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Short communication

Immunomodulatory properties of *Concholepas concholepas* hemocyanin against francisellosis in a zebrafish model

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ABSTRACT

The development of vaccines for aquaculture has been an important milestone in providing a continuous and sustainable production. Most of the vaccines currently on the market for aquaculture include oil as adjuvant. Nevertheless, several studies reported an occurrence of side effects after their use in farmed fish. As a result, there is a need for new and improved adjuvants that can stimulate the immune system while causing as few side-effects as possible. Hemocyanins are versatile macromolecules with strong immunogenic and immunomodulatory properties. Due to these characteristics, hemocyanin from *Concholepas concholepas* (CCH) has been biochemically characterized and evaluated as vaccine adjuvant in mice and humans. Francisellosis is a chronic granulomatous disease, which can result in high mortality depending on the host. The disease is caused by the facultative intracellular Gram-negative bacteria *Francisella noatunensis*, which remains an unsolved problem for the aquaculture, as no efficient vaccines are available. The aim of the present work was to investigate the immunoregulatory properties of CCH against francisellosis in an experimental zebrafish model. When immunized with CCH, zebrafish were protected from subsequent challenge with a lethal dose of *Francisella noatunensis* subsp. *orientalis*. Subsequently the mRNA expression levels of several immune-related genes were studied, including *mhcii, il12a, tnfα* and *ifng1-1*. Taken together, the data report the immunoregulatory properties of CCH and its potential use as a vaccine adjuvant for aquaculture.

1. Introduction

The majority of fish vaccines currently on the market consist of formulations of inactivated bacteria or viruses in combination with mineral oil as an adjuvant. Adjuvants are a highly heterogeneous group of compounds with one thing in common: the ability to enhance the immune response. There are several adjuvants available, which are based on mineral oil emulsions, products from bacteria, endotoxins, paraffinic or vegetables oils [1]. Unfortunately, several studies report side effects after their use in farmed fish including inflammation, granulomas, pigmentation at the site of infection and connective tissue in internal organs [2]. Thus, there is a need for new and improved adjuvants that can stimulate the immune system in order to prevent diseases, while causing fewer or no side-effects. Hemocyanins are large multi-subunit oxygen carrier glycoproteins freely dissolved in the hemolymph of numerous arthropods and mollusks. These are versatile macromolecules with strong immunogenic and immunomodulatory properties [3,4]. The hemocyanin isolated from the gastropod *Megathura crenulata*, KLH is the most studied due to its immunomodulatory properties and has been used as a protein carrier, conjugated to haptens, as well as for tumor-associated antigens [5]. However, the bioavailability of KLH is limited, which has prompted the interest in finding new candidates with similar immunological properties. Therefore, hemocyanins from *Concholepas concholepas (CCH)* [3] and *Fissurella latimarginata* (FLH) [6], among others [7–9], have been biochemically characterized and evaluated according to their immunomodulatory properties, presenting hemocyanins as an interesting alternative to oil adjuvants.

The evaluation of new adjuvants with enhanced immunogenic properties may improve the vaccine development against intracellular pathogens. Intracellular pathogens represent today an

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emerging threat to the aquaculture, as both, innate and adaptive immune responses are needed for sufficient protection. In contrast, infections caused by extracellular pathogens are mainly controlled by the use of vaccines inducing the production of antibodies in combination with cytotoxic T-cells [10]. Intracellular pathogens, such as *Francisella*, have been shown to cause a severe economic impact in aquaculture due to the lack of vaccines [11]. Francisellosis is a chronic granulomatous disease caused by the facultative intracellular bacteria *Francisella noatunensis (Fn),* which can result in high mortality depending on the host [12]. *Francisella noatunensis* consists of two subspecies, which appear to be adapted to different host temperatures: *F. noatunensis* subsp. *orientalis* (*Fno*) causes disease in "warm-water" fish, like tilapia [13], and *F. noatunensis* subsp. *noatunensis* (*Fnn*) which causes disease in fish living in colder waters, like salmon and cod [12]. In general, the disease is characterized by the formation of multifocal white nodules in spleen, kidney and other organs [14]. The bacterium is not only resistant to innate immunity components (complement), but is also able to penetrate, replicate, and survive in tilapia and Atlantic cod head kidney-derived macrophages [14,15]. Currently, there is no efficient treatment or vaccine available against fish francisellosis, but attempts using attenuated strains of *Fn* created by mutation have provided promising results in tilapia [16] and zebrafish [17].

Zebrafish (*Danio rerio*) has for decades been the ectothermic vertebrate used for genetic dissection, vertebrate development and now is becoming a choice model for the study of a range of diseases including cancer and infectious disease [18,19]. A *Francisella* zebrafish infection model has previously been established, showing that zebrafish infected with *Francisella* sp. undergo an acute disease process and succumb to infections in a dose-dependent manner [20,21]. The aim of the present work was therefore to investigate the immunomodulatory properties of CCH against francisellosis in an experimental zebrafish model.

