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Title: Transgene and immune gene expression following intramuscular injection of Atlantic salmon (Salmo salar L.) with DNA-releasing PLGA nano- and microparticles

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Abstract: The use of poly-(D,L-lactic-co-glycolic) acid (PLGA) particles as carriers for DNA delivery has received considerable attention in mammalian studies. DNA vaccination of fish has been shown to elicit durable transgene expression, but no reports exist on intramuscular administration of PLGA-encapsulated plasmid DNA (pDNA). We injected Atlantic salmon (Salmo salar L.) intramuscularly with a plasmid vector containing a luciferase (Photinus pyralis) reporter gene as a) naked pDNA, b) encapsulated into PLGA nano- (~320nm) (NP) or microparticles (~4μm) (MP), c) in an oil-based formulation, or with empty particles of both sizes. The ability of the different pDNA-treatments to induce transgene expression was analyzed through a 70-day experimental period. Anatomical distribution patterns and depot effects were determined by tracking isotope labeled pDNA. Muscle, head kidney and spleen from all treatment groups were analyzed for proinflammatory cytokines (TNFα, IL-1β), antiviral genes (IFN-α, Mx) and cytotoxic T-cell markers (CD8, Eomes) at mRNA transcription levels at days 1, 2, 4 and 7. Histopathological examinations were performed on injection-site samples from days 2, 7 and 30. Injection of either naked pDNA or the oil-formulation was superior to particle treatments for inducing transgene expression at early time-points. Empty particles of both sizes were able to induce proinflammatory immune responses as well as degenerative and inflammatory pathology at the injection site. Microparticles demonstrated injection-site depots and an inflammatory pathology comparable to the oil-based formulation. In comparison, the distribution of NP-encapsulated pDNA resembled that of naked pDNA, although encapsulation into NPs significantly elevated the expression of antiviral genes in all tissues. Together the results indicate that while naked pDNA is most efficient for inducing transgene expression, the encapsulation of pDNA into NPs up-regulates antiviral responses that could be of benefit to DNA vaccination.

Highlights

- Plasmid DNA was encapsulated into PLGA nano- and microparticles
- Naked and encapsulated pDNA was injected into muscle of Atlantic salmon
- Naked pDNA delivery is superior for inducing transgene expression
- Nano- and microparticles induce significant expression of inflammatory genes
- Nanoparticles carrying pDNA significantly up-regulate antiviral genes

Table(s)

Table 1 – An overview of the preparation protocol and main particle characteristics for pDNA-loaded PLGA nanoparticles (NP-(^{125}I-f-pDNA)) and microparticles (MP-(^{125}I-f-pDNA)). (S): preparation by sonication. (H): preparation by homogenization. Preparation of empty particles followed the same protocol, but pDNA was excluded from the W_1 phase.

	NP-(^{125}I-f-pDNA)	MP-(^{125}I-f-pDNA)
First water phase (W_1)	pDNA (3.5 mg) in 600 µl dH_2O with 0.1% PVA	
Oil phase (O)	300 mg PLGA in 6 ml chloroform (5% w/v)	
First emulsion (W_1/O)	S: 30 sec, 35% (262.5 W)	S: 35 sec, 30% (225 W)
Second water phase (W_2)	15 ml dH_2O with 2% PVA	
Second emulsion (O/W_2)	S: 1 min, 30%	H: 45 sec, 9500 rpm min^{-1}
Washing	5000 x g, 15000 x g and 25000 x g	500 x g
Particle yield (%)	78	89
Encapsulation efficiency (%)	27	24
Loading (µg pDNA/mg PLGA)	3.24	2.85
Mean size	320 nm	3-4 µm
PLGA injected (mg)	3.5	4

Table 2 - Experimental groups and group nomenclature.

Treatment groups	**Nomenclature**
(^{125}I-f-pDNA) encapsulated in nanoparticles	NP-(^{125}I-f-pDNA)
(^{125}I-f-pDNA) encapsulated in microparticles	MP-(^{125}I-f-pDNA)
Empty nanoparticles	NP
Empty microparticles	MP
Plasmid DNA in PBS	pDNA
(^{125}I-f-pDNA) in PBS	^{125}I-f-pDNA
PBS	PBS
(^{125}I-f-pDNA) in Freund's incomplete adjuvant	FIA-(^{125}I-f-pDNA)

Table 3 - Primers for quantitative polymerase chain reaction (QPCR). (*): primers obtained from Natasha Hynes (TNF-α) and Jaya Kumari (Eomes).

Primer		Oligonucleotides, 5´ to 3´	GenBank accession number	Concentration (nM)	Amplification efficiency (%)	Amplicon size (bp)
EF1A[1]	Fw	CACCACCGGCCATCTGATCTACAA	AF321836	150	93	78
	Rv	TCAGCAGCCTCCTTCTCGAACTTC		150		
Luc[2]	Fw	TGGGCTCACTGAGACTACATCA	M15077.1	900	100	64
	Rv	CGCGCCCGGTTTATCATC		900		
TNFα*	Fw	TGTCCATCAAGCCACTACACTC	BT049358	250	84	129
	Rv	GCACTCACACACCCTGTCATT		250		
IFNα[1]	Fw	TGGGAGGAGATATCACAAAGC	NM_001123570	250	89	163
	Rv	TCCCAGGTGACAGATTTCAT		250		
IL-1β[1]	Fw	GCTGGAGAGTGCTGTGGAAGA	AY617117	200	104	73
	Rv	TGCTTCCCTCCTGCTCGTAG		200		
CD8α[3]	Fw	CGTCTACAGCTGTGCATCAATCAA	AY693391	200	83	266
	Rv	GGCTGTGGTCATTGGTGTAGTC		200		
Eomes*	Fw	ACCTCTCGTCGTCAGATAGTG	NM_001204100	200	82	204
	Rv	GGACCGGTGAGTCTTTTCTTC		200		
Mx[4]	Fw	TGCAACCACAGAGGCTTTGAA	NM_001139918	200	92	79
	Rv	GGCTTGGTCAGGATGCCTAAT		200		

Table 4 - Histopathological observations in tissue-sections of muscle from the injection site. Muscle degeneration and inflammation are classified as either moderate (+) or strong (++), depending on the extent of the pathology.

Sampling	Group	Total number of fish	Hemorrhage	Muscle degeneration		Inflammation	
				+	++	+	++
D2	NP-(^{125}I-f-pDNA)	3	2	2	0	0	0
	MP-(^{125}I-f-pDNA)	3	2	3	0	2	0
	NP	2	1	1	1	0	0
	MP	3	2	0	2	0	0
	pDNA	3	1	1	2	0	0
	^{125}I-f-pDNA	3	2	0	0	0	0
	PBS	2	1	2	0	0	0
	FIA-(^{125}I-f-pDNA)	1	-	1	-	-	-
D7	NP-(^{125}I-f-pDNA)	3	2	2	0	2	0
	MP-(^{125}I-f-pDNA)	3	0	3	0	2	0
	NP	2	0	1	1	2	0
	MP	2	1	2	0	2	0
	pDNA	3	1	2	0	1	0
	^{125}I-f-pDNA	3	2	1	0	0	0
	PBS	2	0	0	0	0	0
	FIA-(^{125}I-f-pDNA)	1	-	-	-	-	-
D30	NP-(^{125}I-f-pDNA)	2	0	0	0	2	0
	MP-(^{125}I-f-pDNA)	2	0	1	1	0	2
	pDNA	2	0	0	0	2	0
	^{125}I-f-pDNA	2	0	0	0	0	0
	PBS	1	0	0	0	0	0
	FIA-(^{125}I-f-pDNA)	1	-	-	-	-	1

Dear Editors,

Please find enclosed a revised manuscript of "**Transgene and immune gene expression following intramuscular injection of Atlantic salmon (*Salmo salar* L.) with DNA-releasing PLGA nano- and microparticles**" by Linn Benjaminsen Hølvold, Børge N. Fredriksen, Jarl Bøgwald and Roy A. Dalmo, which we are re-submitting for consideration for publication in *Fish and Shellfish Immunology*.

Our study is the first on intramuscular injection of DNA-releasing PLGA particles in Atlantic salmon. We aimed to evaluate the effect that encapsulation of pDNA into PLGA nano- and microparticles would have not only on transgene expression, but also on immune responses that might modulate the efficacy of the expressed transgene, as well as tissue distribution and injection site depots. DNA vaccination has been shown to induce protective immune responses in Atlantic salmon, and research on vaccine strategies to further improve the efficacy of DNA vaccines should therefore be of interest to the readers of *Fish and Shellfish Immunology*.

The manuscript has been revised in accordance with the received comments from reviewers.

All authors contributed to the work described in the paper, and take responsibility for it. Further, none of the described work has been published elsewhere.

We would be grateful if you would consider this revised manuscript for publication in your journal.

Sincerely,

Linn Benjaminsen Hølvold

Transgene and immune gene expression following intramuscular injection of Atlantic salmon (*Salmo salar* L.) with DNA-releasing PLGA nano- and microparticles

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Key words: PLGA/Nanoparticles/Microparticles/Transgene expression/Immune responses

Abstract

The use of poly-(D,L-lactic-*co*-glycolic) acid (PLGA) particles as carriers for DNA delivery has received considerable attention in mammalian studies. DNA vaccination of fish has been shown to elicit durable transgene expression, but no reports exist on intramuscular administration of PLGA-encapsulated plasmid DNA (pDNA). We injected Atlantic salmon (*Salmo salar* L.) intramuscularly with a plasmid vector containing a luciferase (*Photinus pyralis*) reporter gene as a) naked pDNA, b) encapsulated into PLGA nano- (~320nm) (NP) or microparticles (~4μm) (MP), c) in an oil-based formulation, or with empty particles of both sizes. The ability of the different pDNA-treatments to induce transgene expression was analyzed through a 70-day experimental period. Anatomical distribution patterns and depot effects were determined by tracking isotope labeled pDNA. Muscle, head kidney and spleen from all treatment groups were analyzed for proinflammatory cytokines (TNFα, IL-1β), antiviral genes (IFN-α, Mx) and cytotoxic T-cell markers (CD8, Eomes) at mRNA transcription levels at days 1, 2, 4 and 7. Histopathological examinations were performed on injection-site samples from days 2, 7 and 30. Injection of either naked pDNA or the oil-formulation was superior to particle treatments for inducing transgene expression at early time-points. Empty particles of both sizes were able to induce proinflammatory immune responses as well as degenerative and inflammatory pathology at the injection site. Microparticles demonstrated injection-site depots and an inflammatory pathology comparable to the oil-based formulation. In comparison, the distribution of NP-encapsulated pDNA resembled that of naked pDNA, although encapsulation into NPs significantly elevated the expression of antiviral genes in all tissues. Together the results indicate that while naked pDNA is most efficient for

inducing transgene expression, the encapsulation of pDNA into NPs up-regulates antiviral responses that could be of benefit to DNA vaccination.

Introduction

PLGA (poly-(D,L-lactic-*co*-glycolic)-acid) nano- and microparticles as adjuvants and carriers for vaccine antigens have been extensively investigated, mainly in mammalian models [1-5]. The biodegradable copolymer produces non-toxic degradation products [3], offers increased predictability of antigen release-rates, potential for intracellular antigen delivery and the ability to encapsulate and co-encapsulate a wide variety of antigens, including DNA vaccines [1, 6, 7].

Luciferase has commonly been applied for the evaluation of DNA vaccines at both transcription and protein level, and appears to express higher in fish than in mammals for a given dose of DNA [8-12]. The encapsulation of plasmid DNA into PLGA particles could protect against the rapid on-site degradation reported in both mice and salmon after intramuscular injection, and has been shown to increase the escape of antigen from endosomes to the cytosol [6, 8, 10, 11, 13].

Depending on size, the particles may create injection-site depots (>5µm), get phagocytized by antigen presenting cells (APCs) (<5µm), be internalized by non-phagocytic cells such as myocytes (<500nm) or escape into the bloodstream and subsequently be cleared by phagocytes in the head kidney, spleen and/or liver [4, 14]. Muscle cells have been shown to slowly accumulate pDNA over time, and could benefit from extracellular pDNA-releasing microparticles as well as intracellular nanoparticle depots [15].

A central attribute of DNA vaccines is the ability to induce cellular as well as humoral immune responses, including cytotoxic T-lymphocyte (CTL) responses and antibody production [16-18]. Unlike vertebrate DNA, bacterial DNA contains stretches of

93 unmethylated CpG sequences that are recognized as danger signals by toll-like receptor 9
94 (TLR9). Upon stimulation this endosomal pattern recognition receptor (PRR) may induce
95 a variety of cytokines. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are
96 hallmark cytokines in driving the inflammatory response in mammals and amongst other
97 properties hold key roles in the migration of effector cells to sites of infection [19]. Both
98 cytokines have been found in a number of teleost species and appear to exert functions
99 similar to what is known from mammals [20-22].

100 Interferon-α (IFN-α) is one of the type I IFNs, key mediators of antiviral
101 responses through the regulation of IFN-stimulated genes and central in linking innate
102 and adaptive immunity [16, 23-25]. The IFN-induced protein Mx has demonstrated
103 antiviral functions in Atlantic salmon (*Salmo salar* L.) and can be used to follow IFN
104 activity, as it has a much longer lifetime and accumulates to higher concentrations [26-
105 28]. Type I IFNs also play an important role in the clonal expansion and generation of
106 specific as well as non-specific memory $CD8^+$ T-cells [29]. Sequences for CD8α and
107 CD8β are known from a variety of teleost species and the cytotoxic activity of $CD8^+$ T-
108 cells has been demonstrated in rainbow trout (*Oncorhynchus mykiss*) [30, 31]. The
109 transcription factor Eomesodermin (Eomes) is critical to the development of $CD8^+$ T-cell
110 effector functions and memory cells in mammals [32, 33]. A recent study indicated
111 similar functions as well as induction by IFN-α in Atlantic salmon [34].

112 Intramuscular injection of naked pDNA generally induces few and transient
113 histopathological changes in fish as well as in mammals [9]. In contrast, the use of PLG
114 microspheres as DNA carriers has been shown to result in a foreign body response in
115 mice, where the infiltrating cells were also the ones that were primarily transfected [35].

Enhanced inflammatory reactions coupled with prolonged availability of pDNA might therefore be beneficial to transgene expression and T-cell responses.

This report is the first on intramuscular injection of Atlantic salmon with PLGA nano- and microparticles carrying pDNA, and the effect of these particles on 1) tissue distribution and retention of pDNA 2) expression of a firefly (*Photinus pyralis*) luciferase reporter gene 3) innate inflammatory (TNF-α, IL-1β) and antiviral (IFN-α, Mx) immune responses, 4) expression of cytotoxic T-cell markers (CD8, Eomes) and 5) injection site histopathology. We hypothesize that PLGA-encapsulated pDNA will induce transgene expression and proinflammatory as well as antiviral responses more efficiently than non-encapsulated pDNA.

2. Experimental

2.1 Materials/Chemicals

Poly(D,L-lactic-co-glycolic) acid (PLGA; 50:50 ratio, M_w of 7-17 kDa), poly vinyl alcohol (PVA, 87-89 % hydrolyzed), D-(+)-trehalose dehydrate, 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (Iodogen; Pierce, Rockford, IL, USA), Freund's incomplete adjuvant (FIA) and quantitative polymerase chain-reaction (QPCR) primers were purchased from Sigma Aldrich. Carrier free Na[^{125}I] was from Perkin-Elmer Norge (Oslo, Norway). Acetone, dichloromethane (DCM) and chloroform were purchased from Merck Biochemicals. Sodium metabisulphite ($Na_2S_2O_5$ > 98 % purity) and potassium iodide (KI > 99.5 % purity) were purchased from Fluka Biochemica. Luciferase lyophilizate was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

2.2 Plasmid DNA

Plasmid R70pRomiLuc (gift from Uwe Fischer, Friedrich-Loeffler-Institut, Germany) contains a firefly luciferase gene under the control of a murine cytomegalovirus immediate early promoter (CMV-IEP). The plasmid was isolated from a culture of *Escherichia coli* (strain DH5α) by use of Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer instructions. Purified plasmid was eluted in Tris-EDTA (TE) buffer (pH=8.0). DNA concentration and quality was determined with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 1% agarose gel-electrophoresis. High quality samples (A_{260}/A_{280} ratio > 1.9 and distinct DNA bands on gel) were stored at -20 ºC until use.

