In vitro and in vivo interaction of macrophages from vaccinated and non-vaccinated channel catfish (Ictalurus punctatus) to Edwardsiella ictaluri

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ABSTRACT

Macrophages from catfish vaccinated with an Edwardsiella ictaluri vaccine and macrophages from non-vaccinated catfish were used in in vitro and in vivo studies with red-fluorescent E. ictaluri to assess phagocytic ability, reactive oxygen and nitric oxide production and bactericidal activity. In the in vitro experiment, macrophages were harvested from vaccinated and non-vaccinated fish and then exposed to red-fluorescent E. ictaluri. Results of this study showed that E. ictaluri can survive and replicate in macrophages from non-vaccinated catfish (relative percent killing, RPK, from 0.011 to 0.620 and from −0.904 to 0.042 with macrophage:bacteria ratios of 1:20 and 1:100, respectively) even in the presence of reactive oxygen and nitrogen products. Macrophages from vaccinated fish were significantly (p < 0.05) more efficient in killing E. ictaluri (RPK from 0.656 to 0.978 and from 0.011 to 0.620 with macrophage:bacteria ratios of 1:20 and 1:100, respectively) and produced significantly (p < 0.05) higher amounts of ROS (10-fold increase) and nitrogen oxide (about 10-fold increase) than macrophages from non-vaccinated fish. In the in vivo experiment, vaccinated and non-vaccinated catfish were injected with red-fluorescent E. ictaluri to allow the interaction between macrophages and other components of the immune system. After 6 h, macrophages were harvested from the fish and seeded in glass chamber slides and bactericidal activity was measured in vitro. Results showed in vivo interaction of other components of the immune system enhanced bactericidal activity of macrophages from vaccinated fish. In another set of experiments, catfish were intraperitoneally injected with fluorescent bacteria opsonized with immune serum or non-opsonized and necropsied in the first 48 h after bacterial challenge to observe localization of E. ictaluri between vaccinated and non-vaccinated catfish. Vaccinated fish were able to control the dispersion of E. ictaluri in the body and red-fluorescent bacteria were observed only in the spleen, anterior and trunk kidney. In non-vaccinated fish E. ictaluri was able to replicate and invade all organs with the exception of the brain. We further determined that macrophages seeded with E. ictaluri could cause infection in non-vaccinated fish upon reinoculation with in vitro infected-macrophages. Overall, the results indicated that macrophages from vaccinated fish are activated and responsible for rapid clearance of infection upon re-exposure to virulent E. ictaluri.

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1. Introduction

Edwardsiella ictaluri are Gram-negative, rod shaped, motile bacteria belonging to the family Enterobacteriaceae akin to the phylogenetically closely related Salmonella spp. [65]. The disease, enteric septicemia of channel catfish (ESC) caused by E. ictaluri, accounts for considerable economic loss to the catfish farming industry world wide [56]. Recent attempts to control ESC through prophylactic immunization have shown that live attenuated E. ictaluri vaccines are efficacious in protecting catfish fingerlings, fry, and eyed-eggs following immersion vaccination [42,43,63]. Several studies have alluded that protective immunity in channel catfish against E. ictaluri is largely mediated by a cellular immune response with humoral antibodies having a secondary function [9,39]. This observation is also supported by the fact that E. ictaluri [45,50], like Salmonella [47], could survive and replicate intracellularly and it is the cell mediated immune response that is primarily important in combating these infections.

Since innate immunity plays a crucial role in defense against invading pathogenic organisms in teleosts, cells of the monocytic macrophage lineage have drawn considerable interest in studies involving mechanisms of host–pathogen interactions in fish. Previous studies have shown that macrophages from channel catfish (Ictalurus punctatus) are able to efficiently phagocyte and
kill E. ictaluri [62] and macrophages from vaccinated fish are more efficient in killing E. ictaluri after opsonization with specific antibodies [38,40]. However, other studies reported that E. ictaluri are able to survive and replicate in catfish macrophages [4,6,44] and neutrophils [25,57]. Since these studies present conflicting results, we used E. ictaluri transformed with the DsRed fluorescent reporter gene to precisely determine several parameters (bacterial internalization and killing efficiency, reactive oxygen species and nitrogen oxide production) in macrophage-mediated phagocytosis of E. ictaluri in real-time, in vivo and in vitro interactions. Our studies were prompted by several studies where fluorescent protein-tagged bacteria have been used successfully to study the kinetics and killing efficiency of phagocytic cells in other systems [10,24,53] because, fluorescent protein production does not compromise bacterial entry or survival in cells and the reporter gene is passed on to bacteria progeny. Using red-fluorescent E. ictaluri, we examined channel catfish macrophage phagocytosis both in vitro and in vivo. The in vivo experiments were particularly designed to permit evaluation of macrophage–E. ictaluri interactions in the catfish internal milieu where various components of the immune system modulate the overall phagocytic process. In this study, we were also interested in understanding how vaccination and/or boosting, used to control the infection of E. ictaluri by assessment of the efficiency of macrophage phagocytosis and by observing the dispersion of fluorescent-tagged E. ictaluri in the body of the fish.

