite its proven safety and level of attenuation *in vivo*, its efficacy as a vaccine was suboptimal, suggesting that the presence of ORF25 is required to trigger KHV-specific protective responses [15].

Given the promising results from previous studies, we set out to investigate the potential of a DNA vaccine encoding the ORF25 for oral and injection vaccination. We hypothesized that since previous studies on DNA vaccination against KHV used constructs expressing soluble proteins, a transmembrane ORF25 would trigger a more appropriate immune response already after a single injection. In the current study, a construct encoding the extracellular soluble domain of the ORF25 (pcDNA3-solORF25), and one encoding a transmembrane ORF25-GFP fusion protein (pcDNA3-tmORF25-GFP) were constructed. We here describe their application as injection as well as oral DNA vaccines using various doses and vaccination regimes. We did not only assess vaccine efficacy upon KHV challenge using a bath and a cohabitation method, closely mimicking the natural route of infection, but we also characterized the local immune response upon injection vaccination. Since moderate degree of protection was observed only after repeated administrations of the DNA plasmid encoding the transmembrane ORF25 protein, further optimization is required to achieve full protection against KHV. Accordingly, the use of additional KHV proteins as vaccine antigens for future (DNA) vaccination strategies will be discussed.

2. Materials and methods

2.1. Animals

European common carp (*Cyrpinus carpio carpio*) R3xR8 that originated from a cross between the Hungarian R8 strain and the Polish R3 strain [29] were used, unless stated otherwise. Specific pathogen free (SPF) carp were bred in the Aquatic Research Facility (ARC) of the Carus animal facility at Wageningen University, the Netherlands. Fish were either raised at the local facility or eggs were transported to the Veterinary Research Institute (VRI, Brno, Czech Republic) 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. As a control, susceptible naïve Koi carp were purchased at the Czech koi breeding center ALCEDOR s.r.o.

For the experiments in Israel, common carp of the Yugoslavian (YxY) strain (~7 g in weight) were used. As a control, susceptible koi carp of mixed varieties were used. Näive koi were obtained at ~10 g weight from Kibbutz Gan Shmuel and Kibbutz Ma'agan Michael fish breeding centers in Israel. Common carp were bred and reared in recirculating water as described previously [30,31], at the fish facility of the Robert H. Smith Faculty of Agriculture, Food and Environment of the Hebrew University of Jerusalem in Rehovot, Israel.

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 the work performed in Israel was approved by the Animal research ethics committee of the Hebrew University of Jerusalem under permit # AG-13059-5. Animal work in Wageningen University was approved by the local experimental animal committee (DEC number 2014098). Animal work at VRI was approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (permission No MZe 1717).

2.2. Cells

Common carp brain (CCB) cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 1% penicillin/streptomycin, 1% L-glutamine, 1% non-essential amino acids, 10% FCS and 3.5 g/L D-glucose at 27 °C, in the presence of 5% CO_2.

2.3. Cloning of KHV ORF25

The full-length KHV ORF25 was amplified from genomic DNA isolated from KHV isolate C250 (kindly provided by Dr. Keith Way, CEFAS, Waymouth, UK) by PCR using proofreading Taq polymerase (Roche). Primers were designed based on alignments of the genome sequences of the following KHV strains; KHV-U (Acc. Nr, DQ657948),

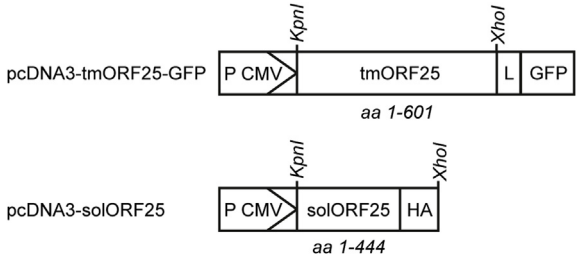

Fig. 1. Schematic representation of the two pcDNA3 constructs encoding ORF25. tmORF25: transmembrane ORF25; solORF25: soluble ORF25; P CMV: cytomegalovirus promotor; restriction sites are indicated in italics; numbers indicate the first and last nucleotide relative to the full-length sequence. C-terminal GFP and HA tags are indicated, as well as the GGSGG linker sequence (L) between the tmORF25 and the GFP sequence.