2.3 Preparation of [^{125}I]-fluorescein-pDNA

Purified pDNA was modified using the nucleic acid labeling kit *Label*IT Fluorescein (MIR 3200, Mirus Corp., Madison, WI, USA) according to manufacturer instructions. Radiolabeling of f-pDNA with carrier-free Na[^{125}I] was performed in a direct reaction with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril as the oxidizing agent. The protocol was in accordance with the Iodogen method of radiolabeling [36], with minor modifications concerning incubation time (1h). Free iodine was removed by filtration on a PD-10 column equilibrated with pDNA in PBS. Radiation was determined by gamma counting (COBRA™ II Auto-Gamma®, ©Packard Instrument Co., Meridan, IL, USA), with specific activity measured to ~ 4.75 million cpm μg^{-1} ^{125}I-f-pDNA.

2.4 Preparation of naked and pDNA-loaded nano- and microparticles

Particles were prepared by a modified version of the double emulsion ($W_1/O/W_2$) solvent evaporation method [2, 5, 37], outlined in table 1. A fraction of ^{125}I-f-pDNA was included in the first water phase (W_1) for determination of encapsulation efficiency and tracing of tissue distribution in *in vivo* experiments. Emulsions were prepared on ice-baths by sonication (S) (Sonics VibraCell VC750, 3 mm tapered micro tip, Sonics & Materials Inc., Newtown, CT, USA) or homogenization (H) (Ultra-turrax® T-25 Basic, IKA®-WERKE, Staufen, Germany). Plasmid was excluded from the W_1 phase for preparation of naked particles. Fifteen ml dH_2O was added to the $W_1/O/W_2$-emulsions before overnight stirring to facilitate solvent evaporation. After centrifugation (Avanti® J-26 XP, BeckmanCoulter®, USA), pellets were resuspended in dH_2O and the fractions were pooled. Trehalose (5 mg ml^{-1} in dH_2O) was used as a lyoprotectant and added to suspension aliquots in a ratio of 1:3. Aliquots were kept at -80˚C for a minimum of 2 h before lyophilization for 72 h at 0.001 hPa, -110 °C (ScanVac CoolSafe™, LaboGene, Denmark). Lyophilizates were stored in airtight containers at 4 °C.

2.5 Particle characterization

Encapsulation of pDNA was determined by the gamma emission in a known amount of particles (COBRA™ II Auto-Gamma®) and measured radioactivity related to the specific radioactivity in the fraction of ^{125}I-f-pDNA stock solution added to the W_1-phase. Encapsulation efficiency was determined as entrapped amount of pDNA relative to the amount initially present in the W_1-phase. Loading (µg pDNA mg^{-1} PLGA) was calculated by relating encapsulated pDNA to the total weight of retrieved particles. Sizing of

nanoparticles was performed by photon correlation spectroscopy (PCS) (NiComp 380 Submicron Particle Sizer, Santa Barbara, USA). Microparticle size was determined by use of a Model 780 AccuSizer (NiComp).

2.6 In vitro particle stability and pDNA release

Five containers of 10 mg lyophilizate dissolved in 1 ml PBS (pH 7.4) with 0.02% sodium azide (NaN_3) were prepared for both nano- and microparticles. The suspensions were incubated at 8˚C on a Stuart® SB3 rotator. Sampling was performed immediately after particles had been dissolved, and then at 3 h, day 1, 2, 4, 7, 14, 21, 30, 40, 50, 60 and 70. At each sampling 100 µl were removed and centrifuged at 15,000 x g for 1 minute. Fifty µl of the supernatant were removed for isotope measurement, and the remaining fraction transferred back to the original container after addition of 50 µl new PBS and full re-suspension of the particle pellets.

2.7 Fish

Unvaccinated pre-smolt Atlantic salmon with a mean weight of 30 g were provided by and kept at Tromsø Aquaculture Research Station. The fish were kept in 200 L plastic tanks supplied with running fresh water (8-10˚C) and fed a commercial feed from Skretting AS (Stavanger, Norway). Adaptation to test conditions was performed for one week prior to immunization. All experiments were in compliance with The Norwegian Welfare of Animals Act.

208 *2.8 Experimental groups and vaccination*

209 A total of 336 fish were distributed among seven tanks. Each tank contained 6 fish from
210 each of the eight experimental groups (Table 2). Prior to tattooing and immunization the
211 fish were sedated with benzocaine using 40 mg L^{-1} (Benzoak Vet., ACD
212 Pharmaceuticals, Leknes, Norway). The PanJet needle free injection system with
213 saturated Alcian blue was used for tattooing. All formulations were administered in 50 µl
214 volumes by injection in the left epaxial muscle. Each injection dose contained 11 µg
215 pDNA, and the amount of NPs and MPs was adjusted to make up for differences in
216 pDNA loading. Injection dose samples were collected from each formulation in order to
217 determine cpm.

218

219 *2.9 Sampling*

220 The fish were starved for 24 h prior to sampling and killed by a double dose (80 mg L^{-1})
221 of benzocaine. At day 1, 2, 4, 7, 14, 30 and 70 post injection one tank was sampled. All
222 fish were weighed. Blood from fish given radioactive formulations was sampled using
223 vacutainers (Becton Dickinson) and stored on ice. Two muscle samples from the
224 injection site were transferred to 10% neutral buffered formalin for storage until
225 histological processing. Muscle samples, spleens and head kidneys were transferred to
226 separate tubes containing 1 ml RNAlater® (Ambion, Austin, TX, USA) and kept at room
227 temperature overnight before storage at -20˚C. To determine tissue distribution in fish
228 given radioactive formulations it was necessary to sample the full carcass. Trunk kidney,
229 organ package (liver, heart, gastrointestinal tract (GIT) and interstitial adipose tissue),
230 head (incl. gills) and remaining muscle (with skin) were collected in separate tubes. One

fish injected only with PBS was similarly sampled at each time point in order to exclude the possibility of radioactive contamination by cohabitation.

2.10 Anatomic distribution and plasmid DNA retention

Determination of *in vivo* retention and biodistribution was achieved by tracking trace amounts of ^{125}I-f-pDNA. From blood samples, 50 µl were removed for analysis. Radioactivity in blood and trunk kidney samples was determined with a COBRA™ II gamma counter. Remaining samples, including spleen and injection site tissue, were analyzed using a Packard Auto-Gamma Scintillation Spectrometer (©Packard Instrument Co., model 5120). Background radiation was measured to be 50 and 100 cpm respectively for the two machines. These were set as baselines, and were subtracted from measured values before data were adjusted according to [^{125}I] half-life. Blood volume was set to 4% of the body weight [38]. Mean values of the injection dose samples gathered during vaccination were used to determine total cpm recovery (%) at day 1. To determine the stability of retention, the daily cpm means were related to recovery at day 1. Daily cpm means were used to determine anatomical distribution.

2.11 RNA isolation and cDNA synthesis

Tissue samples (20-30 mg) were removed from RNAlater® and homogenized in lysis buffer from the RNeasy mini kit (Qiagen) using tubes with ceramic beads (Precellys) and a Precellys® 24 Homogenizer (Bertin Technologies). Parameters were set for 5300 x g x 3 spins, 10 s. Subsequent RNA extractions and on-column purification with the RNase-Free DNase Set from Qiagen were performed according to manufacturer instructions.

RNA was eluted in 50 µl RNase-free water and stored at -80˚C until use. RNA concentration and quality was determined using NanoDrop® (NanoDrop Technologies) and 1% agarose gel electrophoresis. High quality samples (260/280 ratio > 1.9 and tight 18S/28S bands) were used for cDNA synthesis. Reverse transcription was performed with the High Capacity RNA-to-cDNA Master-mix from Applied Biosystems in accordance with manufacturer instructions. Each reaction contained 400 ng RNA in 20 µl. Samples were diluted to 80 µl with RNase-free water and stored at -80˚C.

2.12 Quantitative reverse transcription polymerase chain reaction (QPCR)

Quantitative PCR was performed according to manufacturer instructions using Fast SYBR® Green Master Mix (Applied Biosystems) and primers previously applied in our laboratory (Table 3). Primer efficiency was determined using a six-point 1:2 dilution series in triplicate, with an initial cDNA concentration of 20 ng. All primers were free of primer-dimers in no-template control and showed a single peak in the dissociation curve. Samples containing 20 ng cDNA were run in triplicate, with each plate including no-template control and an inter-run calibrator. Analysis was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) with the thermal cycler profile set to 95˚C for 20 s, 40 cycles of 95˚C for 3 s and 40 cycles of 60˚C for 30 s. Dissociation curves were performed on all samples. The 7500 Software Relative Quantification Study Application (v2.0.4, Applied Biosystems) was used to determine C_T values, with walking baseline and threshold set manually to be equal for all genes. Relative gene expression was determined by the Pfaffl method [39], comparing saline injected fish to treated groups. To determine relative expression of luciferase the highest day 1 transcript average was

used as control. Head kidney and spleen samples from day 2 and 7 were also checked for luciferase transcripts.

2.13 Histology

Muscle samples from days 2, 7 and 30 post injection were chosen for histology. The service of routine histological processing and staining with hematoxylin and eosin (HE) was purchased from the National Veterinary Institute (Oslo, Norway). Sections were studied by light microscopy for signs of hemorrhages, tissue degradation and inflammation. Using the changes in day 2 saline injected fish as a baseline, observed changes were classified as either moderate (+) or strong (++). Moderate changes were determined as largely limited to the needle trajectory, with inflammatory cells primarily associated with degenerated tissue and/or dispersed among intact muscle cells. Strong changes were seen as degeneration and/or inflammation spreading beyond the needle trajectory, with few or no intact muscle cells present within the cellular infiltrate. The histopathology scores were performed as blind testing by two people.

2.14 Statistical analysis

Microsoft Excel (Microsoft™) was used for arrangement of data as well as calculation of biodistribution and relative gene expression. Statistical analyses for real-time PCR were performed with IBM SPSS (Statistical Package for the Social Sciences, version 19.0). All data were natural log transformed prior to analysis, and normality verified by the Shapiro-Wilk test. Levene's test of equality of variances was used to check for homoscedasticity. Homoscedastic data were analyzed by one-way ANOVA with Tukey

as the post hoc test. Where homoscedasticity was violated, the Welch test of equality of means was applied, followed by Dunnett T3 post hoc. Graphs were constructed with Microsoft Excel and SPSS.

3. Results

3.1 Particle characterization

The encapsulation efficiency and loading of ^{125}I-f-pDNA were low for both particles sizes. The different particle characteristics are summarized in table 1, where empty particles have been excluded due to minor size differences. The mean size of nanoparticles was 320 nm by intensity distribution, with more than 90% measuring less than 500 nm. The number weight mean diameter of microparticles was 3-4 µm, with 80% measuring less than 5 µm and more than 90% smaller than 10 µm.

3.2 In vitro particle stability and pDNA release

Neutral pH and a temperature of 8˚C were chosen in order to simulate *in vivo* conditions. Initial burst release was 81% for NP-(^{125}I-f-pDNA) and 49% for MP-(^{125}I-f-pDNA). Both groups subsequently displayed a slow and sustained release, with a 96% and 69 % accumulated release at day 70 (Fig. 1).

3.3 Anatomical distribution and depot of vaccine formulations

No radioactivity was registered in blood later than day 7 post injection. The average total cpm recovery at day 1 ranged from 15% for NP-(^{125}I-f-pDNA) to 39% for ^{125}I-f-pDNA

(results not shown). Radioactivity was primarily recovered from the injection site, organs and trunk kidney. High degrees of similarities in tissue distribution were observed for NP-(^{125}I-f-pDNA) and ^{125}I-f-pDNA (Fig. 2A, B). Likewise, MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) had similar distribution profiles and injection site depots (Fig. 2C, D). The trunk kidney was the primary site of recovery for NP-(^{125}I-f-pDNA) and ^{125}I-f-pDNA, containing almost 80% of total radiation at day 70. NP-(^{125}I-f-pDNA) showed higher injection site retention (5%) at day 70 compared to ^{125}I-f-pDNA (1%). The respective values for MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) were 34% and 26%, with retention in trunk kidney reaching 32% and 27% at day 70.

3.4 Quantitative reverse transcription polymerase chain reaction (QPCR)

3.4.1 Expression of luciferase reporter gene

FIA-(^{125}I-f-pDNA) induced the highest luciferase transcript average in muscle at day 1 and was chosen as calibrator for this tissue. FIA-(^{125}I-f-pDNA) also induced the highest individual levels of luciferase transcripts throughout the experiment, with significant expression at day 1, 4 and 14 ($p \leq 0.032$) (Fig. 3). Naked plasmids consistently induced higher expression than the particle formulations, with significance for pDNA at day 1-7 ($p \leq 0.042$) and ^{125}I-f-pDNA at day 4 ($p = 0.018$). No significant differences were found at days 30 and 70. Low luciferase transcript levels were detected in head kidney, with pDNA and ^{125}I-f-pDNA inducing significant expression compared to NP-^{125}I-f-pDNA at day 2 ($p \leq 0.016$, results not shown). No expression was detected in the spleen.

346 *3.4.2 Proinflammatory cytokines IL-1β and TNF-α*

347 The particle formulations significantly up-regulated IL-1β in muscle samples at all time-
348 points; NP-(^{125}I-f-pDNA) ($p \leq 0.002$), NP ($p \leq 0.004$), MP-(^{125}I-f-pDNA) ($p \leq 0.003$),
349 MP ($p \leq 0.015$) (Fig. 4A). From day 2 the up-regulation was significant compared to all
350 non-particle groups. MP formulations consistently induced the highest expression. FIA-
351 (^{125}I-f-pDNA) significantly up-regulated IL-1β in muscle at day 7 ($p = 0.000$). Significant
352 expression of TNF-α in head kidney was only found at day 4, with NP-(^{125}I-f-pDNA) (p
353 $= 0.034$) and MP ($p = 0.008$) (Fig. 4B). No significant expression of TNF-α was found in
354 the spleen or muscle.

355

356 *3.4.3 IFN-α and Mx*

357 NP-(^{125}I-f-pDNA) significantly up-regulated IFN-α at the injection site at day 4 ($p =$
358 0.005). Levels in head kidney were generally low, with significant up-regulation by ^{125}I-
359 f-pDNA ($p = 0.000$) at day 2. NP-(^{125}I-f-pDNA) ($p \leq 0.003$) and NP ($p \leq 0.036$)
360 significantly up-regulated IFN-α in spleen at day 1 and 2 (results not shown). Levels of
361 Mx at the injection site increased for all groups from day 1 to 7. NP-(^{125}I-f-pDNA)
362 induced significant expression at day 2-7 ($p = 0.000$), and was superior to all groups at
363 day 4 and 7 ($p \leq 0.020$). MP-(^{125}I-f-pDNA) significantly up-regulated Mx in muscle at
364 day 2 and 4 ($p \leq 0.006$) (Fig. 5A). NP-(^{125}I-f-pDNA) appeared the most potent treatment
365 for inducing Mx in head kidney, but no significance was found (Fig. 5B). Mx expression
366 increased in spleen for all groups from day 1 to 7, with significance for NP-(^{125}I-f-pDNA)
367 at day 4 ($p = 0.013$) (Fig. 5C).