2. Materials and methods

2.1. Bacterial strain culture and transformation

The characteristics of the virulent E. ictaluri strain AL-93–58 used in this study were previously reported [28]. Bacteria were cultured in brain heart infusion (BHI) broth or agar at 27 °C. Gram stain and API 20E test strips (bioMerieux, Hazelwood, MO, USA) were used to confirm the purity of the bacterial cultures. E. ictaluri AL-93–58 were transformed with the pDsRed vector (Clontech, Mountain View, CA, USA) carrying the DsRed (Discosoma spp. coral fluorescent protein) reporter gene and an ampicillin resistance marker. A standard CaCl2 transformation protocol [31] was used with the following modifications: the heat-shock step was done at 35 °C and the E. ictaluri were cultured at 27 °C. pDsRed-transformed E. ictaluri were selected by culturing in SOB agar plates with 100 μg/ml ampicillin. Colonies that fluoresced red were selected. The transformed E. ictaluri were tested both with API 20E test strips and by PCR using E. ictaluri specific primers (indicated below). pDsRed-transformed E. ictaluri were mixed with 20% glycerol and stored at −80 °C. In experiments described below, the term red-fluorescent E. ictaluri will be used when referring to the pDsRed-transformed E. ictaluri AL-93–58 to distinguish it from the untransformed E. ictaluri parent.

2.2. Fish and vaccination protocol

Channel catfish (I. punctatus) were reared in 38-L flow-through tanks with constant aeration, a water temperature of 26 ± 2 °C and a 12:12 h light/dark photoperiod and acclimated for 2 weeks prior to experimentation. The average weight of the fish was 72.8 ± 17 g and length was 17.6 ± 0.5 cm. One-hundred and forty fish were immersed vaccinated with the modified-live E. ictaluri RE-33 [21,41] vaccine strain (~7 × 10⁸ CFU bacteria in 1 L water for 30 min). Fish were booster vaccinated similarly 3 weeks following the initial vaccination and from 4 weeks afterward, fish were used for the experiments. An equal number of non-vaccinated (control) fish were held in 1 L water without vaccine for 30 min (at day 0 and day 21). All subsequent experiments were conducted with fish obtained from the above pools of vaccinated and/or non-vaccinated fish.

2.3. Effect of opsonization on bacterial survival

Approximately 14 ml of sera collected from 10 vaccinated and 10 non-vaccinated fish were separately pooled. An aliquot of serum from vaccinated fish was heat-inactivated by incubation at 54 °C for 30 min. For opsonization, 1.7 × 10⁹ CFU/ml red-fluorescent E. ictaluri were incubated for 30 min at room temperature in serum from vaccinated or non-vaccinated fish diluted 1:1 with PBS as follows: (1) untreated bacteria, (2) bacteria opsonized with serum from vaccinated fish, (3) bacteria opsonized with heat-inactivated serum from vaccinated fish, and (4) bacteria opsonized with serum from non-vaccinated fish. Bacterial plate counts were made following serial 10-fold dilution in phosphate buffered saline solution (PBS) and growth on SOB agar with ampicillin.

2.4. Macrophage isolation and identification

Macrophages were isolated from catfish by peritoneal lavage according to the protocol described by Jenkins and Klesius [16]. Briefly, catfish were injected intraperitoneally with 150 μl of squalene (Sigma, St. Louis, MO, USA) and macrophages were harvested 4–5 days after treatment by peritoneal lavage with 20 ml of sterile ice-cold PBS. Harvested macrophages were centrifuged at 500 × g for 15 min, washed once more by centrifugation in 12 ml of ice-cold PBS, and resuspended in 5 ml of channel catfish macrophage medium (CCMM) prepared according to Booth et al. [6]. Antibiotics, gentamicin 100 μg/ml, streptomycin 100 μg/ml and penicillin 100 U/ml were added to the CCMM medium when required. Samples of harvested macrophages were stained with Hema-3 (Biochemical Sciences Inc., Swedesboro, NJ, USA) for total macrophage counts. Viability was assessed with trypan blue exclusion (Sigma) and observed microscopically using a hemacytometer.