KHV-I (Acc. Nr. DQ177346) and KHV-J (AP008984). All primers used for cloning can be found in Table 1, and a schematic representation of the constructs can be found in Fig. 1. The obtained products were verified on a 1% agarose gel and were purified using S400 Sephacryl columns (GE Healthcare). For the transmembrane ORF25 fused to GFP (tmORF25-GFP) construct, the purified PCR product amplifying the full-length ORF25 sequence was ligated using T4 ligase (Promega) in the pcDNA3-GFP vector [32] between the *KpnI* and *XhoI* sites generating the pcDNA3-tmORF25-GFP. Cloning in this vector allowed for insertion of the tmORF25 upstream of the GFP sequence separated by a short linker sequence encoding the amino acids GGSGG, as described previously [32]. For the soluble ORF25 (solORF25) construct, the purified PCR product encoding for amino acid 1–444 followed by a C-terminal HA tag, with the first amino acid being the first methionine, was ligated in the pcDNA3 vector between the *KpnI* and *XhoI* sites generating the HA-tagged pcDNA3-solORF25-. Constructs were verified by sequencing and plasmids were purified using the endotoxin-free plasmid isolation midi kit (Invitrogen) prior to *in vitro* transfection or *in vivo* i.m. injection.

2.4. Transfection of CCB cells and staining of tmORF25-GFP

CCB cells were seeded on glass cover slips in 12 well-plates at a density of 250.000/well. Cells were transfected with 1 μg pcDNA3-tmORF25-GFP or pcDNA3-GFP and 4 μL of FuGENE HD transfection reagent (Promega) using the manufacturer's guidelines. After 48 h cells were rinsed twice with Tris-buffered saline (TBS, 10 mm TRIS, 135 mm NaCl, pH 7.5), fixed with 4% paraformaldehyde (PFA) in TBS for 10 min at RT and blocked with 1% bovine serum albumin (BSA) in TBS. Serum from carp that were vaccinated once with 0.5 μg pcDNA3-tmORF25-GFP plasmid/g fish and subsequently exposed to KHV, as further described in paragraph 2.6, was used to visualize tmORF25 surface expression. Serum was collected from survivor fish 30 days after the cohabitation challenge. Serum was diluted 1:50 in PBS and was incubated on the slides for 45 min. Subsequently, slides were stained with an antibody against carp Igm (WCI12; [33], diluted 1:100 in TBS containing 1% BSA) and PE-conjugated goat-anti-mouse (Invitrogen, 1:200). Cells were visualized using an EVOS fl LED fluorescence microscope (Advanced Microscopy Group (AMG)).

2.5. Virus

Wild type KHV virus was used for the challenges performed in Israel and was kindly provided by KoVax, Ltd. (Israel). KHV (Isolate Hedrick KHV 261) was used for the challenges performed in the Czech Republic. KHV isolate Hedrick KHV 261 was propagated in CCB cells. Virus titres were determined using the method of Reed and Muench [34] and are displayed as plaque-forming units (pfu).

2.6. Vaccination and challenge experiments

In the first experiment, carp (R3xR8 strain) of 24–30 g (n = 20/group) received 0.5 μg DNA/g of fish by i.m. injection, either once or three times with a three-weeks interval. Carp were anesthetized in 0.3 g/L of Tricaine Methane Sulfonate (TMS; Crescent Research Chemicals) before vaccination. Vaccination groups included the pcDNA3 empty vector, pcDNA3-solORF25 and pcDNA-tmORF25-GFP. Susceptible koi carp were included as unhandled (non-vaccinated) controls. Carp were challenged 3 months after the first vaccination by bath (2 h exposure to 0.7×10^4 pfu/mL) or by cohabitation (1:5 ratio of shedders to vaccinated carp). Shedders were susceptible koi carp infected on forehand by intraperitoneal (i.p.) injection of 150 μL of undiluted virus suspension (approximately 5000 pfu) of wild-type KHV (strain Hedrick KHV 261) and mortalities were recorded daily for 30 days.

In the second experiment, carp (YxY strain) of ~7 g were vaccinated by i.m. injection of 1 μg of DNA/g of fish (pcDNA3-GFP or pcDNA3-tmORF25-GFP, n = 15/group) or by i.m. injection of 20 μl ($> 0.7 \times 10^5$ pfu/ml) of the KV3 live attenuated KHV vaccine (KoVax, Ltd). Immersion vaccination with the KV3 vaccine for 1 h in 2L water containing $> 0.7 \times 10^5$ pfu/ml of KV3 vaccine was used as a positive vaccine control (n = 12/group) and unhandled (non-vaccinated) fish as a negative control. Fish were challenged by cohabitation two months later as described above.

In a third experiment, carp (YxY strain) of 2 g (n = 30/group, divided over duplicate tanks) received 1.5 μg DNA/g of fish by i.m. injection (pcDNA3-GFP or pcDNA3-tmORF25-GFP) or 2.5 μg of DNA/g of fish by oral gavage, either once or three times with a 72 h interval. DNA plasmids (pcDNA3-GFP or pcDNA3-tmORF25-GFP) were encapsulated in alginate microspheres as described previously [35]; this issue) and administered in 10 μL by oral gavage using a 10 μL pipette. Carp were challenged by cohabitation 3 months later.