368

3.4.4 CD8 and Eomes

There was no significant up-regulation of CD8 in any tissue. Several muscle samples showed no CD8 expression, and no statistical analyses could therefore be performed. No expression of CD8 could be detected at day 2 in head kidney samples from fish injected with ^{125}I-f-pDNA. Interestingly, these samples induced the only significant up-regulation of Eomes observed within any tissue ($p = 0.000$) (results not shown).

3.6 Histopathology

Tissue sections of injection site samples were examined by light microscopy for signs of hemorrhages, muscle degeneration and inflammation (Table 4). PBS caused only minor hemorrhages and tissue degeneration at day 2 (Fig. 6A), indicating that later changes were likely the results of the different treatments. Hemorrhages were most pronounced at day 2 in all groups. Administration of PBS and ^{125}I-f-pDNA caused moderate muscle degeneration, but no inflammation. Both tissue degeneration and inflammation were observed for pDNA (Fig. 6C), but both pathologies were more frequent with particles formulations. MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) both demonstrated strong inflammation at day 30 (Fig. 6B, D), with a high influx of inflammatory cells that for FIA-(^{125}I-f-pDNA) was concentrated around possible oil-droplets. The histopathological changes caused by particles seemed to depend on particle size (nano vs. micro) rather than content (empty vs. pDNA).

4. Discussion

The use of PLGA particle constructs as carriers for DNA vaccine delivery has so far received little attention in fish studies [1, 40]. We have investigated distribution/depot, transgene and immune gene transcription as well as injection site histopathology following intramuscular injection of Atlantic salmon with PLGA particles carrying pDNA encoding luciferase. The double emulsion solvent evaporation method [2, 37] was used to prepare nano- and microparticles with mean diameters of 320 nm and 4 µm, respectively. Low pDNA encapsulation appears to be a recurring problem, and our results (<30% for both particle sizes) are supported by other studies [12, 35].

One of the challenges of conventional DNA vaccination is the rapid tissue clearance and on-site degradation of DNA, which may result in reduced pDNA uptake and transgene expression [8, 10]. Although oil adjuvants are not commonly applied for intramuscular vaccine delivery, FIA-(^{125}I-f-pDNA) provided a positive control to measure the depot potential of nano- and microparticles. The high burst release observed for NP-(^{125}I-f-pDNA) during the *in vitro* release study (Fig. 1) likely explains the strong similarities between this group and ^{125}I-f-pDNA (Fig. 2A, B), a similarity reflected also in the injection site histopathology (Table 4). In comparison, the injection site retention of MP-(^{125}I-f-pDNA) remained similar to that of FIA-(^{125}I-f-pDNA) throughout the study (Fig. 2C, D). The duration of the MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) depots likely contributed to the severe histopathological inflammations observed in tissue sections from both groups at day 30 (Fig. 6B, D). The transfection of infiltrating cells has been shown to take place in both fish and mammals [35, 37, 41], and transient inflammatory responses may thus contribute to increased transgene expression. Long lasting

415 inflammations such as seen with MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) are, however,
416 undesirable in a product meant for consumption. It would appear that nanoparticles may
417 be favorable in order to avoid potential tissue damage, but the small sample sizes meant
418 we could only obtain an indication of the histopathological influence of the various
419 treatments.

420 Although the similarities in injection site retention were not reflected in the
421 expression of the luciferase transgene (Fig. 3), the observation that FIA-(^{125}I-f-pDNA)
422 induced the highest individual levels of luciferase transcripts suggests that increased
423 cellular infiltration along with a high injection site pDNA depot may be of benefit to
424 transgene expression in Atlantic salmon. The significantly lower expression obtained
425 with encapsulated pDNA could be a result of the preparation conditions, as the $W_1/O/W_2$
426 method has been shown to cause a partial reduction in the content of supercoiled (SC)
427 DNA topoform that contributes to a lower transfection efficiency [1, 8, 37, 42]. The
428 process of emulsification required lower shear forces and shorter duration of preparation
429 and may thus have preserved a larger portion of SC DNA in FIA-(^{125}I-f-pDNA). We did
430 not, however, address whether the encapsulation of ^{125}I-f-pDNA resulted in an altered SC
431 content. The absence of significant differences in luciferase expression at day 30 and 70
432 still indicated a certain level of stability in particle groups that could result from a
433 continued release of bioactive pDNA even at later time-points.

434 Another explanation for the low transcription levels obtained with encapsulated
435 pDNA-Luc may lie with the inflammatory cytokine responses. Induced not only by
436 stimulation of PRRs but also by PLGA itself [43, 44], IL-1β and TNF-α have both
437 demonstrated inhibitory effects on transgene expression even at low concentrations,

contributing to a reduced transfection efficiency of encapsulated DNA *in vivo* as opposed to *in vitro* [2, 37, 45]. The nano- and microparticle formulations significantly up-regulated IL-1β at the injection site at all time-points (Fig. 4A), and while the expression of TNF-α was generally low a synergistic effect of different cytokines on the inhibition of transgene expression has been reported [45]. In comparison, the levels of IL-1β in fish injected with FIA-(^{125}I-f-pDNA) were not found significant until 7 days post administration, indicating that PLGA may provide a more potent inflammatory stimulus than the oil adjuvant even in the absence of pronounced inflammatory histopathology.

Viral challenge studies in fish have shown that specific protection may be acquired even with very low doses of DNA, and innate antiviral responses also appear to play a more critical role in fish than mammals upon exposure to viruses [46, 47]. Whereas luciferase is unlikely to induce a differentiation of antigen specific $CD8^+$ T-cells due to low immunogenicity in *in vivo* studies [48, 49], type I IFNs play an important role in the clonal expansion of $CD8^+$ T-cells and may enhance CTL responses when immunogenic transgene products are expressed [29]. Whereas the expression of IFN-α was generally low and transient, NP-(^{125}I-f-pDNA) still appeared the most potent inducer of antiviral responses and significantly up-regulated the expression of Mx in all tissues (Fig. 5). This result supports a previous study on the influence of particle size on the cytokine profile induced after administration of particle associated CpG DNA [50]. We did observe that formulations with naked pDNA significantly up-regulated both Eomes and IFN-α in head kidney at day 2, but no statistical correlation between these genes was found for any other group, tissue or time-point.

This study is the first on intramuscular injection of Atlantic salmon with PLGA particles carrying pDNA. Our particle formulations did not induce transgene expression as efficiently as injection of naked plasmids, but appeared to provide a continued release of bioactive pDNA even at the end of the study. A strong expression of transgene may not always be necessary in order to mount a significantly protective immune response, and an efficient up-regulation of innate antiviral responses in particular might enhance the immunogenicity of an antiviral vaccine. Both particle sizes proved superior to naked pDNA injection for the induction of IL-1β as well as an influx of inflammatory cells at the injection site, but only encapsulation into nanoparticles significantly increased the expression of IFN-α and Mx. Together these results indicate that PLGA nanoparticles as carriers for plasmid vectors encoding viral antigens might enhance the responses to DNA vaccines.

Acknowledgements

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Ref. No.: FSIM-D-12-00522

Response to review of manuscript with title "Transgene and immune gene expression following intramuscular injection of Atlantic salmon (Salmo salar L.) with DNA-releasing PLGA nano- and microparticles".

General comments on the revised manuscript:

For this re-submission revisions have been confined to the introduction and discussion sections of the manuscript, in addition to some figure revisions in response to reviewer's queries. As the study was not designed to evaluate DNA vaccine efficacy but rather the potential influence of various factors on the expression of a reporter gene, we found it right not to make too much speculation as to how our results might transfer to an actual DNA vaccine trial. An effort has instead been made to clarify the various results and observations in terms of their potential impact – positive or negative – on the expression of the reporter gene.

New additions as well as altered sections of the text have been highlighted in red for easier recognition, and line-references have also been provided in the answers to reviewer queries following below.

Query 1:

In the abstract there are several "pDNA". The authors should clarify what "p" stands for in the first one.

Clarification has been made in line (L) 28.

Query 2:

The authors have used a luciferase gene as a reporter gene in the present study. If the authors have been tried a luciferase assay in this project, the authors should show the results of the assay. If not, the author should describe the reason why a luciferase gene has been chosen as a reporter gene.

No luciferase assay was included in the current project. Luciferase (Luc) was still chosen as it is one of the most common reporter genes and has frequently been applied for the evaluation of transgene expression in fish at both protein and transcription level (L77-79). The application of a Luc-gene hence makes it easier to evaluate and relate the work performed for this manuscript to previously published studies.

Query 3:

In the results of histopathology, it is difficult to understand the histological differences between treated samples and controls for those who are not familiar with the inflamed tissue.

a) Please describe the detailed features of muscle degeneration and inflammation in the manuscript or figure legend.

More detailed descriptions of the various histopathological features including muscle cell degeneration and inflammation have been provided in the figure legend.

b) Please add the enlarged figures for the inflammatory cells and add the description of the detailed features for the inflammatory cells in fig 6D.

Detailed features, such as cell types, cannot be provided for the observed inflammatory cells as no immunohistochemical assays were conducted to verify their specific nature. A higher magnification micrograph has been provided for one of the original, lower magnification sections to show the high variation in infiltrating cells.

c) Please add scale bars for the panels in histological figures.

Scale bars have been added.

Query 4:

Although several homolog genes were used as markers for pro-inflammatory, antiviral response and cytotoxic T-cell markers in the present study, the authors should show some proofs that those genes had similar roles in Atlantic salmon by the referring of previous other work, or other way.

The introduction has been altered to include specific references to studies on function and/or presence of the various cytokines and markers in fish species. For TNF-α and IL-1β the additions can be found in L95-99. Studies on the activities of IFN-α and Mx in teleosts were referenced in the previous draft. Descriptions are now given in L100-106. Brief references to the existence and activity of CD8 in fish have also been made (L106-108), while reference to a very recent study on the expression and function of Eomesodermin in Atlantic salmon has been included in L108-111-

Query 5:

The authors should add the evidence-based descriptions that the early response (1-7 days post vaccination) of immune-related genes showed in the fig. 4 and 5 would be of benefit to DNA vaccination in the discussion part.

Whereas some mention had been made to the benefit of these early responses, we agree that the original manuscript did not clarify the findings and evaluations of these in a satisfactory manner. The discussion has therefore to some extent been rewritten to clarify potential benefits *as well as* negative effects of the various early immune responses.

The role of proinflammatory cytokines in the migration of inflammatory cells to the site of injection has been specified in the description of biological function (L95-97), whereas the potential influence of these cytokines on DNA vaccines and transgene expression is described in L434-442. Likewise, the antiviral functions of IFN-α and Mx and the relation to CD8 and Eomes expression can be found in the introduction, as described for query 4. In the discussion, L448-449 and L451-453 reference the importance of innate antiviral responses in teleosts and presents the potential benefit to later, acquired

responses. In L455-457 a reference is given to the potential influence of PLGA particle size on the resulting antiviral response.

Figure(s)

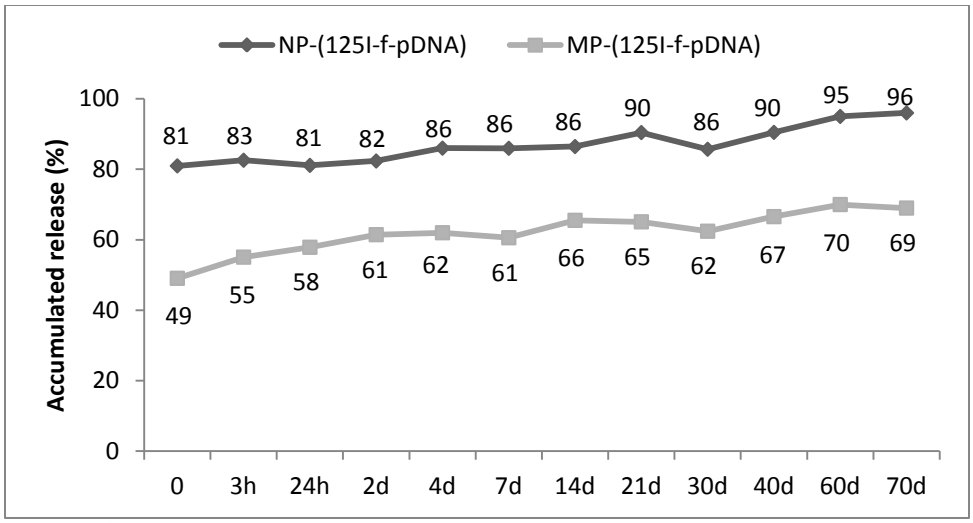

Figure 1 - Release of ^{125}I-f-pDNA from nano- and microparticles through 70 days of incubation at 8° C, presented as the mean accumulated release (%) of five samples at each time-point.

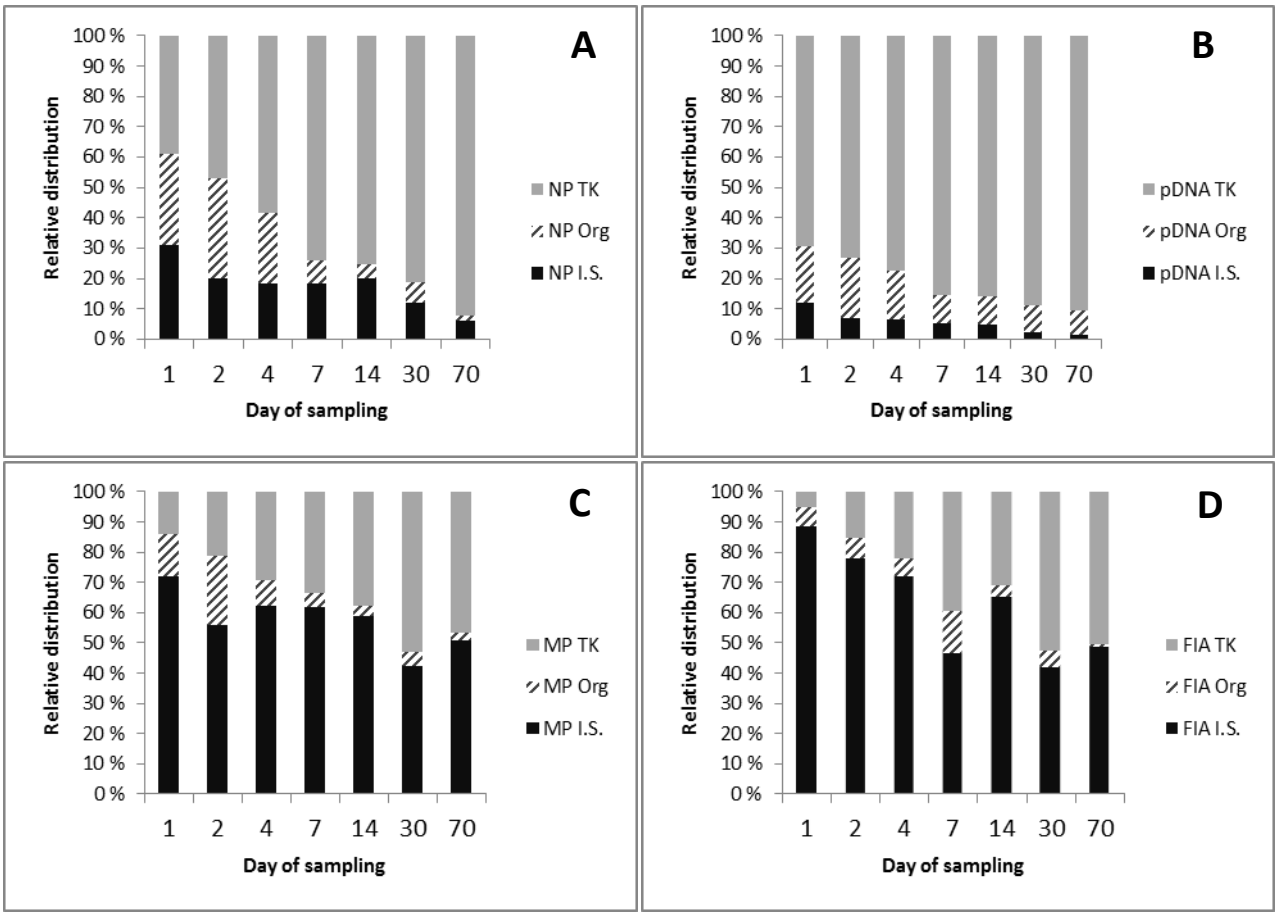

Figure 2 – Radioactivity recovered from samples of trunk kidney (TK), organs (Org) and injection site (I.S.). The stacked bars represent the relative distribution of pDNA among these organs in groups A) NP-(^{125}I-f-pDNA), B) ^{125}I-f-pDNA, C) MP-(^{125}I-f-pDNA) and D) FIA-(^{125}I-f-pDNA). Five fish were sampled at each time-point.