2.5. Phagocytosis and killing assay in vitro

(Note: The term phagocytosis in this study refers to internalization of bacteria and distinguishes from the term killing). Macrophage collections from groups (vaccinated and non-vaccinated) of five catfish were pooled and aliquoted into 16-well fibronectin-coated, glass chamber slides at 1 × 10⁵ cells/well and incubated in CCMM without antibiotic for 2 h at 28 °C with 5% CO2 in a humidified chamber. Following incubation, the macrophages were treated with red-fluorescent E. ictaluri according to the following regimens: (1) bacteria not opsonized, (2) bacteria opsonized with serum from vaccinated catfish, (3) bacteria opsonized with heat-inactivated serum from vaccinated catfish, and (4) bacteria opsonized with serum from non-vaccinated catfish. The ratio of macrophage:bacteria in each chamber well was 1:20 or 1:100 and the slides were incubated in the humidified chamber for 1 h. Each treatment was conducted in triplicate. Following incubation, the medium was discarded and the wells gently washed with warm PBS and replenished with fresh CCMM with antibiotics. The slides were monitored at 4 h and thereafter at intervals of 24 h for a total period of 96 h with an inverted fluorescent microscope (Olympus 1X 70, Center Valley, PA, USA) equipped with a digital camera (Olympus DP11). A sample of macrophages was aspirated from each well with a sterile pipette at 4 h and thereafter at 24 h intervals up to 96 h. Aspirated macrophages were briefly treated with 2.5 g/L trypsin (Invitrogen Corp., Carlsbad, CA, USA) and 0.2 g/L versene (Invitrogen) to break cell aggregates, placed in a hemacytometer chamber and observed with...
a fluorescent microscope (Olympus BX 41) equipped with a digital camera (Olympus DP 70) at a 600× magnification. The number of macrophages with or without engulfed bacteria was counted. Data were recorded at each time point based on a total count of 200 macrophages for each treatment replicate.

2.6. Bacteria killing assay

Bacteria killing assay was done according to the procedure described by Booth et al. [6], with modifications. Macroorganisms from vaccinated and non-vaccinated fish were incubated for 48 h at 28 °C with red-fluorescent *E. ictaluri*, either (1) untreated or (2) opsonized with immune serum from vaccinated catfish, in a macrophage:bacteria ratio of 1:1 in a 96-well plate containing CCMM without antibiotics. After 1 h, extracellular bacteria were killed by incubation with CCMM containing 100 µg/ml of gentamicin, 100 µg/ml streptomycin, and 100 U/ml penicillin for 1 h. Macrophages were then cultured in the same medium containing 10 µg/ml gentamicin, 10 µg/ml streptomycin, and 10 U/ml penicillin. Macrophages contained in three wells were harvested and lysed with 1% Triton X-100 (Sigma, St. Louis, MO, USA) at 12 h and thereafter at intervals of 24 h up to a total of 48 h. The lysed macrophages were centrifuged at 5000 g for 5 min, washed twice with sterile PBS and plated on SOB agar with ampicillin. Plates were incubated overnight at 27 °C.

2.7. Reactive oxygen species production

The chemiluminescence assay was conducted according to the procedure described previously [22] with modifications. A subsample of macrophages obtained from the in vitro phagocytosis experiments was used. Briefly, macrophages were washed twice in ice-cold PBS, resuspended in Hank's balanced salt solution without phenol red, calcium chloride and magnesium sulphate (HBSS) (Gibco, Invitrogen Corp., Carlsbad, CA, USA) and seeded at 1 × 10⁵ cells/well in a 96-well opaque Lumimeter plate (Promega, Madison, WI, USA). After 30 min, (1) untreated *E. ictaluri*, (2) *E. ictaluri* opsonized with serum from vaccinated fish, (3) *E. ictaluri* opsonized with heat-inactivated serum from vaccinated fish, or (4) *E. ictaluri* opsonized with serum from non-vaccinated fish were added to each respective well to contain a macrophage:bacteria ratio of 1:1 in a volume of 20 µl/well. Controls included wells with macrophages stimulated with zymosan, HBSS alone, macrophages stimulated with opsonized bacteria, and macrophages stimulated with zymosan and HBSS alone. Chemiluminescence was measured with a luminometer (Turner Designs, Sunnyvale, CA) using the following cycling parameters: an initial denaturation step at 95 °C for 5 min, followed by incubation at 28 °C for 30 s. The signal was measured at 0, 15, 30 s intervals and the area under the curve was recorded for 10 s. The chemiluminescence was calculated as the ratio of the area under the curve for each treatment group.

2.8. Nitrogen oxide production

Nitric oxide production by macrophages was measured by the Griess reaction [32] with a commercial kit (Promega) according to the protocol provided. A subsample of macrophages harvested for the in vitro phagocytosis experiment was washed and resuspended in cold HBSS. Aliquots of 100 µl of the macrophage suspension adjusted to 1 × 10⁶ cells were transferred into three microcentrifuge tubes and 100 µl of *E. ictaluri* were added to each tube to provide a macrophage:bacteria ratio of 1:2.0. At intervals of 0.5, 1.2, 2.5, and 3 h following bacterial challenge, the reaction was stopped by placing the tube on ice and 50 µl of the macrophage/bacteria suspension transferred to a well of a 96-well flat-bottom plate. Each test was performed in triplicate. Sulfanilamide in a volume of 50 µl was added to each test well and to wells containing the nitrite reference standard provided in the test kit and run in parallel. The test plates were incubated at room temperature for 10 min in the dark. The Griess reagent [N-1-naphthylethylenediamine dihydrochloride (NED)] equilibrated to room temperature was added in a volume of 50 µl to each well and the plates incubated for 10 min in the dark. The colorimetric-reactions were measured with a Bio-Rad (BioRad, Hercules, CA, USA) microplate reader at an absorbance of 540 nm. The average absorbance per test sample for each time interval was determined and compared with the nitrite standard reference curve generated for each assay.