2.7. Histological analysis

Carp were i.m. injected with 20 μg of DNA (pcDNA3 or pcDNA3-tmORF25-GFP, n = 3 per plasmid) to examine the expression of the GFP-fused tmORF25 protein. Fish were euthanized in 0.6 g/L (Crescent Research Chemicals) and bled through the caudal vein 14 days later. The site of injection was isolated, snap-frozen in liquid nitrogen and stored at −80 °C until further processing. Cryosections (5 μm) from muscle sections were mounted on poly-lysine slides, fixed for 15 min at 4 °C with 4% PFA in PBS, stained with DAPI (4′,6-Diamidino-2-Phenylindole, Dilactate) to visualize cell nuclei (Thermo Scientific), and mounted with Vectashield (Vectorlabs). Sections were imaged using a EVOS fl LED fluorescence microscope (Advanced Microscopy Group (AMG)).

2.8. RNA isolation and cDNA synthesis

Carp were vaccinated by i.m. injection with 1 μg DNA/g fish and were sacrificed 3 and 5 days later to analyze the local response to the vaccine. Carp were euthanized in 0.6 g/L tricaine methane sulfonate (TMS, Crescent Research Chemicals) and bled through the caudal vein. Muscle at the injection site was isolated, snap frozen in liquid nitrogen, and stored at −80 °C until further processing.

Total RNA was isolated from muscle tissue using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions including on-column DNase treatment using the RNase-free DNase set (Qiagen) and an additional Proteinase-K (Qiagen) treatment. RNA concentrations were measured using a Nanodrop-1000 (Thermo Scientific), the integrity was verified on a 1% agarose gel and RNA was stored at −80 °C until further use. Prior to cDNA synthesis, 1 μg total RNA was subjected to a second DNase treatment 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.

2.9. 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 as described previously [36,37]. Briefly, 5 μL of 25 times diluted cDNA was mixed with 2 μL of forward and reverse gene-specific primers (2.1 μM of each primer) and 7 μL of 2x ABsolute qPCR SYBR Green Mix (Thermo Scientific) as detection chemistry. Thirty-five cycles were used for the detection of all selected genes. The list of primers can be found in Table 1. The take-off (Ct) 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 [38]. The relative expression ratio (R) of each sample was calculated according to the Pfaffl method [39] based on the take-off deviation of sample versus each of the unhandled controls at time point 0 h, and normalized relative to the *s11* protein of the *40s* subunit (referred to as *40s*) as reference gene.

3. Results

3.1. Expression of KHV tmORF25-GFP in vitro and in vivo

For the *in vitro* validation of the tmORF25-GFP, CCB cells were transfected with the pcDNA3-tmORF25-GFP construct or with pcDNA3-GFP. A strong GFP signal was detected 48 h after transfection with either of the two constructs. Surface expression of tmORF25 was confirmed upon labelling using serum from fish that were vaccinated with the pcDNA3-tmORF25-GFP construct and that survived a subsequent bath challenge with KHV (Fig. 2A, upper panel). No reactivity was observed in CCB cells transfected with the pcDNA3-GFP construct (Fig. 2A, lower panel), confirming the specific of the reaction. Surface labelling using control serum from pcDNA3-injected carp showed a similar pattern to Fig. 2A, lower panel (not shown).

For the *in vivo* validation of the same construct, muscle tissue at the site of injection and spleen of fish that were injected i.m. with 20 μg of plasmid were examined. Tissues were processed for cryosectioning and expression of ORF25-GFP was clearly observed locally in the muscle as well as in the spleen 14 days after injection of the pcDNA3-tmORF25-GFP plasmid (Fig. 2B–C). Together this data shows that ORF25-GFP was expressed and detected *in vivo* both locally and systemically.

3.2. Protection against KHV after i.m. and oral DNA vaccination using ORF25

To investigate whether a single i.m. DNA injection is sufficient to protect carp against KHV, and to compare the efficacy of the constructs encoding either the soluble ORF25 or the native transmembrane ORF25, we tested the pcDNA3-solORF25 as well as the pcDNA3-tmORF25-GFP plasmids. To this end carp received 0.5 μg DNA/g of fish, either once or three times with 3-weeks intervals and were challenged three months after the first injection (six weeks after the 3rd injection). Fish injected with the same dose of the empty pcDNA3 plasmid served as control. For the KHV challenge, we chose to infect by either bath or cohabitation since these best resemble natural routes of infection. Strikingly, large differences in survival rate were observed between the cohabitation and bath challenge (Fig. 3A). After cohabitation challenge, mortalities were generally high. The highest survival (relative percent of survival (RPS) of 26) was observed in the groups vaccinated with either 1 or 3 injections of the tmORF25 construct. In the groups injected with the solORF25 construct, survival was less than 20%. After bath challenge, highest survival was observed in the group receiving three injections of the tmORF25 construct (RPS of 89), followed by the solORF25-injected group (RPS of 84). In the groups injected once, survival was generally lower; the highest survival was observed again in the tmORF25-injected group (RPS of 45). Based on these results, we selected the pcDNA3-tmORF25-GFP construct for follow up experiments and decided to optimize the dose of the single injection regime, since this is preferred over multiple injections. Despite the generally higher mortality observed upon cohabitation challenge, we decided to continue with this challenge method in all follow-up experiments, since this better reflects a natural mode of infection.