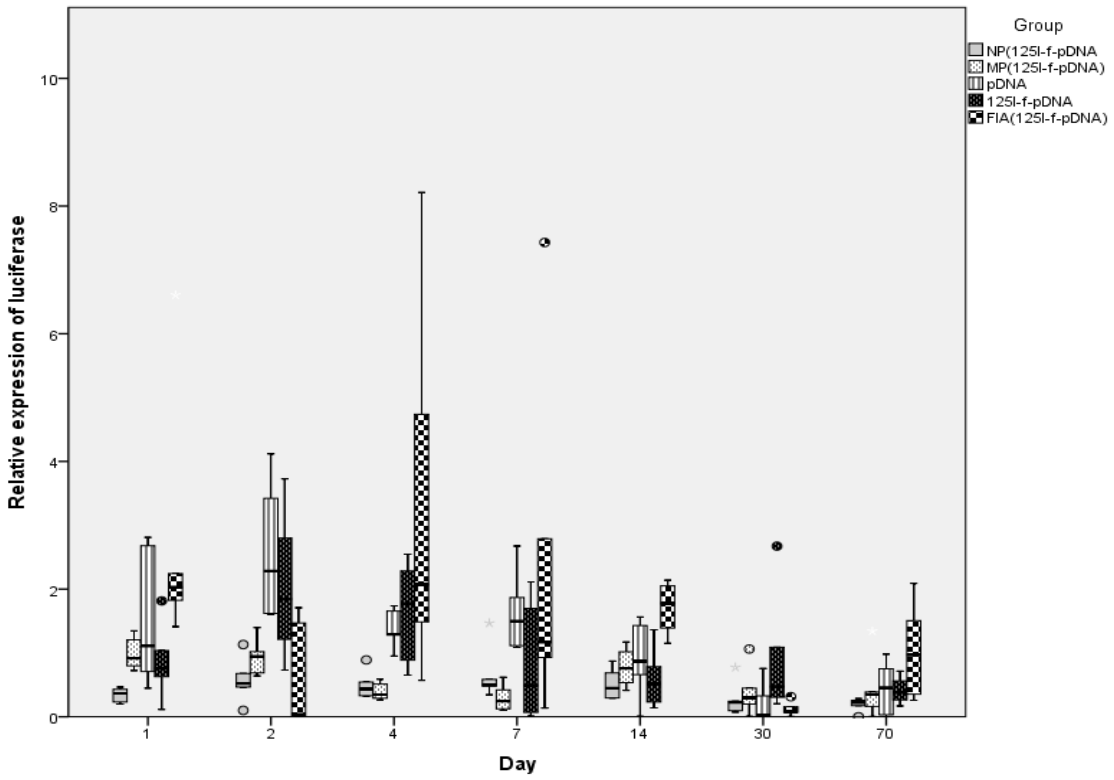

Figure 3 - Relative expression of luciferase mRNA transcripts in muscle from the injection site through the 70 day sample period. The box-plot shows the median value (line) and maximum and minimum values (whiskers), with the box representing 50% of the samples. Circles show outliers. The highest cpm-average at day 1 was found in group FIA-(^{125}I-f-pDNA) and was set as calibrator. Six fish were sampled and analyzed at each time-point.

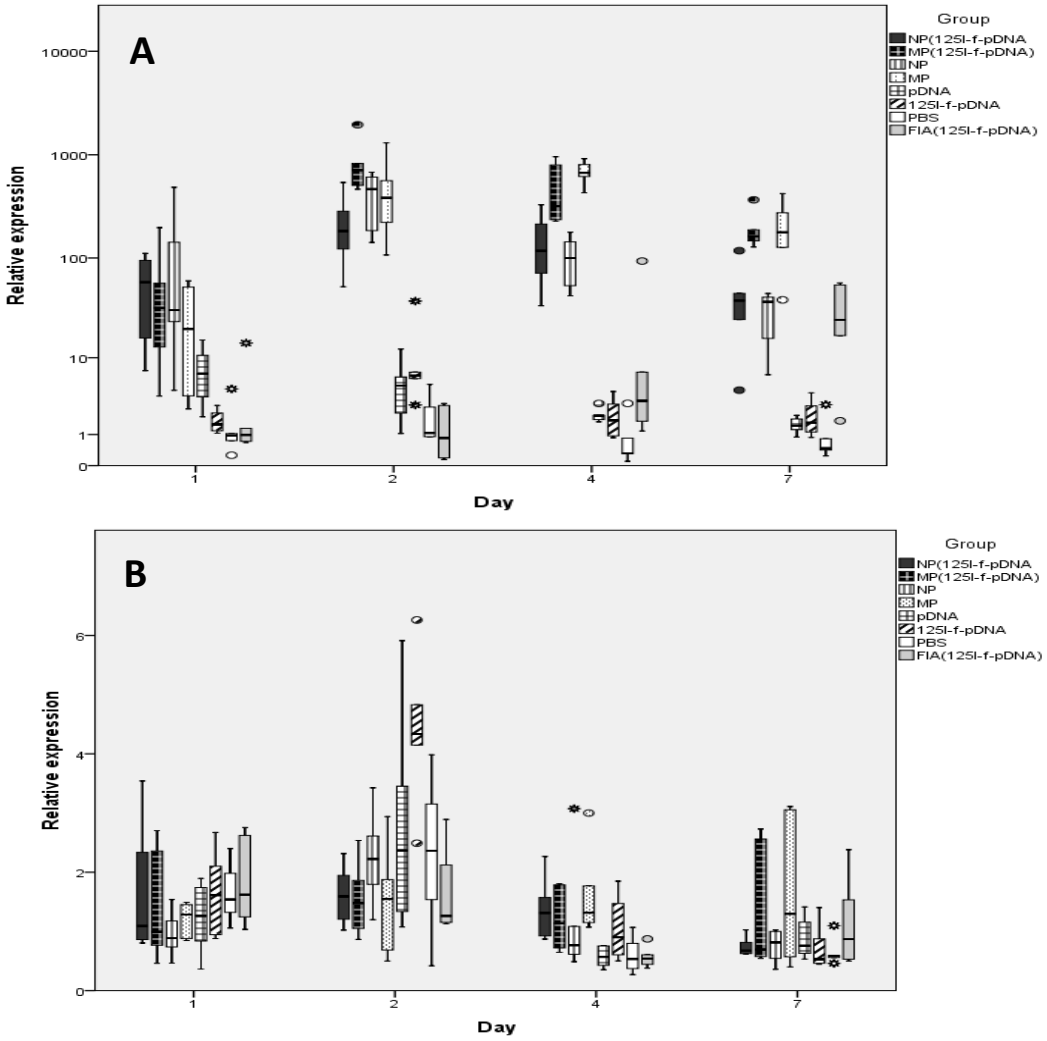

Figure 4 – Relative expression of proinflammatory cytokines; A) IL-1β in muscle from the injection site and B) TNF-α in head kidney. The box-plot shows the median value (line) and maximum and minimum values (whiskers), with the box representing 50% of the samples. Circles show outliers, and asterisks (*) extreme outliers. Six fish were sampled and analyzed at each time-point.

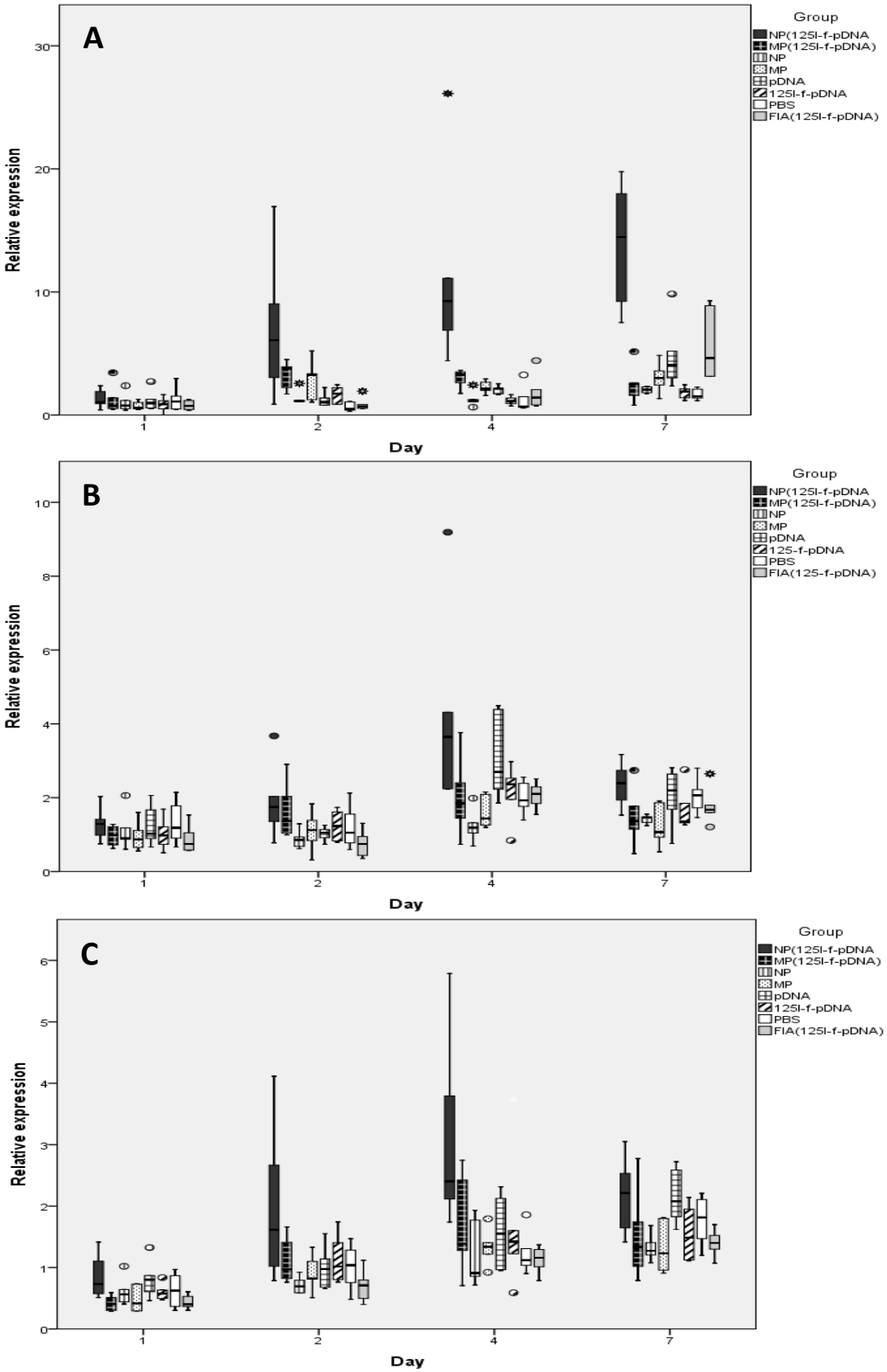

Figure 5 - Expression of the antiviral protein Mx in A) injection site muscle, B) head kidney and C) spleen. The box-plots show median values (line) and maximum and minimum values (whiskers), with the boxes representing 50% of the samples. Circles show outliers, and asterisks (*) extreme outliers. Six fish were sampled and analyzed at each time-point.

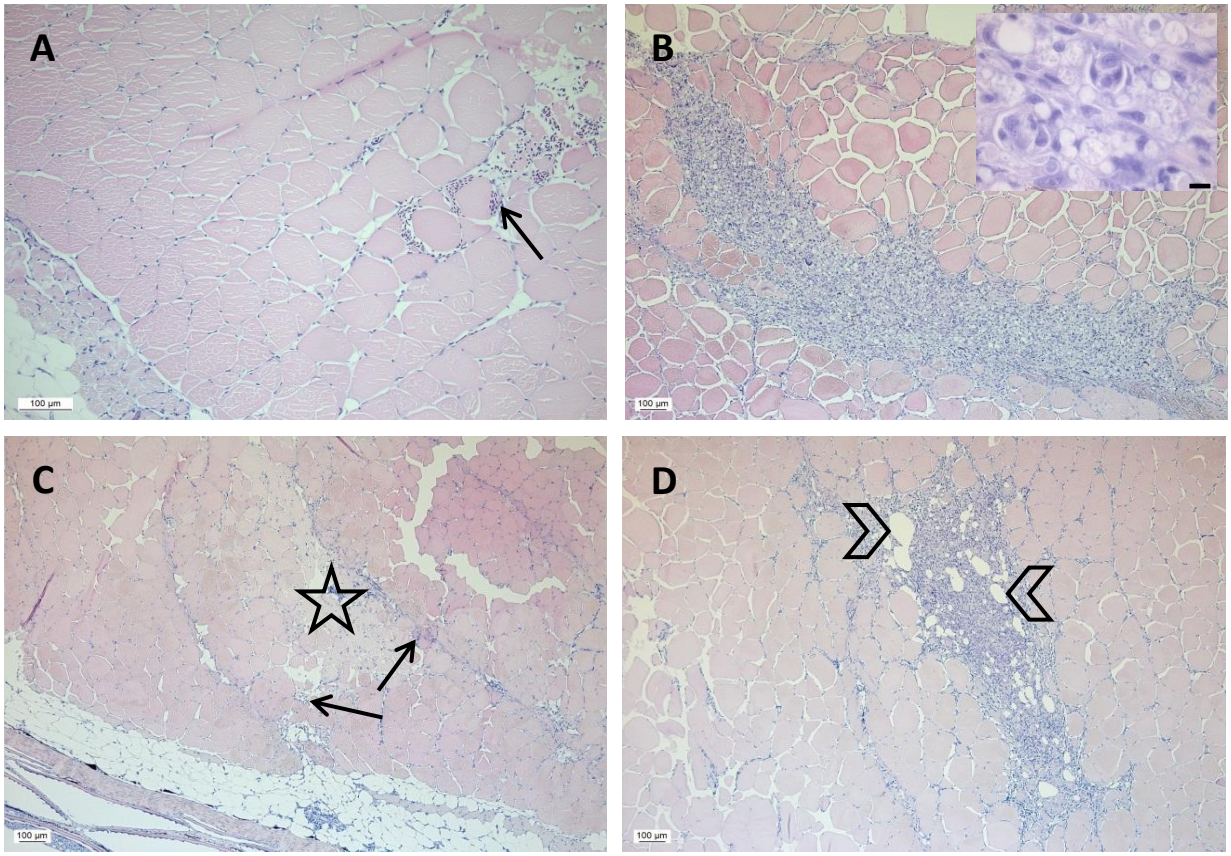

Figure 6 – All pictures show transversal sections of skeletal muscle from the injection site, stained with hematoxylin and eosin (HE). A) Minor hemorrhages (arrow) and muscle cell degeneration in negative control (PBS) group observed at day 2. B) Massive cellular infiltrate in the myocyte fibre and surrounding interstitium at day 30 in tissue of fish injected with MP-(^{125}I-f-pDNA). Magnification of cellular infiltrate is shown with a 10.0 µm scale-bar. C) Focal muscle cell degeneration (star) and minor hemorrhages (arrows) can be seen at day 7 in samples from fish injected with pDNA. D) Large oil-droplets (arrowheads) surrounded by degenerative muscle fibers and inflammatory cells at day 30 after injection of FIA-(^{125}I-f-pDNA).