2.9. In vivo–in vitro killing assay

Twenty vaccinated (A) and 20 non-vaccinated fish (B) were injected intraperitoneally with 150 µl of squalene. Five days later five fish in each of group (A) and (B) were injected intraperitoneally with 5 × 10⁸ CFU of red-fluorescent *E. ictaluri* pretreated as follows: (1) *E. ictaluri* non-opsonized, (2) *E. ictaluri* opsonized with serum from vaccinated catfish, (3) *E. ictaluri* opsonized with heat-inactivated serum from vaccinated catfish, and (4) *E. ictaluri* opsonized with serum from non-vaccinated catfish. Macroorganisms were harvested 6 h following injection and placed in 16-well glass chamber slides at 1 × 10⁵ cells/well and incubated in CCMM with antibiotics at 28 °C for up to 6 days. The counts of macroorganisms with internalized bacteria were made as previously described for the in vitro phagocytosis assay. The experiment was repeated two times. Only the efficiency of macroorganisms in killing *E. ictaluri* was calculated in this experiment. The efficiency of internalization of *E. ictaluri* by macroorganisms (that is, the percentage of macroorganisms with bacteria that were inoculated in the wells) was not compared among treatments due to potentially interfering processes, such as the ability of bacteria to move from the peritoneal cavity to other organs, difference in rates of bacterial internalization and killing efficiency by macroorganisms, and the traffic of macroorganisms between the peritoneal cavity and other organs, that happened during the 6 h period between the injection of *E. ictaluri* in the fish and the harvest of macroorganisms.

2.10. Localization of *E. ictaluri* in fish

Vaccinated and non-vaccinated catfish were injected intraperitoneally with ~5 × 10⁸ CFU of red-fluorescent *E. ictaluri* pretreated as follows: (1) non-opsonized bacteria or (2) bacteria opsonized with serum from vaccinated catfish. Nine vaccinated and non-vaccinated fish were used for each treatment regimen. Three fish from each respective treatment were picked at random at intervals of 12, 24 and 48 h and sampled. Fish were euthanized by immersion in 300 mg/L of tricaine methanesulfonate (Western Chemical, Ferndale, WA, USA) and samples were taken from gills, skin, fins, muscle, anterior and posterior kidney, spleen, liver, stomach, intestine, heart and brain. Thin sections of tissues were prepared and examined by fluorescence microscopy and processed for histological examination (Department of Pathobiology, College of Veterinary Medicine, Auburn University). Thin sections of identical tissues were prepared and stained with hematoxylin and eosin. Random samples from five fish were examined by bacteriological culture and select colonies used for examination by PCR amplification using *E. ictaluri* specific primers (forward F1: TTACGTGCAAYATHGAYGG and reverse R3: ACTTCIGTIGCGTART-CIGCRTCYTG). PCR was done in a final volume of 50 µl with 25 µl of 1× Taq PCR Master Mix (QIAGEN, Valencia, CA), 1 µl (50 µM) of each primer, one bacterial colony, and 23 µl of nanopure water. A negative control without template was included with each PCR. Amplification was performed in a TGradient thermocycler (Whatman Biometra, Göttingen, Germany) using the following cycling parameters: an initial denaturation step at 95 °C for 5 min, followed by...
by 10 cycles at 94°C for 30 s, annealing at 60–50°C (decreasing 1°C/cycle) for 30 s, and extension at 72°C for 1 min. Another 22 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min.

2.11. Trojan horse experiment

This experiment was performed to determine whether red-fluorescent *E. ictaluri* surviving within macrophages following in vitro phagocytosis could subsequently establish an infection in vivo when the same macrophages were injected into the cognate fish. Five non-vaccinated catfish were used in this experiment and from 1.3 to 3.7 × 10⁶ macrophages were harvested from each fish. Red-fluorescent *E. ictaluri* were initially opsonized with serum collected from vaccinated catfish and introduced into the corresponding wells of a 16-well glass chamber slide seeded with macrophages from each respective fish used in this experiment. The bacteria to macrophage ratio was maintained at 1:10 and the mixture of bacteria/macrophage incubated at 28°C for 1 h. The media in the wells were replenished with fresh CCMM with antibiotics and incubated for 1 h to kill any unphagocytosed bacteria. The red-fluorescent *E. ictaluri* infected-macrophages were harvested and washed twice in PBS and each respective macrophage preparation was injected intraperitoneally into the cognate fish. The percentage of injected macrophages with internalized *E. ictaluri* was 7.2 ± 2.3 (approximately 2.5 × 10⁶ macrophages). A section of gill tissue was excised from each fish (after anesthetizing with tricaine methanesulfonate) at intervals of 24 h following injection and examined with the fluorescent microscope for presence of red-fluorescent *E. ictaluri*. One fish was euthanized on day 3 post-injection and sampled as described above. Thereafter, remaining fish were sampled at daily intervals until the last fish was euthanized and sampled on day 7 post-injection.