In our second experiment, fish were i.m. injected once with 1 μg DNA/g of fish. This time, the KV3 (KoVax, Ltd, Israel) vaccine was included as positive control and fish were either i.m. injected with 20 μL (> 1400 pfu/fish) of the KV3 solution or immersed according to the recommended protocol. All groups were challenged two months later by cohabitation. The groups vaccinated with the KV3 vaccine, either by i.m. injection or by immersion, showed very good protection (RPS of 88) (Fig. 3B). In contrast, no survival was observed in the group vaccinated with the pcDNA3-tmORF25-GFP plasmid. These results indicate that vaccination through the i.m. can protect carp against KHV, at least when using the live attenuated KV3 vaccine, whereas i.m. DNA vaccination needs further optimization.

In a third experiment, we performed a preliminary assessment of the efficacy of the pcDNA3-tmORF25-GFP vaccine when delivered orally. Fish received one or three administrations (with a 72 h interval) of 2.5 μg of alginate-encapsulated DNA/g of fish or one i.m injection of 1.5 μg DNA/g of fish. Upon cohabitation challenge three months after vaccination, no protection was observed in all tested groups (Fig. 3C). Overall, we conclude that i.m. and oral DNA vaccination against KHV using a constructs encoding tmORF25, using the chosen vaccine doses and regimes, does not confer strong protection against a cohabitation challenge with KHV.

3.3. Effects of pcDNA3-tmORF25-GFP injection on expression of immune-related genes

Given that a single injection of DNA vaccine induced only limited protection, we set out to examine the local response to the DNA vaccine. We analysed the expression of a selected panel of immune-related genes in muscle of carp injected with 1 μg/g of fish of the pcDNA3-tmORF25-GFP vaccine. Samples from the site of injection were taken after 3 and 5 days. Our data show that although the expression of pro-inflammatory molecules and interferon-stimulated genes (ISGs) *mx1, vip2* and *isg15.2* was increased up to 100 folds at 3 or 5 days post-injection (dpi), the levels were not significantly different from those observed in the control group not expressing the ORF25 (pcDNA3-GFP) (Fig. 4). A similar pattern was observed for genes linked to adaptive immune responses; despite a 40-fold upregulation of *Igm* at 5 dpi in carp receiving the pcDNA3-tmORF25-GFP plasmid, this upregulation was also observed in the pcDNA3-GFP-injected group. Overall, we conclude that given the absence of a significant ORF25-specific response, the observed early local response is likely due to both the damage-related inflammation caused by the injection as well as the plasmid backbone.

4. Discussion

While effective inactivated, live recombinant or attenuated experimental vaccines have been described for KHV [9–14,16], owing to safety concerns, the only commercially available live attenuated vaccine is restricted for use in Israel. To circumvent some of the concerns associated with the use of live recombinant or attenuated viral vaccines, there is a strong drive for the development of alternative types of vaccines such as subunit vaccines or DNA vaccines. Recently, the CLYNAV DNA vaccine against Pancreatic Disease in Atlantic salmon (*Salmo salar*) has been granted marketing authorization in Europe [40], becoming the first commercially available DNA vaccine on the European market. This development opens up a whole new era, as well as a