COVER LETTER - Ref. No.: FSIM-D-12-00522

We are pleased to have the referees' comments, and we have now undertaken the revision that hopefully fulfills the requirements to get the manuscript accepted.

In general, we have met the criticisms made by the referees, and performed the revision accordingly. In addition we have made some minor additional improvements based on new input by the authors. Please note that there are two corresponding authors.

Best wishes,
Roy A. Dalmo

Ref. No.: FSIM-D-12-00522

Response to review of manuscript with title "Transgene and immune gene expression following intramuscular injection of Atlantic salmon (*Salmo salar* L.) with DNA-releasing PLGA nano- and microparticles".

General comments on the revised manuscript:

For this re-submission revisions have been confined to the introduction and discussion sections of the manuscript, in addition to some figure revisions in response to reviewer's queries. As the study was not designed to evaluate DNA vaccine efficacy but rather the potential influence of various factors on the expression of a reporter gene, we found it right not to make too much speculation as to how our results might transfer to an actual DNA vaccine trial. An effort has instead been made to clarify the various results and observations in terms of their potential impact – positive or negative – on the expression of the reporter gene.

New additions as well as altered sections of the text have been highlighted in red and yellow for easier recognition, and line-references have also been provided in the answers to reviewer queries following below.

Query 1:

In the abstract there are several "pDNA". The authors should clarify what "p" stands for in the first one.

Clarification has been made in line (L) 28.

Query 2:

The authors have used a luciferase gene as a reporter gene in the present study. If the authors have been tried a luciferase assay in this project, the authors should show the results of the assay. If not, the author should describe the reason why a luciferase gene has been chosen as a reporter gene.

No luciferase assay was included in the current project. Luciferase (Luc) was still chosen as it is one of the most common reporter genes and has frequently been applied for the evaluation of transgene expression in fish at both protein and transcription level (L77-79). The application of a Luc-gene hence makes it easier to evaluate and relate the work performed for this manuscript to previously published studies.

Query 3:

In the results of histopathology, it is difficult to understand the histological differences between treated samples and controls for those who are not familiar with the inflamed tissue.

a) Please describe the detailed features of muscle degeneration and inflammation in the manuscript or figure legend.

More detailed descriptions of the various histopathological features including muscle cell degeneration and inflammation have been provided in the figure legend.

b) Please add the enlarged figures for the inflammatory cells and add the description of the detailed features for the inflammatory cells in fig 6D.

Detailed features, such as cell types, cannot be provided for the observed inflammatory cells as no immunohistochemical assays were conducted to verify their specific nature. A higher magnification micrograph has been inserted for one of the originals (6B) to show the high variation in infiltrating cells.

c) Please add scale bars for the panels in histological figures.

Scale bars have been added.

Query 4:

Although several homolog genes were used as markers for pro-inflammatory, antiviral response and cytotoxic T-cell markers in the present study, the authors should show some proofs that those genes had similar roles in Atlantic salmon by the referring of previous other work, or other way.

The introduction has been revised accordingly to include specific references to studies on function and/or presence of the various cytokines and markers in fish species. For TNF-α and IL-1β the additions can be found in L95-99. Studies on the activities of IFN-α and Mx in teleosts were referenced in the original manuscript. Descriptions are now given in L100-106. Brief references to the existence and activity of CD8 in fish have also been made (L106-108), while reference to a very recent study on the expression and function of Eomesodermin in Atlantic salmon has been included in L108-111.

Query 5:

The authors should add the evidence-based descriptions that the early response (1-7 days post vaccination) of immune-related genes showed in the fig. 4 and 5 would be of benefit to DNA vaccination in the discussion part.

Whereas some mention had been made to the benefit of these early responses, we agree that the original manuscript did not clarify the findings and evaluations of these in a satisfactory manner. The discussion has therefore to some extent been rewritten to clarify potential benefits *as well as* negative effects of the various early immune responses.

The role of proinflammatory cytokines in the migration of inflammatory cells to the site of injection has been specified in the description of biological function (L95-97), whereas the potential influence of these cytokines on DNA vaccines and transgene expression is described in L434-442. Likewise, the antiviral functions of IFN-α and Mx and the relation to CD8 and Eomes expression can be found in the introduction, as described for query 4. In the discussion, L448-449 and L451-453 reference the importance of innate antiviral responses in teleosts and presents the potential benefit to later, acquired

responses. In L455-457 a reference is given to the potential influence of PLGA particle size on the resulting antiviral response.

Reviewer #2: I consider that author's answers for my comments were sufficient, and revisions made the manuscript clearer.

The present study is now worthwhile publishing in Fish and shellfish immunology.

However, there are two comments for the revised manuscript as shown in below.

1. The authors used a term "vaccination" for pDNA delivery in the present study. Is this "vaccination" appropriate term for the experiment performed in the presents study?

Response: We have changes the term “DNA vaccination” to “genetic engineering”

2. For "Histopathology" in the section of results. The authors’ classified histological changes post injection. However, there is a low objectivity in this analysis. Therefore, the authors should make the all calcification index clear by showing all images of histological classification, if possible.
I suppose that the result, "The histopathological changes caused by particles seemed to depend on particle size (nano vs. micro) rather than content (empty vs. pDNA)." is very important for the present study. Therefore, the authors should show detailed results for this analysis.

Response: A calcification index is a measure of accumulation of calcium salts in a body tissue and thus not very relevant for this study, we think. However in certain cases, a calcification process may occur during inflammation and would preferably be observed and diagnostized by X-ray analysis. We have addressed the histopathological score vs. different particle sizes (table 4), but it is difficult to give exact quantitative score, as histological examination is a qualitative method. We are sorry that we cannot meet this criticism by applying other quantitative measures.

Transgene and immune gene expression following intramuscular injection of Atlantic salmon (*Salmo salar* L.) with DNA-releasing PLGA nano- and microparticles

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Key words: PLGA/Nanoparticles/Microparticles/Transgene expression/Immune responses

Abstract

The use of poly-(D,L-lactic-*co*-glycolic) acid (PLGA) particles as carriers for DNA delivery has received considerable attention in mammalian studies. DNA vaccination of fish has been shown to elicit durable transgene expression, but no reports exist on intramuscular administration of PLGA-encapsulated plasmid DNA (pDNA). We injected Atlantic salmon (*Salmo salar* L.) intramuscularly with a plasmid vector containing a luciferase (*Photinus pyralis*) reporter gene as a) naked pDNA, b) encapsulated into PLGA nano- (~320nm) (NP) or microparticles (~4μm) (MP), c) in an oil-based formulation, or with empty particles of both sizes. The ability of the different pDNA-treatments to induce transgene expression was analyzed through a 70-day experimental period. Anatomical distribution patterns and depot effects were determined by tracking isotope labeled pDNA. Muscle, head kidney and spleen from all treatment groups were analyzed for proinflammatory cytokines (TNFα, IL-1β), antiviral genes (IFN-α, Mx) and cytotoxic T-cell markers (CD8, Eomes) at mRNA transcription levels at days 1, 2, 4 and 7. Histopathological examinations were performed on injection-site samples from days 2, 7 and 30. Injection of either naked pDNA or the oil-formulation was superior to particle treatments for inducing transgene expression at early time-points. Empty particles of both sizes were able to induce proinflammatory immune responses as well as degenerative and inflammatory pathology at the injection site. Microparticles demonstrated injection-site depots and an inflammatory pathology comparable to the oil-based formulation. In comparison, the distribution of NP-encapsulated pDNA resembled that of naked pDNA, although encapsulation into NPs significantly elevated the expression of antiviral genes in all tissues. Together the results indicate that while naked pDNA is most efficient for

inducing transgene expression, the encapsulation of pDNA into NPs up-regulates antiviral responses that could be of benefit to DNA vaccination.

Introduction

PLGA (poly-(D,L-lactic-*co*-glycolic)-acid) nano- and microparticles as adjuvants and carriers for vaccine antigens have been extensively investigated, mainly in mammalian models [1-5]. The biodegradable copolymer produces non-toxic degradation products [3], offers increased predictability of antigen release-rates, potential for intracellular antigen delivery and the ability to encapsulate and co-encapsulate a wide variety of antigens, including DNA vaccines [1, 6, 7].

Luciferase has commonly been applied for the evaluation of DNA vaccines at both transcription and protein level, and appears to express higher in fish than in mammals for a given dose of DNA [8-12]. The encapsulation of plasmid DNA into PLGA particles could protect against the rapid on-site degradation reported in both mice and salmon after intramuscular injection, and has been shown to increase the escape of antigen from endosomes to the cytosol [6, 8, 10, 11, 13].

Depending on size, the particles may create injection-site depots (>5µm), get phagocytized by antigen presenting cells (APCs) (<5µm), be internalized by non-phagocytic cells such as myocytes (<500nm) or escape into the bloodstream and subsequently be cleared by phagocytes in the head kidney, spleen and/or liver [4, 14]. Muscle cells have been shown to slowly accumulate pDNA over time, and could benefit from extracellular pDNA-releasing microparticles as well as intracellular nanoparticle depots [15].

A central attribute of DNA vaccines is the ability to induce cellular as well as humoral immune responses, including cytotoxic T-lymphocyte (CTL) responses and antibody production [16-18]. Unlike vertebrate DNA, bacterial DNA contains stretches of

93 unmethylated CpG sequences that are recognized as danger signals by toll-like receptor 9
94 (TLR9). Upon stimulation this endosomal pattern recognition receptor (PRR) may induce
95 a variety of cytokines. Interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are
96 hallmark cytokines in driving the inflammatory response in mammals and amongst other
97 properties hold key roles in the migration of effector cells to sites of infection [19]. Both
98 cytokines have been found in a number of teleost species and appear to exert functions
99 similar to what is known from mammals [20-22].

100 Interferon-α (IFN-α) is one of the type I IFNs, key mediators of antiviral
101 responses through the regulation of IFN-stimulated genes and central in linking innate
102 and adaptive immunity [16, 23-25]. The IFN-induced protein Mx has demonstrated
103 antiviral functions in Atlantic salmon (*Salmo salar* L.) and can be used to follow IFN
104 activity, as it has a much longer lifetime and accumulates to higher concentrations [26-
105 28]. Type I IFNs also play an important role in the clonal expansion and generation of
106 specific as well as non-specific memory $CD8^+$ T-cells [29]. Sequences for CD8α and
107 CD8β are known from a variety of teleost species and the cytotoxic activity of $CD8^+$ T-
108 cells has been demonstrated in rainbow trout (*Oncorhynchus mykiss*) [30, 31]. The
109 transcription factor Eomesodermin (Eomes) is critical to the development of $CD8^+$ T-cell
110 effector functions and memory cells in mammals [32, 33]. A recent study indicated
111 similar functions as well as induction by IFN-α in Atlantic salmon [34].

112 Intramuscular injection of naked pDNA generally induces few and transient
113 histopathological changes in fish as well as in mammals [9]. In contrast, the use of PLG
114 microspheres as DNA carriers has been shown to result in a foreign body response in
115 mice, where the infiltrating cells were also the ones that were primarily transfected [35].

Enhanced inflammatory reactions coupled with prolonged availability of pDNA might therefore be beneficial to transgene expression and T-cell responses.

This report is the first on intramuscular injection of Atlantic salmon with PLGA nano- and microparticles carrying pDNA, and the effect of these particles on 1) tissue distribution and retention of pDNA 2) expression of a firefly (*Photinus pyralis*) luciferase reporter gene 3) innate inflammatory (TNF-α, IL-1β) and antiviral (IFN-α, Mx) immune responses, 4) expression of cytotoxic T-cell markers (CD8, Eomes) and 5) injection site histopathology. We hypothesize that PLGA-encapsulated pDNA will induce transgene expression and proinflammatory as well as antiviral responses more efficiently than non-encapsulated pDNA.

2. Experimental

2.1 Materials/Chemicals

Poly(D,L-lactic-co-glycolic) acid (PLGA; 50:50 ratio, M_w of 7-17 kDa), poly vinyl alcohol (PVA, 87-89 % hydrolyzed), D-(+)-trehalose dehydrate, 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril (Iodogen; Pierce, Rockford, IL, USA), Freund's incomplete adjuvant (FIA) and quantitative polymerase chain-reaction (QPCR) primers were purchased from Sigma Aldrich. Carrier free Na[^{125}I] was from Perkin-Elmer Norge (Oslo, Norway). Acetone, dichloromethane (DCM) and chloroform were purchased from Merck Biochemicals. Sodium metabisulphite ($Na_2S_2O_5$ > 98 % purity) and potassium iodide (KI > 99.5 % purity) were purchased from Fluka Biochemica. Luciferase lyophilizate was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

2.2 Plasmid DNA

Plasmid R70pRomiLuc (gift from Uwe Fischer, Friedrich-Loeffler-Institut, Germany) contains a firefly luciferase gene under the control of a murine cytomegalovirus immediate early promoter (CMV-IEP). The plasmid was isolated from a culture of *Escherichia coli* (strain DH5α) by use of Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer instructions. Purified plasmid was eluted in Tris-EDTA (TE) buffer (pH=8.0). DNA concentration and quality was determined with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and 1% agarose gel-electrophoresis. High quality samples (A_{260}/A_{280} ratio > 1.9 and distinct DNA bands on gel) were stored at -20 ºC until use.

2.3 Preparation of [^{125}I]-fluorescein-pDNA

Purified pDNA was modified using the nucleic acid labeling kit *Label*IT Fluorescein (MIR 3200, Mirus Corp., Madison, WI, USA) according to manufacturer instructions. Radiolabeling of f-pDNA with carrier-free Na[^{125}I] was performed in a direct reaction with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril as the oxidizing agent. The protocol was in accordance with the Iodogen method of radiolabeling [36], with minor modifications concerning incubation time (1h). Free iodine was removed by filtration on a PD-10 column equilibrated with pDNA in PBS. Radiation was determined by gamma counting (COBRA™ II Auto-Gamma®, ©Packard Instrument Co., Meridan, IL, USA), with specific activity measured to ~ 4.75 million cpm μg^{-1} ^{125}I-f-pDNA.

2.4 Preparation of naked and pDNA-loaded nano- and microparticles

Particles were prepared by a modified version of the double emulsion ($W_1/O/W_2$) solvent evaporation method [2, 5, 37], outlined in table 1. A fraction of ^{125}I-f-pDNA was included in the first water phase (W_1) for determination of encapsulation efficiency and tracing of tissue distribution in *in vivo* experiments. Emulsions were prepared on ice-baths by sonication (S) (Sonics VibraCell VC750, 3 mm tapered micro tip, Sonics & Materials Inc., Newtown, CT, USA) or homogenization (H) (Ultra-turrax® T-25 Basic, IKA®-WERKE, Staufen, Germany). Plasmid was excluded from the W_1 phase for preparation of naked particles. Fifteen ml dH_2O was added to the $W_1/O/W_2$-emulsions before overnight stirring to facilitate solvent evaporation. After centrifugation (Avanti® J-26 XP, BeckmanCoulter®, USA), pellets were re-suspended in dH_2O and the fractions were pooled. Trehalose (5 mg ml^{-1} in dH_2O) was used as a lyoprotectant and added to suspension aliquots in a ratio of 1:3. Aliquots were kept at -80˚C for a minimum of 2 h before lyophilization for 72 h at 0.001 hPa, -110 °C (ScanVac CoolSafe^{TM}, LaboGene, Denmark). Lyophilizates were stored in airtight containers at 4 °C.