2. Materials and methods

2.1. Hemocyanin

Soluble *Concholepas* hemocyanin in PBS (0.1 M sodium phosphate, 0.15 M NaCl [pH 7.2]), obtained under sterile and pyrogen-free conditions was provided by Biosonda Company (Santiago, Chile).

2.2. Strains, media and labeling

Francisella noatunensis subsp. *orientalis* 07–285 A, isolated from diseased tilapia *Oreochromis niloticus* in Costa Rica, was cultivated at 27 °C as previously described [21]. The number of colony forming units (CFU) for each experiment was estimated by plating 10 μL from a 10-fold serial dilution of the bacterial suspensions onto Eugon Chocolate Agar (ECA) plates [22].

2.3. Francisella infections of adult zebrafish

The immunization and infection of adult zebrafish was performed as reported by Lagos et al. [21]. Six experimental tanks of 15 fish each, three tanks per group (45 fish), were anesthetized by immersion in water containing 100 mg/mL Tricaine methanesulfonate (MS-222, Sigma-Aldrich) and immunized once with either 5 μg CCH or PBS by intraperitoneal injection (i.p.) (15 μl of suspension). After 21 days, fish were challenged by i.p. injection with an acute dose of 1×10^6 CFU *Fno*. The fish were closely monitored and mortality recorded twice a day. All zebrafish experiments were approved by NARA (the Norwegian Animal Research Authority). Waste water was decontaminated by chlorination and tested for sterility before disposal.

2.4. RNA isolation and quantitative real-time PCR

Kidney and spleen were collected at 1, 7 and 21 days after immunization (dpi), as well as 1 and 7 days after challenge (dpc). The qPCR experiments were conducted in duplicate, each sampling point consisting of 6 fish per group. The qPCR conditions were as previously described [19]. Primer sets are listed in Table 1S. The genes *zgc:158463 (18S)* and *eef1a1l1 (elongation factor-1 alpha*) were used as reference genes for normalization of the relative transcription levels. The normalized immune response data of CCH injected fish were standardized against the transcription levels of PBS injected fish for each time point. Relative expression levels were calculated using the Pfaffl method [23], with efficacy correction for each primer.

2.5. Dissection and histological sample preparation

Anesthetized fish were euthanized and dissected under a light microscopy. Briefly, the skin was cut with a scalpel from the anal fin along the belly of the fish to the operculum. Once open, the absence of adhesion or connective tissue in internal organs (intestine, spleen and liver) was verified. For qPCR analysis, spleen and kidney were collected and maintained in RNAlater until processing. For histological preparation, whole fish were fixed in formalin solution. The fish were embedded and stained (hematoxylin and Schiff's reagent), as previously described [17]. Imaging analysis was performed using a Nikon eclipseTE300 microscope and a Leica DFC320 camera. Images were acquired using LAS version 3.6.

2.6. Statistical analysis

Data (mean ± SD) were analyzed (Prism 6.0; GraphPad Software Inc.) using unpaired, two-tailed *t*-tests for comparisons between 2 groups, and one-way ANOVA with Turkey's multiple comparisons method ($^*p < 0.03$, $^{**}p < 0.001$, $^{***}p < 0.001$). Kaplan Meier survival curves were used to analyze percent for survival, and the statistical significance of differences between groups were $^{***}p$ value < 0.001 using Gehan-Breslow-Wilcoxon test and Log-rank test.

3. Results

3.1. CCH protect adult zebrafish challenged with an acute dose of Francisella noatunensis subsp. orientalis

Of the 45 fish immunized with CCH, no fish died post immunization and no evidence of discomfort due to injection was observed in any fish. Four weeks post injection, both immunized and PBS control fish were challenged with a dose of 1×10^6 CFU *Fno*. Fish immunized with CCH displayed a significantly reduced mortality compared to the control group (Fig. 1A), in which most mortalities occurred between 2 and 7 dpc. The control group infected with *Fno* showed signs characteristic of francisellosis, such as loss of appetite, lethargy and reduced swimming. The mortality in the control group increased rapidly during the first week, with only a 20% survival at 7 dpc, in contrast to a 62% survival rate in the CCH immunized group. Francisellosis is characterized by the formation of multifocal white nodules mainly in spleen and kidney. Therefore, these organs were investigated by histologic examination. Histological examination of the spleens isolated from the control group at 7 dpc, showed the formation of granuloma-like structures containing small coccoid bacteria. On average 4 to 5 granulomas were observed in the spleen of each fish, with a