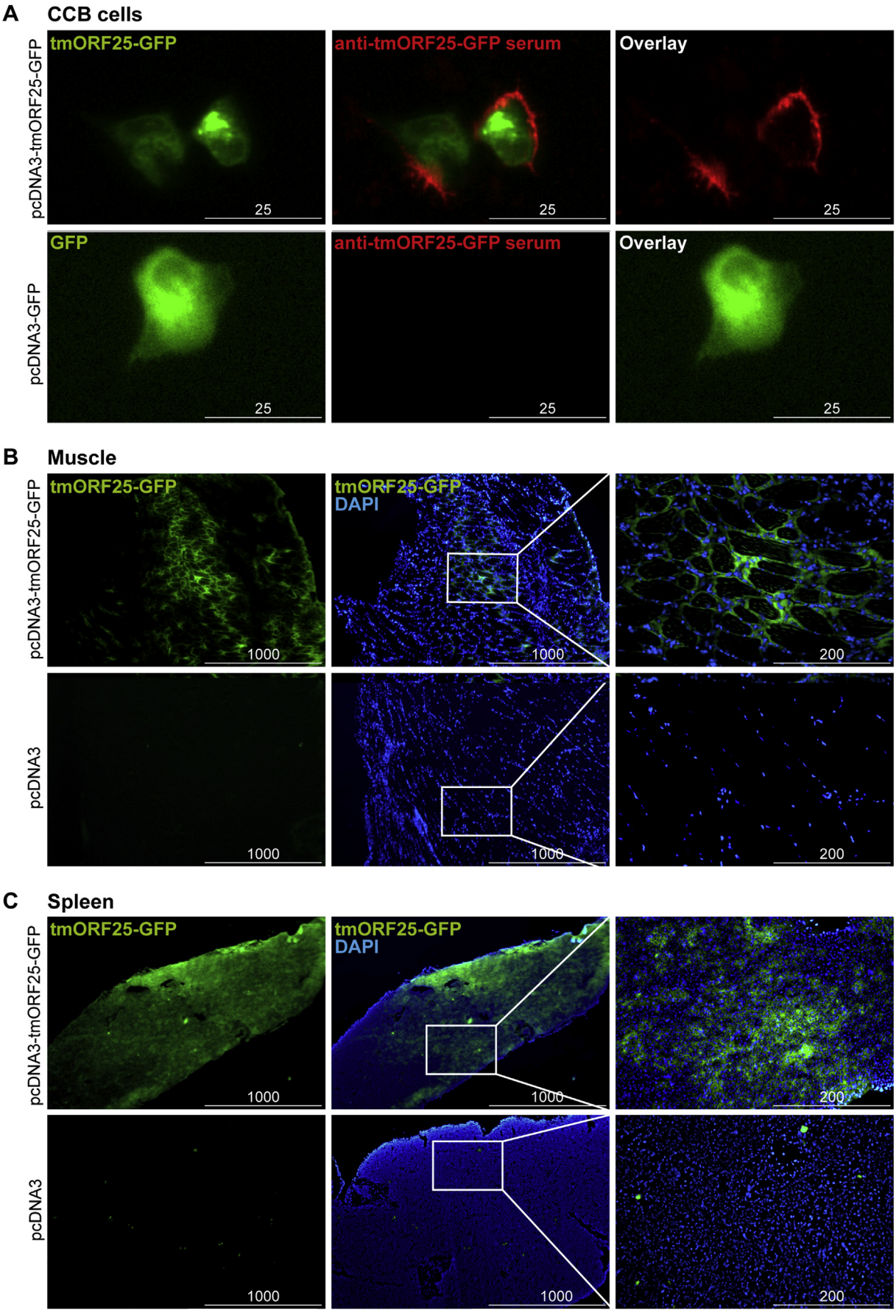

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Fig. 2. Detection of tmORF25-GFP *in vitro* and *in vivo*. (A) CCB cells were transfected with pcDNA3-tmORF25-GFP or pcDNA3-GFP; 48 h later cells were fixed with PFA and analysed for surface expression of tmORF25-GFP. Upon labelling with serum from pcDNA3-tmORF25-GFP-vaccinated fish that survived a subsequent KHV bath challenge, surface expression of tmORF25 was detected only in pcDNA3-tmORF25-GFP transfected cells (upper panel) and not in pcDNA3-GFP transfected cells (lower panel). **(B–C)** For *in vivo* validation of the tmORF25-GFP construct, carp were injected with 20 μg of either pcDNA3 or pcDNA3-tmORF25-GFP and muscle **(B)** and spleen **(C)** were collected 14 days after injection. Direct fluorescence detection of the tmORF25-GFP in 5 μm PFA-fixed cryosections revealed clear tmORF25-GFP expression (upper panels). A counterstaining with DAPI was included to visualize the nuclei. Images were acquired using an EVOS fl LED fluorescence microscope (Advanced Microscopy Group (AMG)). Scale bars indicate μm.

renewed interest in the development and application of targeted DNA vaccines for aquaculture species.

In the current study, we describe two experimental DNA vaccines encoding the KHV ORF25 protein, either as a soluble (pcDNA3-solORF25) or as a transmembrane GFP-fused protein (pcDNA3-tmORF25-GFP). The potential for ORF25 to serve as antigen for DNA vaccination of carp against KHV was previously shown in a study by Zhou et al. [25]. In the latter study, koi carp received three i.m injections of either 1, 10 or 50 μg of DNA encoding for a soluble form of ORF25. Upon i.p. challenge with KHV, survival rates of 80% were obtained already with the lowest concentration of plasmid, and reached 87.5% with the highest concentration of the ORF25 DNA vaccine.