2.12. Statistical analysis

A one- and two-way ANOVA and Tukey's post hoc test, run with the statistical package for social sciences (SPSS-12.0) program (SPSS Inc., Chicago, IL, USA), were used for analysis of data obtained from the in vitro and in vivo macrophage-mediated bacterial phagocytosis assays and for the ROS and nitric oxide production assays (on the highest data-points obtained). A one-way ANOVA and a Tukey's post hoc test were run separately for macrophages from vaccinated and non-vaccinated fish to determine the effect of the four bacteria treatments. A two-way ANOVA was run on cumulative data (macrophages from vaccinated fish + macrophages from non-vaccinated fish) to determine differences between source of macrophages (vaccinated and non-vaccinated fish), treatment of *E. ictaluri* effect and macrophage source by *E. ictaluri* treatment interaction was present. An arcsin square root transformation was performed on data expressed as percentages or as ratios. A p-value ≤ 0.05 was considered statistically significant.

Because the percentage of macrophages with bacteria was different among treatments at the beginning of the experiments, the relative percent killing (RPK) was calculated to compare the efficiency of bacterial killing by macrophages among treatments at the end of each phagocytic assay using the following formula:

\[
\text{RPK} = \frac{\text{MbBe}(\%) - \text{MbEe}(\%)}{\text{MbBe}(\%)}
\]

where MbBe = the percent of macrophages with bacteria at beginning of experiment, MbEe = the percent of macrophages with bacteria at end of experiment. An ANOVA and Tukey's test were run on the calculated RPK for replicate treatments and a negative RPK was assigned a value of 0.0001 for the purpose of statistical analysis.

3. Results

3.1. Phagocytosis and killing assay in vitro

Opsonization of *E. ictaluri* with serum from vaccinated fish significantly (p < 0.05) increased the phagocytosis by macrophages from both vaccinated and non-vaccinated fish when exposed at a macrophage:bacteria ratio of 1:20 or 1:100 (Table 1). Opsonization of *E. ictaluri* with heat-inactivated serum from vaccinated fish increased the phagocytosis efficiency, but this increase was not always significantly different compared to non-opsonized *E. ictaluri*, or *E. ictaluri* opsonized with normal serum. There was no significant difference between *E. ictaluri* opsonized with normal serum and non-opsonized *E. ictaluri*. The results of the two-way ANOVA showed that there was a significant (p = 0.01) difference in the phagocytosis efficiency between macrophages from vaccinated and non-vaccinated fish (Table 1). The phagocytosis efficiency significantly (p < 0.05) increased when the macrophage:bacteria ratio was increased from 1:20 to 1:100. When macrophages were exposed to bacteria at a ratio of 1:20 or 1:100, macrophages from vaccinated fish were significantly (p < 0.05) more efficient in killing bacteria compared to macrophages from non-vaccinated fish, as evidenced by a decrease in percentage of bacteria within macrophages from 0 to 48 h (Table 1). Macrophages from both vaccinated and non-vaccinated fish when mixed in a macrophage:bacteria ratio of 1:100, showed a significant (p < 0.05) decrease in killing efficiency compared to a ratio of 1:20 (Table 1). Based on the results of the two-way ANOVA, the source of macrophages and the interaction between macrophage source and treatment of *E. ictaluri* significantly influenced bacterial activity with macrophages from vaccinated fish when the macrophage:bacteria ratio was 1:100. Treatment of the bacteria with serum alone did not affect bacterial survival (data not shown). Lysed macrophages from non-vaccinated fish were seen from 36 h post-exposure to bacteria and the number of lysed cells increased with time (data not shown). Motile, red-fluorescent *E. ictaluri* were initially observed only around lysed macrophages, suggesting that these bacteria were released from the lysed macrophages. No lysed macrophages were observed among cells from vaccinated catfish.

3.2. Bacterial killing assay

Red-fluorescent *E. ictaluri*, either untreated or opsonized with serum from vaccinated fish, were able to replicate in macrophages from non-vaccinated fish as revealed by bacterial plate counts (only the bacterial counts with opsonized *E. ictaluri* are reported in Fig. 1, the trends in bacterial counts with untreated *E. ictaluri* were similar but the value of CFU/ml was lower, data not shown) and by the increase in intensity of red fluorescence of *E. ictaluri* within macrophages when observed by fluorescence microscopy. Red-fluorescent *E. ictaluri*, either untreated or opsonized with serum from vaccinated fish, were unable to replicate within macrophages from vaccinated fish, and the bacterial numbers in macrophages decreased with time as observed by plate counts (Fig. 1).

3.3. Reactive oxygen species production

The production of reactive oxygen species (ROS) by catfish macrophages from vaccinated and non-vaccinated catfish exposed to *E. ictaluri* treated under different regimens is presented in Fig. 2. Macrophages from vaccinated and non-vaccinated fish produced significantly (p < 0.05) more ROS when they phagocytized *E. ictaluri*.
compared with macrophages alone (i.e., with no bacteria). Macrophages from vaccinated fish produced significantly (p < 0.05) higher amounts of ROS compared to macrophages from normal fish. In macrophages from vaccinated fish, ROS production was significantly (p < 0.05) higher in cells exposed to opsonized E. ictaluri with vaccinated serum compared to the other three treatments. The production of ROS by macrophages from non-vaccinated fish was significantly (p < 0.05) different when compared between macrophages that phagocytized E. ictaluri opsonized with vaccinated serum and non-opsonized bacteria.