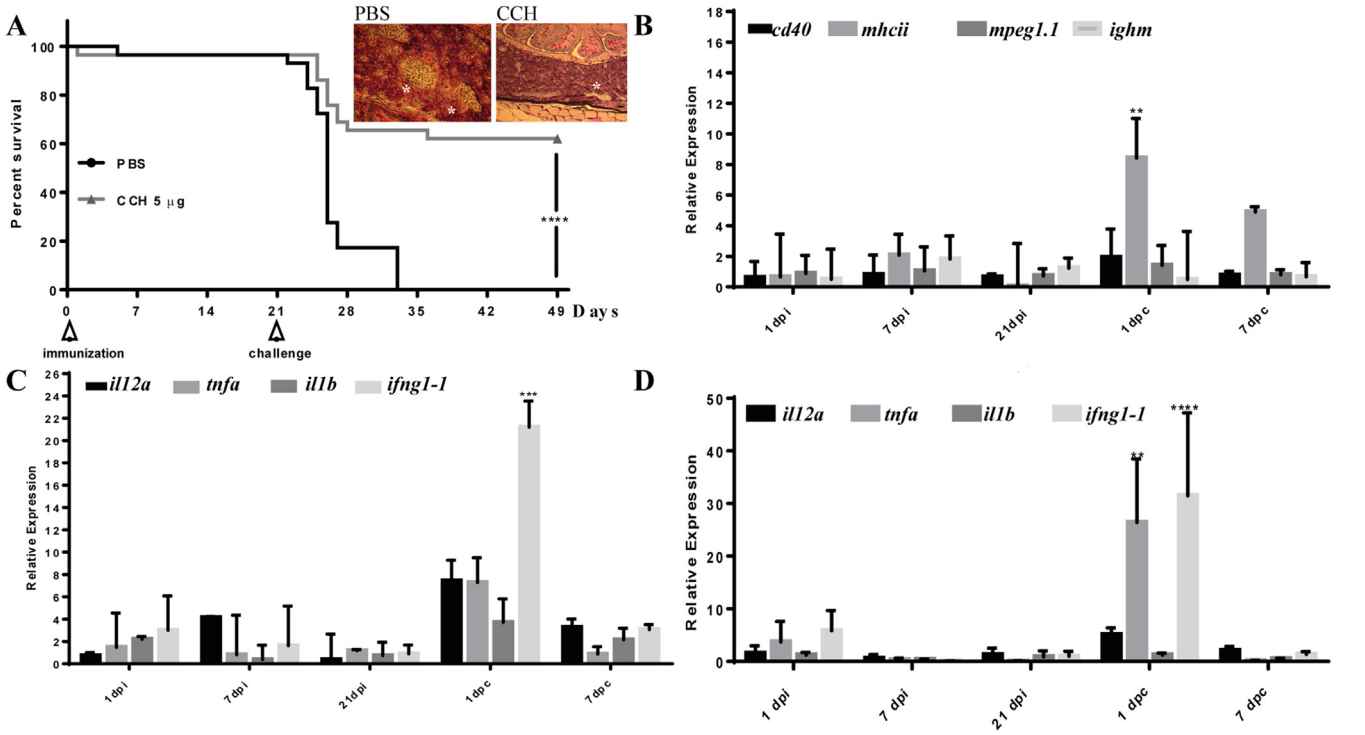

Fig. 1. Cumulative survival and immune response of adult zebrafish immunized with CCH and challenge with *Francisella noatunensis* subsp. *orientalis*. **A.** Kaplan-Meier representation of cumulative survival of adult zebrafish immunized with a dose of 5 µg CCH or PBS by i.p. injection, and challenged with 10^6 CFU *Fno*, *p-value* 0,0001 (Long-rank test). The insert show histological examination by hematoxylin-eosin (HE) staining of spleen from PBS injected (PBS) and CCH immunized (CCH) fish challenged with *Fno* at 7 dpc. Asterisk indicate granulomatous structures. **B.** Transcriptional response of cell markers such as *cd40, mhcii, mpeg1.1* and *ighm* were analyzed at 1, 7 and 21 days post immunization (dpi) and at 1 and 7 days post challenge (dpc) in kidney of adult zebrafish. **C, D.** The cytokines such *il12a, tnfa, il1b* and *ifng1-1* were analyzed in kidney (**C**) and spleen (**D**) from immunized adult zebrafish at 1, 7 and 21 dpi and at 1 and 7 dpc. Bars represent the mean ± SD relative expression levels compared to the control (PBS-injected). Relative expression was normalized to the expression of *eef1a1l1*. Asterisk indicates significant upregulation (**** < 0,0001; ***<0,001; ** < 0,01).

diameter from 50 to 200 µm. Some encapsulated granulomas were also observed in immunized fish, however, the response seemed milder and the granulomas were smaller and more organized (between 1 and 2 with a diameter of 20–50 µm per animal) (Fig. 1 A, insert). A granulomatous response to infection with *Francisella* in zebrafish has previously been documented by others, and white multifocal noodles are commonly observed in the kidney and spleen of tilapia infected with *Fno* [20]. Pathologic processes, pigmentation or connective tissues were not found in control or immunized fish the day before challenge, suggesting that the granulomas revealed in the histological analysis at 7 dpc are caused by *Fno* and not by CCH. This data indicate the apparent absence of side effects of CCH immunization.