Based on the results by Zhou et al., in the current study we set out to optimize a vaccination protocol that required fewer injections and that would confer protection upon a challenge that better resembled the natural route of infection. To this end, we investigated the protection induced by pcDNA3-solORF25 and pcDNA3-tmORF25-GFP, using two routes of vaccination (i.m. injection and oral gavage), different vaccines doses and two challenge routes (bath and cohabitation). In agreement with the study by Zhou et al., in our first vaccination trial, we observed the strongest protection after three i.m. injections of either of the constructs when carp were challenged by bath, but not when

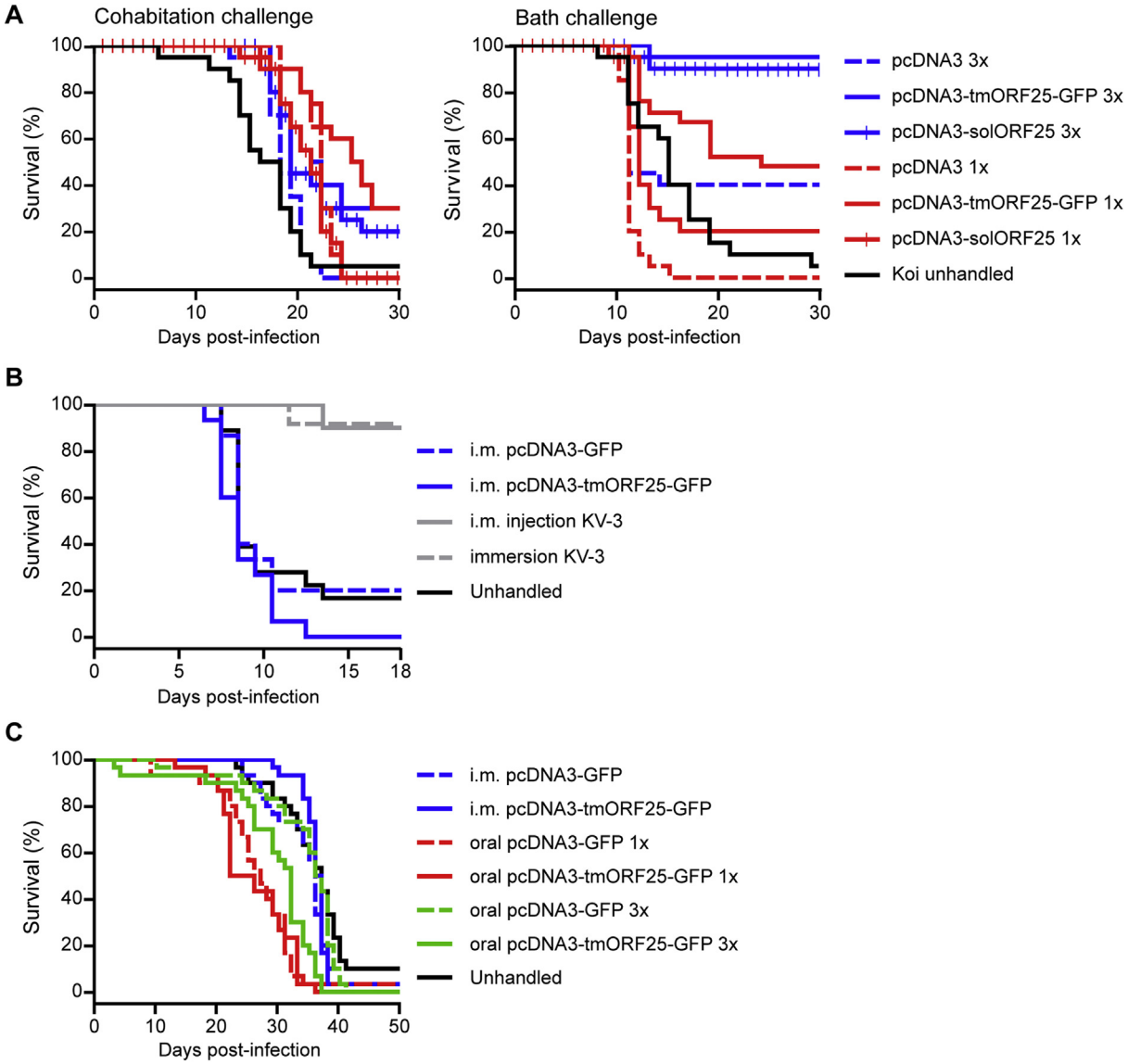

Fig. 3. Survival of carp i.m. and orally vaccinated using ORF25 DNA vaccines. (A) Carp of 24–30 g (n = 20/group) were injected with 0.5 μg DNA/g of fish of the indicated plasmids, either once (1x) or three times (3x), with a 3-week interval. Carp were challenged 3 months after the first injection (six weeks after the 3rd injection) by cohabitation or by bath. **(B)** Carp of 7 g (n = 15/group) were injected with 1 μg DNA/g of fish of the indicated plasmids. The control groups (n = 12/group) were vaccination with the KV3 vaccine by i.m. injection (20 μL, 1400 pfu) or by immersion (200 μL/2L, 7000 pfu/L). Carp were challenged 2 months after vaccination by cohabitation. **(C)** Carp of 2 g (n = 30/group) were injected i.m. with 1.5 μg DNA/g of fish of the