3.4. Nitric oxide production

The production of nitric oxide by macrophages from vaccinated and non-vaccinated catfish exposed to E. ictaluri treated under different regimens is presented in Table 2. Macrophages from both vaccinated and non-vaccinated fish released significantly (p < 0.05) more nitric oxide when exposed to bacteria compared to macrophages without bacteria. Macrophages from vaccinated fish produced significantly (p < 0.05) more nitric oxide than from non-vaccinated fish in all treatment regimens except when bacteria were opsonized with vaccinated serum. Macrophages from both vaccinated and non-vaccinated fish generated significantly (p < 0.05) higher levels of nitric oxide when exposed to bacteria opsonized with serum compared to bacteria opsonized with serum from non-vaccinated fish, and NoS = bacteria opsonized with no serum (only PBS).

3.5. In vivo–in vitro killing assay

The relative efficiency of the macrophages to kill E. ictaluri following exposure to bacteria in vivo in both vaccinated and non-vaccinated fish is presented in Table 3. Macrophages from non-vaccinated fish were not efficient at killing E. ictaluri regardless of pre-treatment of bacteria (i.e., opsonization or not) (Table 3). Lysed macrophage from non-vaccinated fish and free bacteria was observed after 36 h following incubation of the lavaged macrophages seeded in 16-well chamber slides. Macrophages from vaccinated fish were not efficient at killing E. ictaluri at 48 h. The percentage of macrophages from vaccinated fish with E. ictaluri was less than 1% at 96 h post-inoculation (data not shown).

3.6. Localization of E. ictaluri in fish

Fluorescent bacteria were present in all organs, except the brain tissue, in non-vaccinated fish injected with non-opsonized red-fluorescent E. ictaluri (Fig. 3a–f). Bacteria isolated from these
organs were positively identified as *E. ictaluri* by culture and confirmed by PCR. The greatest amount of bacteria (highest fluorescence intensity) was observed in the head (a) and trunk (b) kidneys, spleen (c) and gills (d). Liver (e), intestine (f), and muscle proximal to the peritoneal cavity (not shown) presented fewer bacteria (lower fluorescence intensity). Non-vaccinated fish injected with red-fluorescent *E. ictaluri* opsonized with serum from vaccinated fish (pictures not shown) presented similar level of fluorescence in all organs compared to non-vaccinated fish injected with non-opsonized bacteria. Vaccinated fish injected with non-opsonized red-fluorescent *E. ictaluri* (Fig. 3g–l) or with bacteria opsonized with immune serum (pictures not shown) revealed the lowest level of fluorescence in all organs with practically no fluorescence observed in gills, liver and intestine.

### 3.7. Trojan horse experiment

Fish injected with macrophages containing fluorescent *E. ictaluri* did not show any red-fluorescent bacteria in the gill tissues for the first 2 days post-exposure. Samples of gills examined on day 3 and thereafter showed a fluorescence signal indicating the presence of red-fluorescent bacteria and the fluorescence intensity increased with time until the experiment was terminated. Fish examined microscopically revealed the presence of red-fluorescent *E. ictaluri* (pictures not shown) with an intensity similar to that observed in the organs of non-vaccinated fish from the previous experiment (localization of *E. ictaluri* in fish – see Fig. 3a–f).

### 4. Discussion

From an evolutionary context, innate immunity plays a crucial role in the defense against microbial pathogens in teleost fish due to lack of affinity maturation of teleost antibody response [5] and slow maturity of the adaptive immune parameters in many fish species [23,29]. A wealth of evidence supports that phagocytic cells of the monocytic–macrophage lineage play a dominant role in defense against invading pathogenic bacteria in fish [27,36]. In this study we used *E. ictaluri* transformed with the red-fluorescent
protein reporter gene to examine mechanisms of catfish macrophage-mediated phagocytosis both in vitro and in vivo. Use of a traceable genetic marker has an advantage in that the reporter gene is transferred to the progeny during bacterial replication and the route and fate of *E. ictaluri* could be precisely tracked. Additionally, the surface chemistry of the bacterium remains unaltered.