2.5 Particle characterization

Encapsulation of pDNA was determined by the gamma emission in a known amount of particles (COBRA™ II Auto-Gamma®) and measured radioactivity related to the specific radioactivity in the fraction of ^{125}I-f-pDNA stock solution added to the W_1-phase. Encapsulation efficiency was determined as entrapped amount of pDNA relative to the amount initially present in the W_1-phase. Loading (µg pDNA mg^{-1} PLGA) was calculated by relating encapsulated pDNA to the total weight of retrieved particles. Sizing of

nanoparticles was performed by photon correlation spectroscopy (PCS) (NiComp 380 Submicron Particle Sizer, Santa Barbara, USA). Microparticle size was determined by use of a Model 780 AccuSizer (NiComp).

2.6 In vitro particle stability and pDNA release

Five containers of 10 mg lyophilizate dissolved in 1 ml PBS (pH 7.4) with 0.02% sodium azide (NaN_3) were prepared for both nano- and microparticles. The suspensions were incubated at 8˚C on a Stuart® SB3 rotator. Sampling was performed immediately after particles had been dissolved, and then at 3 h, day 1, 2, 4, 7, 14, 21, 30, 40, 50, 60 and 70. At each sampling 100 µl were removed and centrifuged at 15,000 x g for 1 minute. Fifty µl of the supernatant were removed for isotope measurement, and the remaining fraction transferred back to the original container after addition of 50 µl new PBS and full re-suspension of the particle pellets.

2.7 Fish

Unvaccinated pre-smolt Atlantic salmon with a mean weight of 30 g were provided by and kept at Tromsø Aquaculture Research Station. The fish were kept in 200 L plastic tanks supplied with running fresh water (8-10˚C) and fed a commercial feed from Skretting AS (Stavanger, Norway). Adaptation to test conditions was performed for one week prior to immunization. All experiments were in compliance with The Norwegian Welfare of Animals Act.

2.8 Experimental groups and genetic engineering (GE)

A total of 336 fish were distributed among seven tanks. Each tank contained 6 fish from each of the eight experimental groups (Table 2). Prior to tattooing and GE the fish were sedated with benzocaine using 40 mg L^{-1} (Benzoak Vet., ACD Pharmaceuticals, Leknes, Norway). The PanJet needle free injection system with saturated Alcian blue was used for tattooing. All formulations were administered in 50 µl volumes by injection in the left epaxial muscle. Each injection dose contained 11 µg pDNA, and the amount of NPs and MPs was adjusted to make up for differences in pDNA loading. Injection dose samples were collected from each formulation in order to determine cpm.

2.9 Sampling

The fish were starved for 24 h prior to sampling and killed by a double dose (80 mg L^{-1}) of benzocaine. At day 1, 2, 4, 7, 14, 30 and 70 post injection one tank was sampled. All fish were weighed. Blood from fish given radioactive formulations was sampled using vacutainers (Becton Dickinson) and stored on ice. Two muscle samples from the injection site were transferred to 10% neutral buffered formalin for storage until histological processing. Muscle samples, spleens and head kidneys were transferred to separate tubes containing 1 ml RNAlater® (Ambion, Austin, TX, USA) and kept at room temperature overnight before storage at -20˚C. To determine tissue distribution in fish given radioactive formulations it was necessary to sample the full carcass. Trunk kidney, organ package (liver, heart, gastrointestinal tract (GIT) and interstitial adipose tissue), head (incl. gills) and remaining muscle (with skin) were collected in separate tubes. One

fish injected only with PBS was similarly sampled at each time point in order to exclude the possibility of radioactive contamination by cohabitation.

2.10 Anatomic distribution and plasmid DNA retention

Determination of *in vivo* retention and biodistribution was achieved by tracking trace amounts of ^{125}I-f-pDNA. From blood samples, 50 µl were removed for analysis. Radioactivity in blood and trunk kidney samples was determined with a COBRA™ II gamma counter. Remaining samples, including spleen and injection site tissue, were analyzed using a Packard Auto-Gamma Scintillation Spectrometer (©Packard Instrument Co., model 5120). Background radiation was measured to be 50 and 100 cpm respectively for the two machines. These were set as baselines, and were subtracted from measured values before data were adjusted according to [^{125}I] half-life. Blood volume was set to 4% of the body weight [38]. Mean values of the injection dose samples gathered during GE were used to determine total cpm recovery (%) at day 1. To determine the stability of retention, the daily cpm means were related to recovery at day 1. Daily cpm means were used to determine anatomical distribution.

2.11 RNA isolation and cDNA synthesis

Tissue samples (20-30 mg) were removed from RNAlater® and homogenized in lysis buffer from the RNeasy mini kit (Qiagen) using tubes with ceramic beads (Precellys) and a Precellys® 24 Homogenizer (Bertin Technologies). Parameters were set for 5300 x g x 3 spins, 10 s. Subsequent RNA extractions and on-column purification with the RNase-Free DNase Set from Qiagen were performed according to manufacturer instructions.

RNA was eluted in 50 µl RNase-free water and stored at -80˚C until use. RNA concentration and quality was determined using NanoDrop® (NanoDrop Technologies) and 1% agarose gel electrophoresis. High quality samples (260/280 ratio > 1.9 and tight 18S/28S bands) were used for cDNA synthesis. Reverse transcription was performed with the High Capacity RNA-to-cDNA Master-mix from Applied Biosystems in accordance with manufacturer instructions. Each reaction contained 400 ng RNA in 20 µl. Samples were diluted to 80 µl with RNase-free water and stored at -80˚C.

2.12 Quantitative reverse transcription polymerase chain reaction (QPCR)

Quantitative PCR was performed according to manufacturer instructions using Fast SYBR® Green Master Mix (Applied Biosystems) and primers previously applied in our laboratory (Table 3). Primer efficiency was determined using a six-point 1:2 dilution series in triplicate, with an initial cDNA concentration of 20 ng. All primers were free of primer-dimers in no-template control and showed a single peak in the dissociation curve. Samples containing 20 ng cDNA were run in triplicate, with each plate including no-template control and an inter-run calibrator. Analysis was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) with the thermal cycler profile set to 95˚C for 20 s, 40 cycles of 95˚C for 3 s and 40 cycles of 60˚C for 30 s. Dissociation curves were performed on all samples. The 7500 Software Relative Quantification Study Application (v2.0.4, Applied Biosystems) was used to determine C_T values, with walking baseline and threshold set manually to be equal for all genes. Relative gene expression was determined by the Pfaffl method [39], comparing saline injected fish to treated groups. To determine relative expression of luciferase the highest day 1 transcript average was

used as control. Head kidney and spleen samples from day 2 and 7 were also checked for luciferase transcripts.

2.13 Histology

Muscle samples from days 2, 7 and 30 post injection were chosen for histology. The service of routine histological processing and staining with hematoxylin and eosin (HE) was purchased from the National Veterinary Institute (Oslo, Norway). Sections were studied by light microscopy for signs of hemorrhages, tissue degradation and inflammation. Using the changes in day 2 saline injected fish as a baseline, observed changes were classified as either moderate (+) or strong (++). Moderate changes were determined as largely limited to the needle trajectory, with inflammatory cells primarily associated with degenerated tissue and/or dispersed among intact muscle cells. Strong changes were seen as degeneration and/or inflammation spreading beyond the needle trajectory, with few or no intact muscle cells present within the cellular infiltrate. The histopathology scores were performed as blind testing by two professionals.

2.14 Statistical analysis

Microsoft Excel (Microsoft™) was used for arrangement of data as well as calculation of biodistribution and relative gene expression. Statistical analyses for real-time PCR were performed with IBM SPSS (Statistical Package for the Social Sciences, version 19.0). All data were natural log transformed prior to analysis, and normality verified by the Shapiro-Wilk test. Levene's test of equality of variances was used to check for homoscedasticity. Homoscedastic data were analyzed by one-way ANOVA with Tukey

as the post hoc test. Where homoscedasticity was violated, the Welch test of equality of means was applied, followed by Dunnett T3 post hoc. Graphs were constructed with Microsoft Excel and SPSS.

3. Results

3.1 Particle characterization

The encapsulation efficiency and loading of ^{125}I-f-pDNA were low for both particles sizes. The different particle characteristics are summarized in table 1, where empty particles have been excluded due to minor size differences. The mean size of nanoparticles was 320 nm by intensity distribution, with more than 90% measuring less than 500 nm. The number weight mean diameter of microparticles was 3-4 µm, with 80% measuring less than 5 µm and more than 90% smaller than 10 µm.

3.2 In vitro particle stability and pDNA release

Neutral pH and a temperature of 8˚C were chosen in order to simulate *in vivo* conditions. Initial burst release was 81% for NP-(^{125}I-f-pDNA) and 49% for MP-(^{125}I-f-pDNA). Both groups subsequently displayed a slow and sustained release, with a 96% and 69 % accumulated release at day 70 (Fig. 1).

3.3 Anatomical distribution and depot of vaccine formulations

No radioactivity was registered in blood later than day 7-post injection. The average total cpm recovery at day 1 ranged from 15% for NP-(^{125}I-f-pDNA) to 39% for ^{125}I-f-pDNA

(results not shown). Radioactivity was primarily recovered from the injection site, organs and trunk kidney. High degrees of similarities in tissue distribution were observed for NP-(^{125}I-f-pDNA) and ^{125}I-f-pDNA (Fig. 2A, B). Likewise, MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) had similar distribution profiles and injection site depots (Fig. 2C, D). The trunk kidney was the primary site of recovery for NP-(^{125}I-f-pDNA) and ^{125}I-f-pDNA, containing almost 80% of total radiation at day 70. NP-(^{125}I-f-pDNA) showed higher injection site retention (5%) at day 70 compared to ^{125}I-f-pDNA (1%). The respective values for MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) were 34% and 26%, with retention in trunk kidney reaching 32% and 27% at day 70.

3.4 Quantitative reverse transcription polymerase chain reaction (QPCR)

3.4.1 Expression of luciferase reporter gene

FIA-(^{125}I-f-pDNA) induced the highest luciferase transcript average in muscle at day 1 and was chosen as calibrator for this tissue. FIA-(^{125}I-f-pDNA) also induced the highest individual levels of luciferase transcripts throughout the experiment, with significant expression at day 1, 4 and 14 ($p \leq 0.032$) (Fig. 3). Naked plasmids consistently induced higher expression than the particle formulations, with significance for pDNA at day 1-7 ($p \leq 0.042$) and ^{125}I-f-pDNA at day 4 ($p = 0.018$). No significant differences were found at days 30 and 70. Low luciferase transcript levels were detected in head kidney, with pDNA and ^{125}I-f-pDNA inducing significant expression compared to NP-^{125}I-f-pDNA at day 2 ($p \leq 0.016$, results not shown). No expression was detected in the spleen.

345 *3.4.2 Proinflammatory cytokines IL-1β and TNF-α*

346 The particle formulations significantly up-regulated IL-1β in muscle samples at all time-
347 points; NP-(^{125}I-f-pDNA) ($p \leq 0.002$), NP ($p \leq 0.004$), MP-(^{125}I-f-pDNA) ($p \leq 0.003$),
348 MP ($p \leq 0.015$) (Fig. 4A). From day 2 the up-regulation was significant compared to all
349 non-particle groups. MP formulations consistently induced the highest expression. FIA-
350 (^{125}I-f-pDNA) significantly up-regulated IL-1β in muscle at day 7 ($p = 0.000$). Significant
351 expression of TNF-α in head kidney was only found at day 4, with NP-(^{125}I-f-pDNA) (p
352 $= 0.034$) and MP ($p = 0.008$) (Fig. 4B). No significant expression of TNF-α was found in
353 the spleen or muscle.

354

355 *3.4.3 IFN-α and Mx*

356 NP-(^{125}I-f-pDNA) significantly up-regulated IFN-α at the injection site at day 4 ($p =$
357 0.005). Levels in head kidney were generally low, with significant up-regulation by ^{125}I-
358 f-pDNA ($p = 0.000$) at day 2. NP-(^{125}I-f-pDNA) ($p \leq 0.003$) and NP ($p \leq 0.036$)
359 significantly up-regulated IFN-α in spleen at day 1 and 2 (results not shown). Levels of
360 Mx at the injection site increased for all groups from day 1 to 7. NP-(^{125}I-f-pDNA)
361 induced significant expression at day 2-7 ($p = 0.000$), and was superior to all groups at
362 day 4 and 7 ($p \leq 0.020$). MP-(^{125}I-f-pDNA) significantly up-regulated Mx in muscle at
363 day 2 and 4 ($p \leq 0.006$) (Fig. 5A). NP-(^{125}I-f-pDNA) appeared the most potent treatment
364 for inducing Mx in head kidney, but no significance was found (Fig. 5B). Mx expression
365 increased in spleen for all groups from day 1 to 7, with significance for NP-(^{125}I-f-pDNA)
366 at day 4 ($p = 0.013$) (Fig. 5C).

367

3.4.4 CD8 and Eomes

There was no significant up-regulation of CD8 in any tissue. Several muscle samples showed no CD8 expression, and no statistical analyses could therefore be performed. No expression of CD8 could be detected at day 2 in head kidney samples from fish injected with ^{125}I-f-pDNA. Interestingly, these samples induced the only significant up-regulation of Eomes observed within any tissue ($p = 0.000$) (results not shown).

3.6 Histopathology

Tissue sections of injection site samples were examined by light microscopy for signs of hemorrhages, muscle degeneration and inflammation (Table 4). PBS caused only minor hemorrhages and tissue degeneration at day 2 (Fig. 6A), indicating that later changes were likely the results of the different treatments. Hemorrhages were most pronounced at day 2 in all groups. Administration of PBS and ^{125}I-f-pDNA caused moderate muscle degeneration, but no inflammation. Both tissue degeneration and inflammation were observed for pDNA (Fig. 6C), but both pathologies were more frequent with particles formulations. MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) both demonstrated strong inflammation at day 30 (Fig. 6B, D), with a high influx of inflammatory cells that for FIA-(^{125}I-f-pDNA) was concentrated around possible oil-droplets. The histopathological changes caused by particles seemed to depend on particle size (nano vs. micro) rather than content (empty vs. pDNA).

4. Discussion

The use of PLGA particle constructs as carriers for DNA vaccine delivery has so far received little attention in fish studies [1, 40]. We have investigated distribution/depot, transgene and immune gene transcription as well as injection site histopathology following intramuscular injection of Atlantic salmon with PLGA particles carrying pDNA-encoding luciferase. The double emulsion solvent evaporation method [2, 37] was used to prepare nano- and microparticles with mean diameters of 320 nm and 4 µm, respectively. Low pDNA encapsulation appears to be a recurring problem, and our results (<30% for both particle sizes) are supported by other studies [12, 35].