indicated plasmids or received 2.5 μg of alginate-encapsulated DNA/g of fish of the same plasmids through oral gavage, either once (1x) or three times (3x), with a 72 h interval. Carp were challenged 3 months after vaccination by cohabitation. Mortality was recorded for the indicated time.

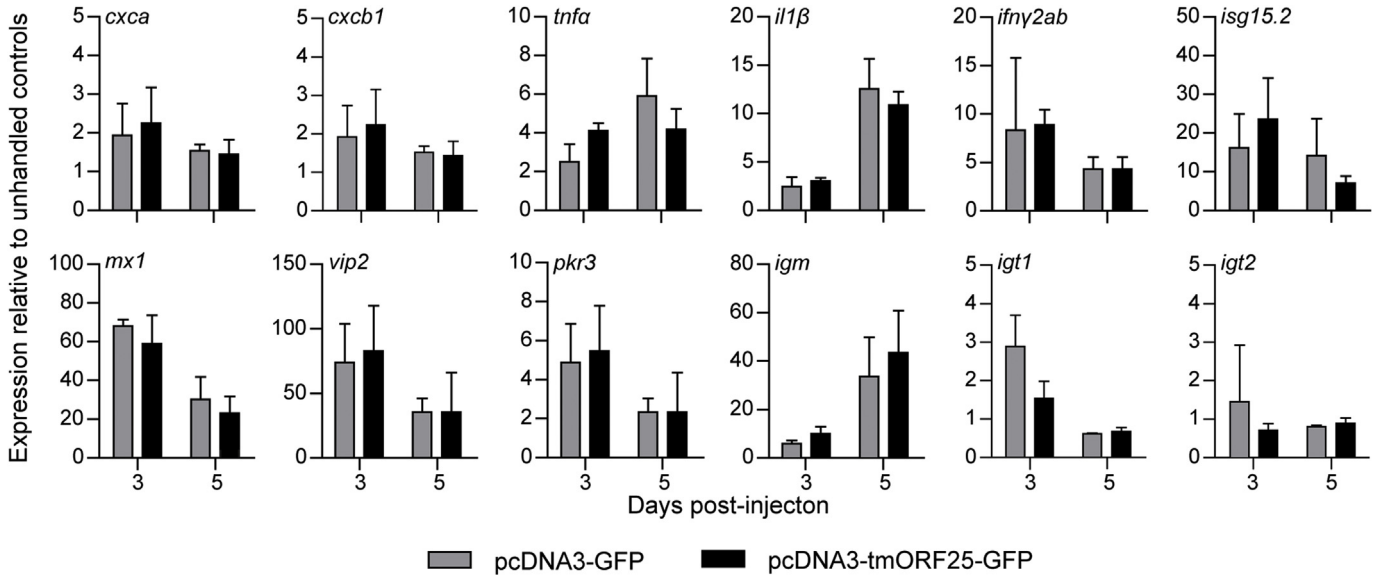

Fig. 4. Gene expression analysis of immune-related genes in the muscle after i.m. injection of pcDNA3-tmORF25-GFP. Carp were injected with 1 μg DNA/g of fish with either pcDNA3-GFP or pcDNA3-tmORF25-GFP. The site of injection was isolated 3 and 5 days post-injection and processed for gene expression analysis. Expression was normalized against the housekeeping gene *s11* of the ribosomal subunit *40s* (referred to as *40s*) and expressed relative to the unhandled controls at $t = 0$. Bars indicate average and SD of $n = 3$ (pcDNA3-GFP) or $n = 4$ (pcDNA3-tmORF25-GFP) fish per time point.

challenged by co-habitation. Nevertheless, considering that a single injection with the tmORF25 construct conferred higher protection upon bath and cohabitation challenge than a single injection with the solORF25 construct, for our subsequent trials we decided to continue with the tmORF25 plasmid. Furthermore, since our aim was also to optimize a vaccination protocol that would confer protection upon a challenge closely resembling the natural route of infection, we decided to continue with the cohabitation challenge for the further optimization of our ORF25 DNA vaccine.