3.2. Immune response

The immune response of the immunized and subsequent challenged fish was assessed by RT-qPCR at different time points during the experiment. The immunization with CCH did not induce a significant effect in the expression of the different cell markers tested in kidney (Fig. 1B). At 1 dpc and 7 dpc, however, a significant upregulation of *mhcii* (*zgc:103700*) was observed. The same markers were studied in spleen, but no differential expression was detected (data not shown). Differential transcription of several cytokines, including *ifng1-1*, *il12a* and *tnfa*, was also observed in the immunized group compared to the control group after challenge (Fig. 1C and D). The immunized group showed a significantly higher transcriptional level of *ifng1-1*, *il12a* and *tnfa* at 1 dpc, but decreased at 7 dpc. However, no significant transcription level of *il1b* was detected compared to the control group. The expression profile of these cytokines was similar for kidney (Fig. 1C) and spleen (Fig. 1D) in all the time points studied.

4. Discussion and conclusion

The aim of the present study was to investigate the immunomodulatory properties of hemocyanin from *C. concholepas* against *Francisella noatunensis* subsp. *orientalis* in adult zebrafish. In mammals, *C. conholepas* hemocyanin has been demonstrated to trigger the innate immune system that leads to the maturation of a Th1-specific adaptive immune response together with a powerful nonspecific immunomodulatory response [5]. However, its use has not been reported in fish.

The development of effective vaccines should be approached by combining immunomodulatory substances together with specific epitopes, thereby maximizing their immunogenicity. Immunomodulatory substances should be able to trigger specific immunological processes without causing strong side effects. Oil adjuvants are able to induce a strong and durable immune response, but their use is shown to cause several side-effects. Thus, there is a need for alternative adjuvants, like CCH. When used for the immunization of adult zebrafish, CCH induced an increased immune gene expression of *il12a, tnfa* and *ifng1-1* compared to the PBS injected control group at 1 and 7 days post immunization (dpi), suggesting a rapid initiation of the protective immune response. A significant upregulation of *mhcii* (*zgc:103700*) was observed in the CCH immunized group after challenge, indicating that *Francisella*, either use antigen presenting cells as the main site of replication or is efficiently presented by MHCII complex [14]. Further experiments are needed to clarify the effect of CCH on the expression of

mhcii in fish. Moreover, a significant upregulation of the expression of *il12a, tnfa* and *ifng1-1* was observed in the group immunized with CCH after challenge. These data suggest that fish immunized with CCH are able to mount a beneficial pro-inflammatory response, characterized by the upregulation of *il12a*, *tnfa*, and *ifng1-1,* to overcome the infection. Both *il-1b* and *tnfa* are pro-inflammatory cytokines excreted by immune cells, which main role are to initiate an anti-infectious response. Studies in several vertebrate models have shown that under infection with *Francisella,* the production of IFN-γ is a key in controlling the infection. Furthermore, IFN-γ knockout mice and mice treated with anti-IFN-γ antibodies succumb to normally sub-lethal doses of *Francisella* [24]. In this study, the CCH immunized group presented a slightly upregulation of IFN-γ at early time points, where the most significant upregulation occurred after challenge, especially at 1dpc. As the majority of the mortalities caused by *Francisella* infection in zebrafish occurred between 3 and 7 dpc (Fig. 1), an IFN-γ upregulation may be the consequence of a higher bacterial load rather than a preventive response. The CCH immunized fish were, however, not fully protected from the infection by *Francisella*, as some tendency of granuloma-like structures were observed by histological analysis. The formation of granulomas in zebrafish immunized against intracellular pathogens has previously been reported, indicating that although a reduction in mortalities is observed, all signs of infection cannot be fully excluded [21]. Thus, a decrease in granuloma size and number could indicate a reduction in the bacterial infection, as observed in the CCH immunized fish. In summary, our results show that CCH acts as a positive immunomodulatory agent able to induce the immune response, protecting zebrafish from an acute high dose of *Fno*. However, further experiments are needed to test the immunomodulatory capacity of CCH, when used as vaccine adjuvant in aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.06.046.

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