One of the challenges of conventional DNA vaccination is the rapid tissue clearance and on-site degradation of DNA, which may result in reduced pDNA uptake and transgene expression [8, 10]. Although oil adjuvants are not commonly applied for intramuscular vaccine delivery, FIA-(^{125}I-f-pDNA) provided a positive control to measure the depot potential of nano- and microparticles. The high burst release observed for NP-(^{125}I-f-pDNA) during the *in vitro* release study (Fig. 1) likely explains the strong similarities between this group and ^{125}I-f-pDNA (Fig. 2A, B), a similarity reflected also in the injection site histopathology (Table 4). In comparison, the injection site retention of MP-(^{125}I-f-pDNA) remained similar to that of FIA-(^{125}I-f-pDNA) throughout the study (Fig. 2C, D). The duration of the MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) depots likely contributed to the severe histopathological inflammations observed in tissue sections from both groups at day 30 (Fig. 6B, D). Inflammatory cell infiltration has been shown to take place in both fish and mammals [35, 37, 41], and transient inflammatory responses may thus contribute to increased transgene expression. Long lasting inflammations such

414 as seen with MP-(^{125}I-f-pDNA) and FIA-(^{125}I-f-pDNA) are, however, undesirable in a
415 product meant for consumption. It would appear that nanoparticles may be favorable in
416 order to avoid potential tissue damage, but the small sample sizes meant we could only
417 obtain an indication of the histopathological influence of the various treatments.

418 Although the similarities in injection site retention were not reflected in the
419 expression of the luciferase transgene (Fig. 3), the observation that FIA-(^{125}I-f-pDNA)
420 induced the highest individual levels of luciferase transcripts suggests that increased
421 cellular infiltration along with a high injection site pDNA depot may be of benefit to
422 transgene expression in Atlantic salmon. The significantly lower expression obtained
423 with encapsulated pDNA could be a result of the preparation conditions, as the $W_1/O/W_2$
424 method has been shown to cause a partial reduction in the content of supercoiled (SC)
425 DNA topoform that contributes to a lower transfection efficiency [1, 8, 37, 42]. The
426 process of emulsification required lower shear forces and shorter duration of preparation
427 and may thus have preserved a larger portion of SC DNA in FIA-(^{125}I-f-pDNA). We did
428 not, however, address whether the encapsulation of ^{125}I-f-pDNA resulted in an altered SC
429 content. The absence of significant differences in luciferase expression at day 30 and 70
430 still indicated a certain level of stability in particle groups that could result from a
431 continued release of bioactive pDNA even at later time-points.

432 Another explanation for the low transcription levels obtained with encapsulated
433 pDNA-Luc may lie with the inflammatory cytokine responses. Induced not only by
434 stimulation of PRRs but also by PLGA itself [43, 44], IL-1β and TNF-α have both
435 demonstrated inhibitory effects on transgene expression even at low concentrations,
436 contributing to a reduced transfection efficiency of encapsulated DNA *in vivo* as opposed

437 to *in vitro* [2, 37, 45]. The nano- and microparticle formulations significantly up-
438 regulated IL-1β at the injection site at all time-points (Fig. 4A), and while the expression
439 of TNF-α was generally low a synergistic effect of different cytokines on the inhibition of
440 transgene expression has been reported [45]. In comparison, the levels of IL-1β in fish
441 injected with FIA-(^{125}I-f-pDNA) were not found significant until 7 days post
442 administration, indicating that PLGA may provide a more potent inflammatory stimulus
443 than the oil adjuvant even in the absence of pronounced inflammatory histopathology.
444 Viral challenge studies in fish have shown that specific protection may be
445 acquired even with very low doses of DNA, and innate antiviral responses also appear to
446 play a more critical role in fish than mammals upon exposure to viruses [46, 47].
447 Whereas luciferase is unlikely to induce a differentiation of antigen specific $CD8^+$ T-cells
448 due to low immunogenicity in *in vivo* studies [48, 49], type I IFNs play an important role
449 in the clonal expansion of $CD8^+$ T-cells and may enhance CTL responses when
450 immunogenic transgene products are expressed [29]. Whereas the expression of IFN-α
451 was generally low and transient, NP-(^{125}I-f-pDNA) still appeared the most potent inducer
452 of antiviral responses and significantly up-regulated the expression of Mx in all tissues
453 (Fig. 5). This result supports a previous study on the influence of particle size on the
454 cytokine profile induced after administration of particle associated CpG DNA [50]. We
455 did observe that formulations with naked pDNA significantly up-regulated both Eomes
456 and IFN-α in head kidney at day 2, but no statistical correlation between these genes was
457 found for any other group, tissue or time-point.
458 This study is the first on intramuscular injection of Atlantic salmon with PLGA
459 particles carrying pDNA. Our particle formulations did not induce transgene expression

as efficiently as injection of naked plasmids, but appeared to provide a continued release of bioactive pDNA even at the end of the study. A strong expression of transgene may not always be necessary in order to mount a significantly protective immune response, and an efficient up-regulation of innate antiviral responses in particular might enhance the immunogenicity of an antiviral vaccine. Both particle sizes proved superior to naked pDNA injection for the induction of IL-1β as well as an influx of inflammatory cells at the injection site, but only encapsulation into nanoparticles significantly increased the expression of IFN-α and Mx. Together these results indicate that PLGA nanoparticles as carriers for plasmid vectors encoding viral antigens might enhance the responses to DNA vaccines.

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Figure

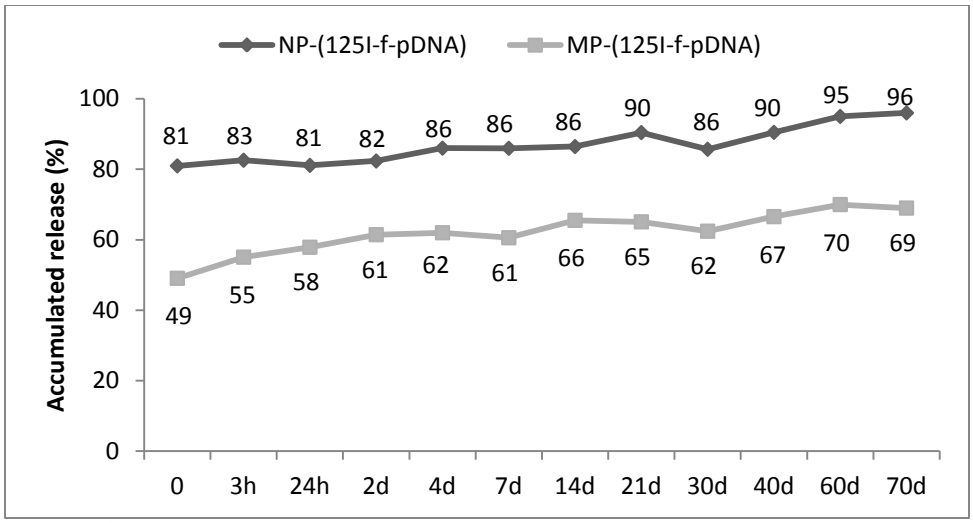

Figure 1 - Release of ^{125}I-f-pDNA from nano- and microparticles through 70 days of incubation at 8° C, presented as the mean accumulated release (%) of five samples at each time-point.

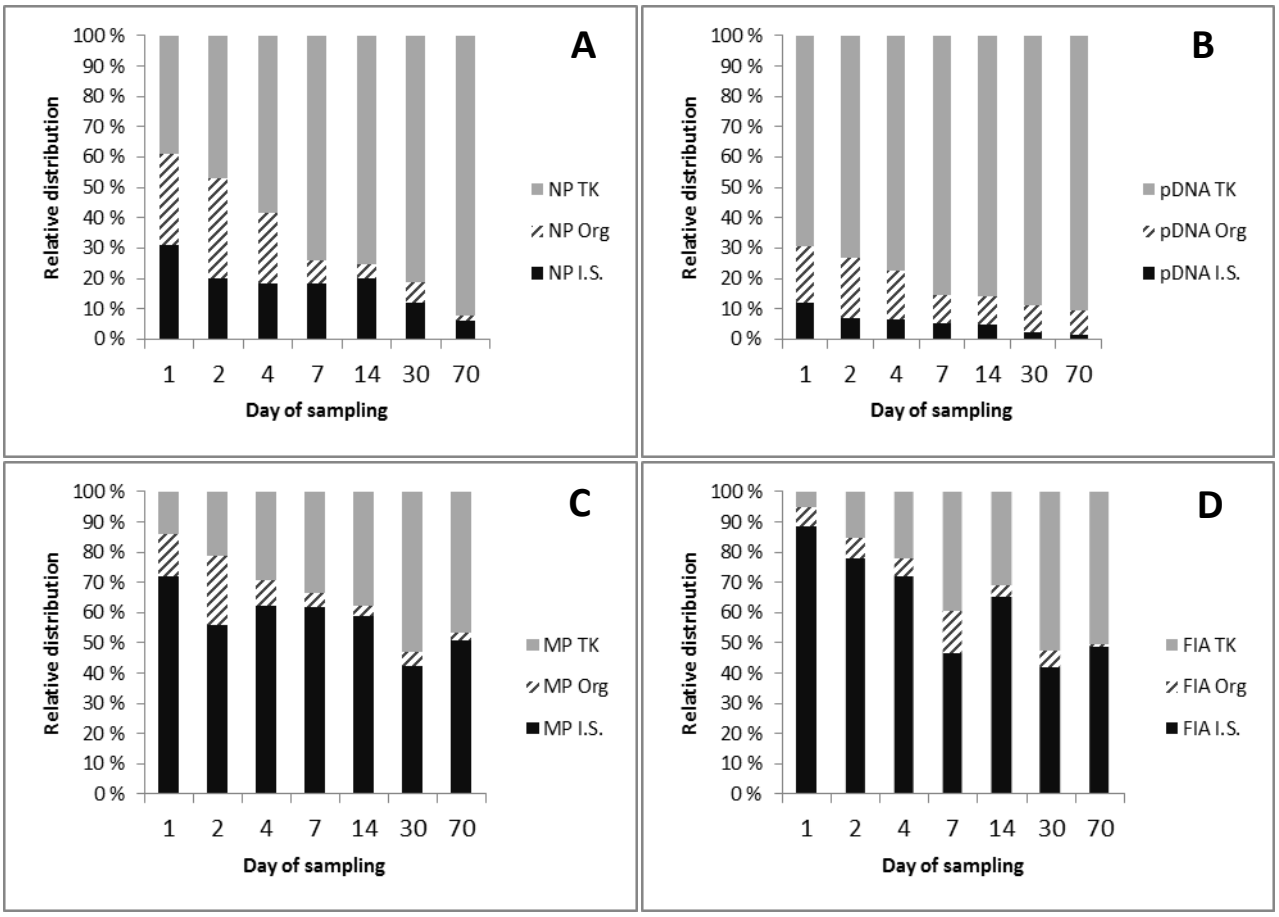

Figure 2 – Radioactivity recovered from samples of trunk kidney (TK), organs (Org) and injection site (I.S.). The stacked bars represent the relative distribution of pDNA among these organs in groups A) NP-(^{125}I-f-pDNA), B) ^{125}I-f-pDNA, C) MP-(^{125}I-f-pDNA) and D) FIA-(^{125}I-f-pDNA). Five fish were sampled at each time-point.

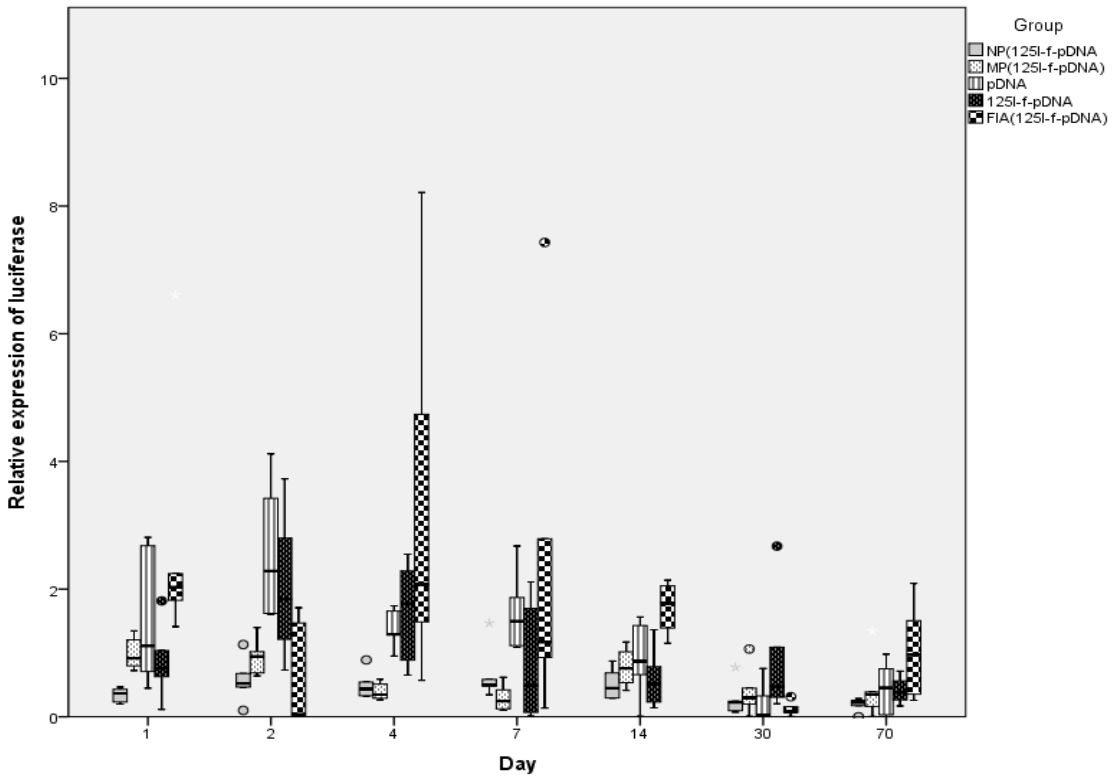

Figure 3 - Relative expression of luciferase mRNA transcripts in muscle from the injection site through the 70 day sample period. The box-plot shows the median value (line) and maximum and minimum values (whiskers), with the box representing 50% of the samples. Circles show outliers. The highest cpm-average at day 1 was found in group FIA-(^{125}I-f-pDNA) and was set as calibrator. Six fish were sampled and analyzed at each time-point.