In a subsequent trials, the protection conferred by i.m. injection was validated by the KV3 vaccine that conferred the same level of protection when delivered through i.m. injection, as it did when administered through the recommended immersion route (90% survival). However, the tmORF25-GFP DNA vaccine, when i.m. injected once, did not confer sufficient protection in any of the follow up experiments. Similar results were observed when the plasmid was delivered orally once or three times (up to 13 μg/fish).

Histological analysis 14 days after i.m. injection of the pcDNA3-GFP-tmORF25 revealed a strong expression of the tmORF25-GFP protein in myocytes at the site of injection and in the spleen of vaccinated carp. This is in agreement with studies in fish showing that part of the injected DNA can be detected in multiple systemic organs within few hours, up until 1 year after i.m. injection [41,42]. Analysis of the local expression of a panel of immune-related genes revealed that injection of pcDNA3-tmORF25-GFP did not induce significant vaccine-specific changes at 3 and 5 days after injection. In fact, changes in expression of various inflammatory and antiviral genes were observed, but these were not different between groups injected with the empty plasmid or with the tmORF25 construct. Recently, we reported the induction of a strong vaccine-specific local response after i.m. injection of the same dose of pcDNA3 plasmid encoding the Spring Viremia of Carp Virus (SVCV) glycoprotein (G) protein, characterized by significant increases in the cytokines *cxcb1, ifnγ2ab, ifnφ1, ifnφ2* and even the adaptive immunity-related genes *igt1* and *zap70* [35]. Since this vaccine was also found to confer full protection against SVCV in juvenile carp, we can speculate that the expression of ORF25 alone might not be sufficient to trigger a strong local as well as systemic response required to achieve protection against KHV.

In the aforementioned ORF25 DNA vaccination study [25] koi carp received three i.m injections (with 3-weeks interval) of a DNA plasmid encoding the soluble ORF25, and were subsequently challenged by i.p. injection. The latter study used a construct encoding a soluble ORF25 protein composed of the core amino acids of the extracellular portion of the ORF25 molecule (amino acid 165–444). In our study we found that the tmORF25 construct conferred a protection similar to the one obtained by the solORF25 construct after three injections, whereas it induced a higher protection than the solORF25 construct after a single injection. The differences between the two studies in the effectiveness of the constructs encoding the soluble ORF25 protein can possibly be ascribed to the fact that our soluble construct encoded amino acid 1–444, thus including also the most N-terminal portion of the protein. Whether and how this (additional) portion of the protein would affect the correct folding of the soluble peptide would require further investigation, but it is unlikely that the additional N-terminal amino acids would have adverse effects on folding or immunogenicity. Furthermore, the approach using the full-length tmORF25 protein should exclude the possibility that important neutralizing-epitopes would be missing. Finally, it cannot be excluded that the route of challenge, i.p. in the former study and cohabitation (or bath) in our study, as well as the time of challenge after vaccination, 2 weeks after the last booster in the former study and 2.5 months in our study, altogether play a crucial role to reveal the effectiveness of the vaccine.

While 40 or more structural proteins have been identified in various KHV strains [18,19], half of them are still uncharacterized, increasing the difficulty to select potential candidates for vaccine design. In a study characterizing the immunogenicity of KHV structural proteins [21], Igm reactivity in sera of carp that survived a KHV infection strongly points towards the fact that not one, but multiple proteins are the target of the KHV-specific antibody response of carp and koi. In particular, the major capsid protein ORF92 and proteins belonging to the ORF25-family (ORF25, ORF65 and ORF148, ORF149) were found to be major targets for the antibody response of carp against KHV [21]. Furthermore, as previously mentioned, neutralizing antibodies against ORF25 [25] and ORF81 [26] were generated when these antigens where used for DNA vaccination. In agreement, it was recently shown that neutralizing antibodies against ORF25 are (at least partly) required for the induction of a protective immune response against KHV [15]. Finally, upon KHV immunization of mice a neutralizing monoclonal antibody was generated against the capsid protein ORF72 [24].

In conclusion, while there is evidence that i.m. DNA vaccination

using an ORF25 DNA vaccine holds promise for the protection against KHV, we were not able to optimize a DNA vaccination protocol that would be effective upon oral administration or upon single injection of the ORF25-based DNA plasmid. Our findings on a challenge route-dependent survival indicate that to optimally evaluate vaccine efficacy, challenge models that comply with the natural route of infection should preferably be used. Considering the complexity of the KHV proteome, we argue that a vaccine approach combining multiple KHV antigenic proteins might be more potent in triggering a protective immune response against a cohabitation challenge with KHV. More specifically, the type-I membrane proteins from the ORF25 family (ORF25, ORF148, ORF149), the glycoprotein ORF81 as well as the capsid proteins ORF72 and ORF92 are strong vaccine candidates that require further investigation.

Competing interests

The authors declare to have no conflict of interests.

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