Our studies clearly showed that *E. ictaluri* are able to survive and replicate within catfish macrophages and can result in macrophage lysis. Macrophages from vaccinated fish were more efficient in phagocytosis and killing of *E. ictaluri* compared to macrophages from non-vaccinated fish, reflecting that other components of the immune system may enhance the macrophage killing efficiency, as observed in the in vivo studies. Additionally, opsonization of *E. ictaluri* with serum from vaccinated fish appeared to augments the phagocytosis by macrophages from both vaccinated and non-vaccinated fish. Previously, Sheldon and Blazer [38], with catfish vaccinated with formalin killed *E. ictaluri*, and Shoemaker et al. [40], using a low dose of live *E. ictaluri* for vaccination, showed that macrophages from vaccinated fish were substantially more efficient in killing *E. ictaluri*. Shoemaker et al. [40] also suggested that opsonization of *E. ictaluri* by serum from immunized fish increased killing by macrophages from vaccinated fish compared to macrophages from non-immunized fish. In our study, we observed a significantly higher killing efficiency in macrophages from vaccinated fish. However, when the macrophage to bacteria ratio was increased to 1:100, there was a significant decrease in killing efficiency regardless of macrophage source. This may be due to the fact that the phagocytized bacteria overwhelmed the effects of lethal components such as the respiratory burst products of macrophages or mitigated the production of potent effectors necessary for killing upon reaching a threshold [52]. Bacteria that escaped the lethal effects of macrophage phagocytosis were able to survive and replicate within macrophages and they were able to cause macrophage lysis, as evidenced in both our in vitro and in vivo studies. In in vitro studies with macrophages derived from head kidney of catfish, Booth et al. [6], observed that *E. ictaluri* numbers increased from two-fold at 4 h to seven-fold at 12 h post-exposure and that opsonization of *E. ictaluri* with normal serum resulted in significantly (p < 0.05) greater internalization compared with *E. ictaluri* pretreated with heat-inactivated serum or no serum. Results of that [6] study prompted them to suggest that *E. ictaluri* may possess a surface ligand capable of recognizing a specific receptor on the macrophage. Other studies have shown that sera from normal catfish are able to opsonize *E. ictaluri* and that opsonized *E. ictaluri* are efficiently internalized by catfish neutrophils [18]. This observation is borne-out by the demonstration of putative receptors on catfish phagocytic–monocytes for components of the complement pathway in catfish serum [15]. Booth et al. [6] also observed lysis of macrophages and release of free *E. ictaluri* 12 h after challenge for cells harboring more than 30 bacteria. At the same time they observed macrophages with only 1–6 bacteria indicating that *E. ictaluri* did not replicate equally, or the efficiency in killing or controlling the replication of *E. ictaluri* was different among macrophages, as also observed in this study. It is well known that Salmonella spp., phylogenetically closely related to *E. ictaluri*, can replicate in macrophages and cause cell apoptosis or necrosis [14,17,30], and that cellular necrosis might facilitate dispersion of the bacteria.

### Table 2

<table>
<thead>
<tr>
<th>Source of macrophages</th>
<th>E. ictaluri Treatment</th>
<th>Nitrite (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated fish</td>
<td>SVF</td>
<td>10.00 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>HISVF</td>
<td>9.83 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>SNVF</td>
<td>8.62 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>NoS</td>
<td>6.11 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>MaO</td>
<td>0.85 ± 0.16</td>
</tr>
</tbody>
</table>

Level of significance with one-way ANOVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>Source of macrophages</th>
<th>Treatment of <em>E. ictaluri</em></th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
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<tr>
<td></td>
<td>p &lt; 0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Superscripts represent significant difference at *p* < 0.05 within treatments (within macrophages from vaccinated and non-vaccinated fish). A two-way ANOVA was run on cumulative data (macrophages from vaccinated fish = macrophages from non-vaccinated fish) to determine differences between source of macrophages (vaccinated and non-vaccinated fish), treatment of *E. ictaluri* effect and macrophage source by *E. ictaluri* treatment interaction.

### Table 3

<table>
<thead>
<tr>
<th>Source of macrophages</th>
<th>E. ictaluri Treatment</th>
<th>In vivo–in vitro (%)</th>
<th>RPK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated fish</td>
<td>SVF</td>
<td>28.2 ± 4.9</td>
<td>11.0 ± 2.8</td>
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<tr>
<td></td>
<td>HISVF</td>
<td>15.1 ± 5.4</td>
<td>10.9 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>SNVF</td>
<td>3.1 ± 1.8</td>
<td>0.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>NoS</td>
<td>7.0 ± 2.9</td>
<td>1.0 ± 0.7</td>
</tr>
</tbody>
</table>

Level of significance with one-way ANOVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>Source of macrophages</th>
<th>Treatment of <em>E. ictaluri</em></th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p &lt; 0.001</td>
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Superscripts represent significant difference at *p* < 0.05 within treatments (within macrophages from vaccinated and non-vaccinated fish). A two-way ANOVA was run on cumulative data (macrophages from vaccinated fish = macrophages from non-vaccinated fish) to determine differences between source of macrophages (vaccinated and non-vaccinated fish), treatment of *E. ictaluri* effect and macrophage source by *E. ictaluri* treatment interaction.