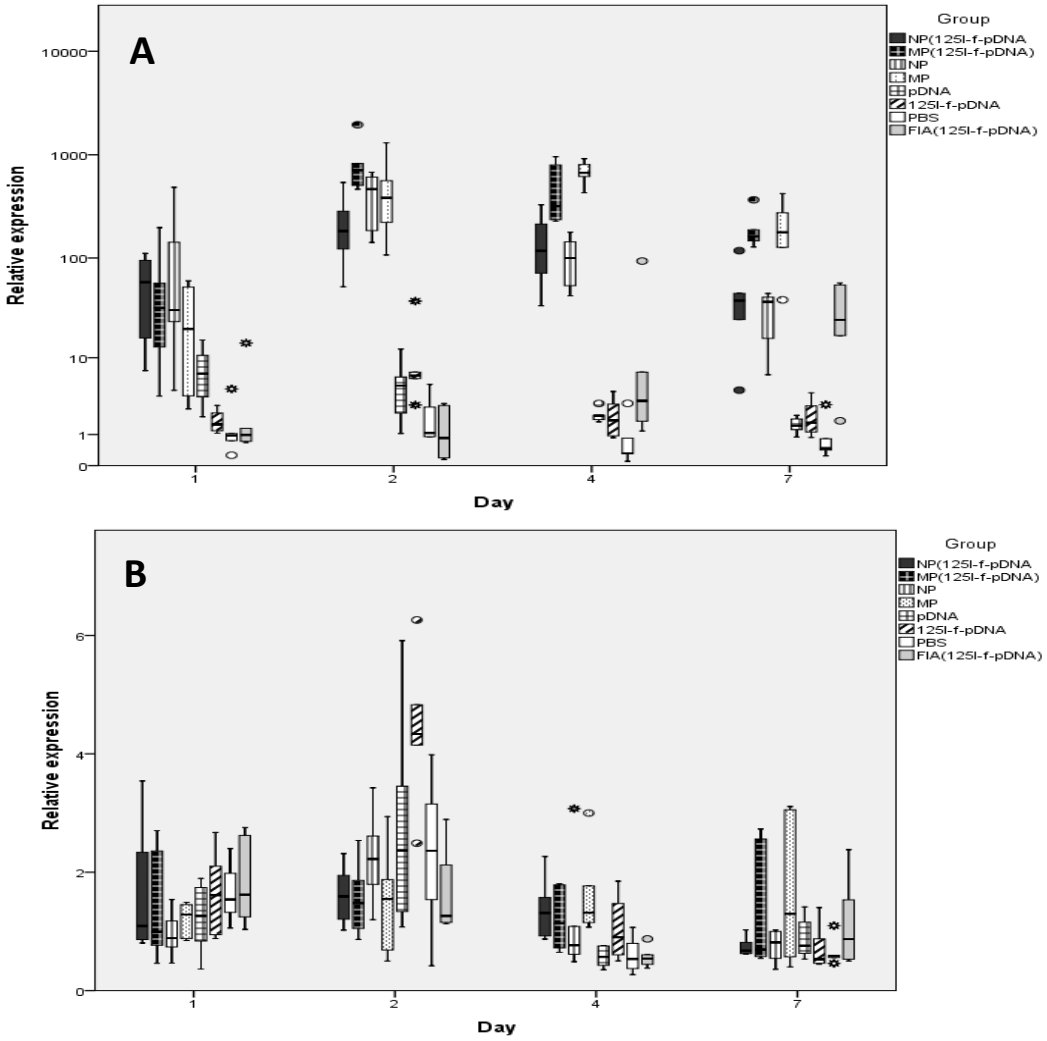

Figure 4 – Relative expression of proinflammatory cytokines; A) IL-1β in muscle from the injection site and B) TNF-α in head kidney. The box-plot shows the median value (line) and maximum and minimum values (whiskers), with the box representing 50% of the samples. Circles show outliers, and asterisks (*) extreme outliers. Six fish were sampled and analyzed at each time-point.

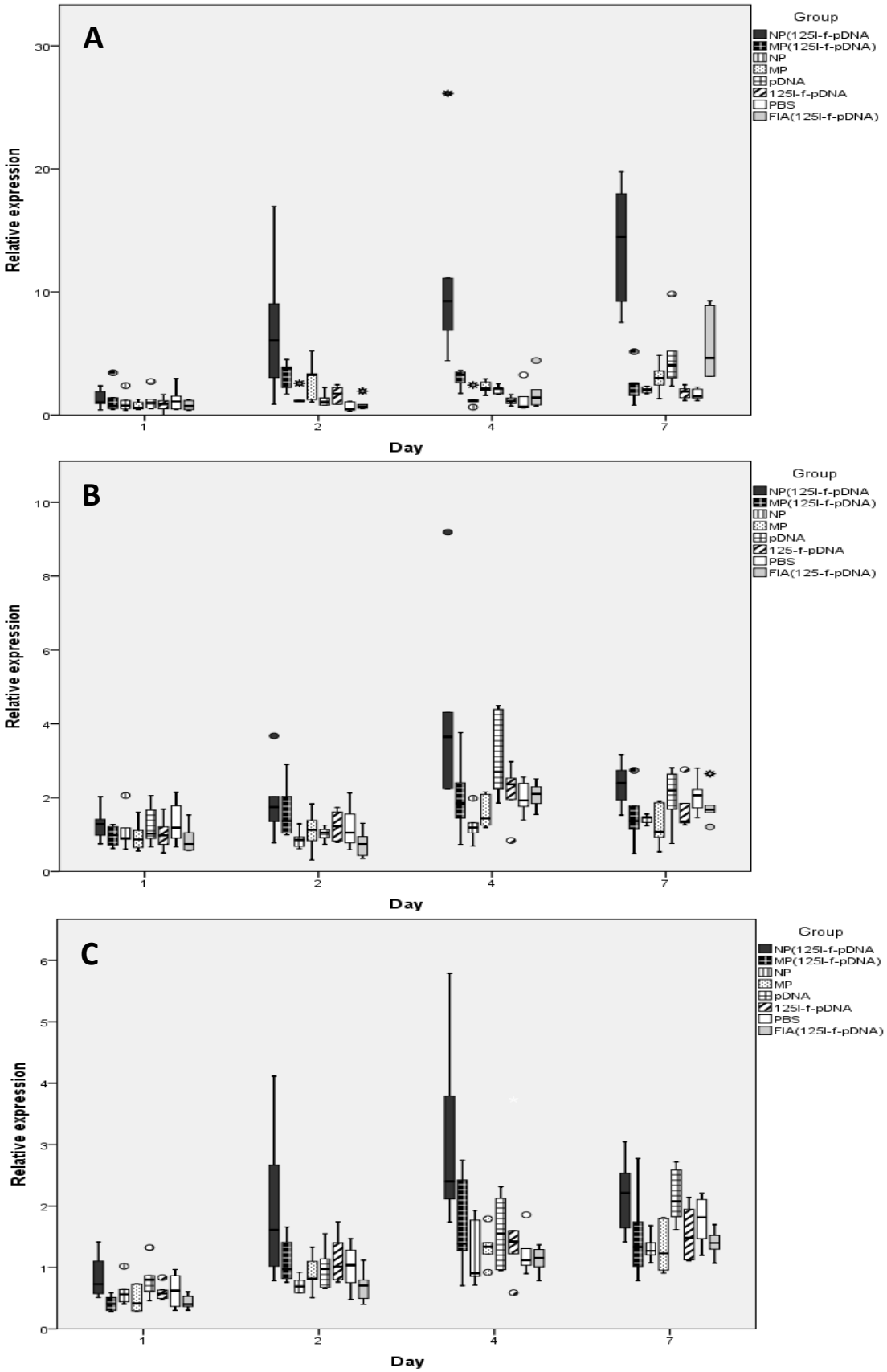

Figure 5 - Expression of the antiviral protein Mx in A) injection site muscle, B) head kidney and C) spleen. The box-plots show median values (line) and maximum and minimum values (whiskers), with the boxes representing 50% of the samples. Circles show outliers, and asterisks (*) extreme outliers. Six fish were sampled and analyzed at each time-point.

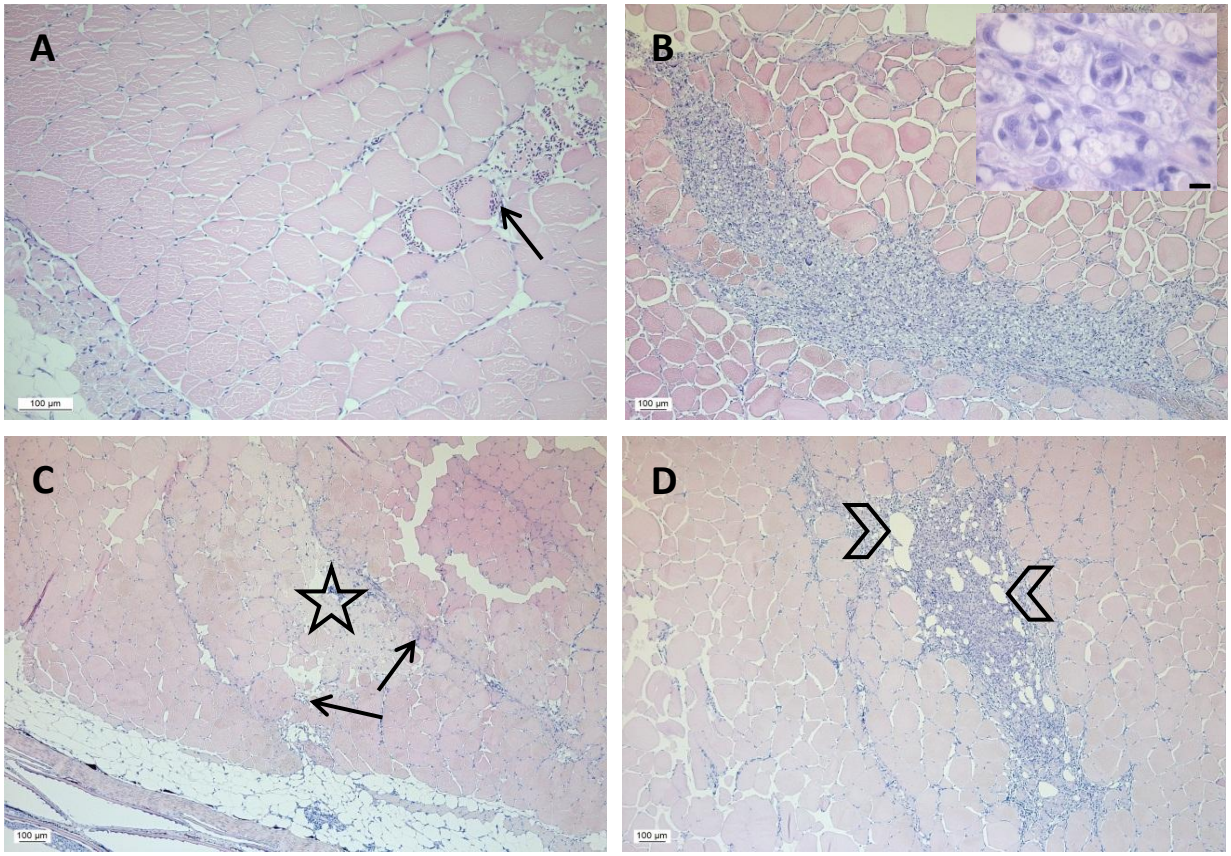

Figure 6 – All pictures show transversal sections of skeletal muscle from the injection site, stained with hematoxylin and eosin (HE). A) Minor hemorrhages (arrow) and muscle cell degeneration in negative control (PBS) group observed at day 2. B) Massive cellular infiltrate in the myocyte fibre and surrounding interstitium at day 30 in tissue of fish injected with MP-(^{125}I-f-pDNA). Magnification of cellular infiltrate is shown with a 10.0 µm scale-bar. C) Focal muscle cell degeneration (star) and minor hemorrhages (arrows) can be seen at day 7 in samples from fish injected with pDNA. D) Large oil-droplets (arrowheads) surrounded by degenerative muscle fibers and inflammatory cells at day 30 after injection of FIA-(^{125}I-f-pDNA).

Table

Table 1 – An overview of the preparation protocol and main particle characteristics for pDNA-loaded PLGA nanoparticles (NP-(^{125}I-f-pDNA)) and microparticles (MP-(^{125}I-f-pDNA)). (S): preparation by sonication. (H): preparation by homogenization. Preparation of empty particles followed the same protocol, but pDNA was excluded from the W_1 phase.

	NP-(^{125}I-f-pDNA)	MP-(^{125}I-f-pDNA)
First water phase (W_1)	pDNA (3.5 mg) in 600 µl dH_2O with 0.1% PVA	
Oil phase (O)	300 mg PLGA in 6 ml chloroform (5% w/v)	
First emulsion (W_1/O)	S: 30 sec, 35% (262.5 W)	S: 35 sec, 30% (225 W)
Second water phase (W_2)	15 ml dH_2O with 2% PVA	
Second emulsion (O/W_2)	S: 1 min, 30%	H: 45 sec, 9500 rpm min^{-1}
Washing	5000 x g, 15000 x g and 25000 x g	500 x g
Particle yield (%)	78	89
Encapsulation efficiency (%)	27	24
Loading (µg pDNA/mg PLGA)	3.24	2.85
Mean size	320 nm	3-4 µm
PLGA injected (mg)	3.5	4

Table 2 - Experimental groups and group nomenclature.

Treatment groups	**Nomenclature**
(^{125}I-f-pDNA) encapsulated in nanoparticles	NP-(^{125}I-f-pDNA)
(^{125}I-f-pDNA) encapsulated in microparticles	MP-(^{125}I-f-pDNA)
Empty nanoparticles	NP
Empty microparticles	MP
Plasmid DNA in PBS	pDNA
(^{125}I-f-pDNA) in PBS	^{125}I-f-pDNA
PBS	PBS
(^{125}I-f-pDNA) in Freund's incomplete adjuvant	FIA-(^{125}I-f-pDNA)

Table 3 - Primers for quantitative polymerase chain reaction (QPCR). (*): primers obtained from Natasha Hynes (TNF-α) and Jaya Kumari (Eomes).

Primer		Oligonucleotides, 5´ to 3´	GenBank accession number	Concentration (nM)	Amplification efficiency (%)	Amplicon size (bp)
EF1A[1]	Fw	CACCACCGGCCATCTGATCTACAA	AF321836	150	93	78
	Rv	TCAGCAGCCTCCTTCTCGAACTTC		150		
Luc[2]	Fw	TGGGCTCACTGAGACTACATCA	M15077.1	900	100	64
	Rv	CGCGCCCGGTTTATCATC		900		
TNFα*	Fw	TGTCCATCAAGCCACTACACTC	BT049358	250	84	129
	Rv	GCACTCACACACCCTGTCATT		250		
IFNα[1]	Fw	TGGGAGGAGATATCACAAAGC	NM_001123570	250	89	163
	Rv	TCCCAGGTGACAGATTTCAT		250		
IL-1β[1]	Fw	GCTGGAGAGTGCTGTGGAAGA	AY617117	200	104	73
	Rv	TGCTTCCCTCCTGCTCGTAG		200		
CD8α[3]	Fw	CGTCTACAGCTGTGCATCAATCAA	AY693391	200	83	266
	Rv	GGCTGTGGTCATTGGTGTAGTC		200		
Eomes*	Fw	ACCTCTCGTCGTCAGATAGTG	NM_001204100	200	82	204
	Rv	GGACCGGTGAGTCTTTTCTTC		200		
Mx[4]	Fw	TGCAACCACAGAGGCTTTGAA	NM_001139918	200	92	79
	Rv	GGCTTGGTCAGGATGCCTAAT		200		

Table 4 - Histopathological observations in tissue-sections of muscle from the injection site. Muscle degeneration and inflammation are classified as either moderate (+) or strong (++), depending on the extent of the pathology.

Sampling	Group	Total number of fish	Hemorrhage	Muscle degeneration		Inflammation	
				+	++	+	++
D2	NP-(^{125}I-f-pDNA)	3	2	2	0	0	0
	MP-(^{125}I-f-pDNA)	3	2	3	0	2	0
	NP	2	1	1	1	0	0
	MP	3	2	0	2	0	0
	pDNA	3	1	1	2	0	0
	^{125}I-f-pDNA	3	2	0	0	0	0
	PBS	2	1	2	0	0	0
	FIA-(^{125}I-f-pDNA)	1	-	1	-	-	-
D7	NP-(^{125}I-f-pDNA)	3	2	2	0	2	0
	MP-(^{125}I-f-pDNA)	3	0	3	0	2	0
	NP	2	0	1	1	2	0
	MP	2	1	2	0	2	0
	pDNA	3	1	2	0	1	0
	^{125}I-f-pDNA	3	2	1	0	0	0
	PBS	2	0	0	0	0	0
	FIA-(^{125}I-f-pDNA)	1	-	-	-	-	-
D30	NP-(^{125}I-f-pDNA)	2	0	0	0	2	0
	MP-(^{125}I-f-pDNA)	2	0	1	1	0	2
	pDNA	2	0	0	0	2	0
	^{125}I-f-pDNA	2	0	0	0	0	0
	PBS	1	0	0	0	0	0
	FIA-(^{125}I-f-pDNA)	1	-	-	-	-	1