Abbreviations: SVF = bacteria opsonized with serum from vaccinated fish, HISVF = bacteria opsonized with heat-inactivated serum from vaccinated fish, SNVF = bacteria opsonized with serum from non-vaccinated fish, NoS = bacteria opsonized with no serum (only PBS), and MaO = macrophages only. The maximum level of nitrite was recorded 3 h from the beginning of the experiment.
Following bacterial engulfment by phagocytic cells, resulting events include encounter of pathogens with phagosomal enzymes and other factors aimed at killing the internalized microbes. Among the well characterized phagosomal lethal mechanisms include the reactive oxygen species (ROS) [36,26], the nitrogen intermediates such as nitric oxide [18,51] and other toxic molecules [52]. In this study we observed that macrophages from vaccinated catfish produced higher amount of ROS and nitric oxide compared to macrophages from non-vaccinated fish. ROS and nitric oxide production were also significantly higher when *E. ictaluri* were opsonized with serum from vaccinated fish compared to other treatment regimens. Similar results were observed in the Griess assay for nitric oxide production. Taken together, these results indicate that catfish macrophages are able to produce both ROS and nitric oxide as it has been observed in previous studies [33,34], but *E. ictaluri* are able to survive inside “naïve” macrophages even in the presence of ROS and nitric oxide. Similar results have been documented for *Salmonella* spp. Several studies demonstrated a positive correlation between the amounts of ROS produced by macrophages and the killing efficiency of *Salmonella* spp. by macrophages, even though the same studies showed that *Salmonella* spp. could still survive in presence of ROS [30,60,61]. It has been proposed that macrophages are involved in the dispersion of *Salmonella* spp. in the body and in the maintenance of the disease [30,55,59] and macrophages are also considered a possible reservoir of *Salmonella* spp. in carrier animals [59]. The results of the Trojan horse experiment indicate that catfish macrophages might also be implicated in the

![Representative views of head kidney (a and g), trunk kidney (b and h), spleen (c and i), gills (d and j), liver (e and k) and intestine (f and l) at 40x magnification from non-vaccinated and vaccinated fish 12 h post-intraperitoneal injection of approximately 5 x 10^8 CFU of non-opsonized red-fluorescent *E. ictaluri*. Pictures from a to f were taken from non-vaccinated fish and pictures from g to l were taken from vaccinated fish. Scale bars represent 2.4 mm.](image-url)
dispersion of *E. ictaluri* in the body and in establishment of the disease. Thune et al. [49] in their study also hypothesized that *E. ictaluri* might spread via migrating macrophages.

In non-vaccinated fish challenged with *E. ictaluri*, bacteria were seen in all organs, with the exception of the brain. Gills, spleen, anterior and posterior kidney presented the higher amount of bacteria, seen as level of fluorescence, and lower bacteria densities were seen in the intestine and liver. The high amount of bacteria observed in the gills indicated the presence of free and dividing bacteria in the blood, as also observed in the Trojan horse experiment where no bacteria were seen in the gills at the beginning of the experiment, but an increased number of bacteria (fluorescence) was seen as early as 3 days after challenge. Vaccinated fish showed a low level of fluorescence in all organs compared to non-vaccinated fish, and almost no fluorescence in the gills, indicating few circulating bacteria. The high amount of bacteria observed in the spleen, anterior and posterior kidney is related to the immune and blood filtering functions of these organs. Similar observations were reported in the study of Karsi et al. [19] where bioluminescent *E. ictaluri* were intracoelomically injected in fish. Using bioluminescent *E. ictaluri* (tagged with the luciferase gene), Karsi et al. [19], demonstrated in a time-course study of catfish exposed by intra-peritoneal injection and by bath immersion, the progressive distribution of *E. ictaluri* by bioluminescent imaging. In their study using non-vaccinated fish [19], they reported that bioluminescent bacteria spread to the whole body within 60–72 h post-infection and intense bioluminescence was found in the anterior and posterior kidney and the spleen.

Several studies with different fish species have shown that bacteria are able to survive within macrophages. For example, *Aeromonas hydrophila* in tilapia (*Oreochromis aureus*) macrophages [22], *Renibacterium salmoninarum* in rainbow trout (*Oncorhynchus mykiss*) macrophages [11], *Photobacterium damselae* subsp. piscicida in macrophages of the gill-head sea bream (*Sparus aurata*) [3], *Vibrio anguillarum* in macrophages of the sea bass (*Dicentrarchus labrax*) [37] and *Vibrio vulniﬁcus* biotype 2 in the macrophages from the European eel (*Anguilla anguilla*) [54]. Mechanisms by which various bacteria subvert or mitigate the bactericidal activity of macrophages are vividly reviewed by Scott et al. [35]. In oncoposin virulent *Edwardsiella tarda*, a closely related species to *E. ictaluri*, it has been found that enzymes including catalase, peroxidase and superoxide dismutase are able to impede the stimulation of ROS in macrophages [12,46]. To our knowledge, no such enzyme systems have been found in *E. ictaluri*. The substantial reduction of red-fluorescent *E. ictaluri* observed in the vaccinated catfish in this study, may have been contributed by the stimulation of a host of defensive immune factors such as cytokines [64], chemokines [7], complement [13] and other components of the immune system present in the internal milieu [29,58].

Overall our studies demonstrated that macrophages from vaccinated fish are efficient in killing *E. ictaluri*. All parameters examined, such as ROS production, nitric oxide production, and in vitro and in vivo killing efficiency, reflected that vaccinated fish have an augmented immune response compared to non-vaccinated fish. These results are in agreement with previous studies that demonstrated that passive immunization did not protect catfish against *E. ictaluri* [20,48,49] and supports the importance of macrophage-mediated immunity against *E. ictaluri* [2,38,40].

Acknowledgements

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Note: Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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