Histamine and mast cell activator compound 48/80 are safe but inefficient systemic adjuvants for gilthead seabream vaccination

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Key words: histamine, compound 48/80, gilthead seabream, vaccination

Abstract

Histamine has a key role in the regulation of inflammatory and innate immune responses in vertebrates. Gilthead seabream (*Sparus aurata* L.), a marine hermaphrodite teleost of great commercial value, was the first fish species shown to possess histamine-containing mast cells (MCs) at mucosal tissues. MCs are highly abundant in the peritoneal exudate of gilthead seabream and compound 48/80 (Co 48/80), often used to promote MC activation and histamine release, is able to promote histamine release from gilthead seabream MCs *in vitro* and *in vivo*. The aim of the present study was to analyze the effect of histamine and Co 48/80 on the immune responses of gilthead seabream. For this purpose, histamine and Co 48/80 were intraperitoneally injected alone or combined with 10^9 heat-killed *Vibrio anguillarum* cells and their effects on head kidney and peritoneal exudate were analyzed. The results indicated that although histamine and Co 48/80 were both able to alter the percentage of peritoneal exudate and head kidney immune cell types, only Co 48/80 increased reactive oxygen species production by peritoneal leukocytes. In addition, histamine, but not Co 48/80, was able to slightly impair the humoral adaptive immune response, i.e. production of specific IgM to *V. anguillarum*. Notably, both histamine and Co 48/80 reduced the expression of the gene encoding histamine receptor H2 in peritoneal exudate leukocytes. These results show for the first time in fish that although systemic administration of histamine and Co 48/80 is safe, neither compound can be regarded as an efficient adjuvant for gilthead seabream vaccination.

1. Introduction

Inflammation, a biological process that consists of the production and release of mediators, changes in the vascular tissues and the recruitment of leukocytes to the inflammatory site, is associated with both tissue damage and infection. Histamine is one of the first biomolecules to reach the inflammation site. Histamine (2-[4-imidazole]-ethylamine) is mainly stored and released by mast cells (MCs) in mammals, reptiles, birds and perciform fish (Crivellato et al., 2015; Mulero et al., 2007). There are two theories on the origin of histamine synthesis in MCs: (i) histamine in MCs is of ancient origin, (ii) histamine production in MCs has had multiple independent origins in different phylogenetic species.

In mammals, MCs are relatively well-known. It is thought that MC precursors leave the bone marrow and, via blood circulation, reach mucosal sites where they mature (Reite, 1996, 1998). Several studies have described not only the role of MCs in inflammation, including the mechanisms through which the release of immune mediators, such as histamine, is induced, but also the MC role in cellular growth modulation and leukocytes differentiation (Dawicki and Marshall, 2007; Marshall, 2004). Although bone marrow and lymph nodes are lacking in fish, this vertebrate group possesses head kidney (HK), spleen, thymus and mucosa-associated lymphoid tissue (Press and Evensen, 1999). HK is a very important hematopoietic organ (Fange, 1986) that shows morphological similarities with the bone marrow of higher vertebrates (Meseguer et al., 1995). MC precursors leave this organ and reach the intestinal mucosa, dermis and gills (also eye, hypothalamus, pancreas and corpuscles of Stannius) (Sfacteria et al., 2015) where they differentiate into mature MCs, presenting a range of phenotypic heterogeneity. Moreover, preliminary studies by our group have shown an enrichment of MCs in the peritoneal exudate (PE) (Gómez-González et al., 2014), as occurs in rat (Diamant, 1990). However, knowledge of the mechanism of MC activation and the release of pro- and anti-inflammatory mediators in fish is still lacking.

The effects of histamine are mediated by four histamine receptors (HR) in mammals (HR H1, H2, H3 and H4) and three in fish (HR H1, H2 and H3), all of which belong to the G-protein-coupled receptor family (Cofiel and Mattioli, 2006; Holstein, 1986; Peitsaro et al., 2000; Peitsaro et al., 2007; Temma et al., 1989). In mammals, the expression of these receptors have been amply studied (Akdis and Simons, 2006) but little information is available about fish HR activation.

The gilthead seabream (*Sparus aurata* L.) is a marine, protandrous hermaphrodite teleost, which has been described as the first fish species known to possess histamine in the granules of its MCs (Mulero et al., 2007). In addition, histamine regulates the respiratory burst of gilthead seabream professional phagocytes and compound 48/80 (Co 48/80), a potent synthetic degranulation agent classically used to induce MC activation in mammals (Lagunoff et al., 1983; Paton, 1951), is able to induce the release of histamine in gills and the contraction of the intestinal smooth muscle *ex vivo*. Moreover, stimulation of gilthead seabream isolated MCs with Co 48/80 induces the release of histamine and increases the transcript levels of the genes encoding IL-1ß and IL-8 following stimulation with bacterial DNA (Gómez-González et al., 2014).

The present study aims analyzing the effect of non lethal doses of histamine and Co 48/80 on the innate and adaptive immune response of gilthead seabream. For this purpose, fish were intraperitoneally (i.p.) injected with heat-killed *Vibrio anguillarum* (hkVa) in combination with histamine or Co 48/80. In addition, the presence of the three known HRs was checked and the impact of the above treatments on their expression was analyzed.

2. Materials and methods

2.1.Animals and experimental design

Healthy specimens of gilthead seabream *Sparus aurata* L. (Actinopterygii, Perciformes, Sparidae) were maintained at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, Mazarrón, Murcia, Spain), where they were kept in 2 m^3 tanks with running seawater (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h), a suitable aeration and filtration system and natural photoperiod and temperature. Fish were fed twice a day with a commercial pellet diet (44% protein, 22% lipids; Skretting) at feeding rate of 1.5% of fish biomass.

In vivo treatments were carried out in December (2014) with mature gilthead seabream males (n = 150) with a body weight (bw) of 205 ± 31 g. Briefly, specimens were i.p. injected with PBS (control fish), histamine (10 mg/kg bw; Sigma-Aldrich) or Co 48/80 (0.25 mg/kg bw; Sigma-Aldrich) alone or combination with 10^9 hkVa (strain R82, serotype 01)/specimen at day 0 (priming) and 49 days post-priming (booster) (Fig.

1). The concentrations of histamine and Co 48/80 used were not lethal for gilthead seabream according to a previous study (Mulero et al., 2007).

Fish were fasted for 24 h before sampling at 1 dpp (day post-priming) and 1 mpb (month post-booster). Specimens (n=5 fish/treatment/sampling time) were tranquilized by 8 µL/L of clove oil and, immediately, anesthetized using 40 µL/L of clove oil and weighed. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80 ºC until use. After that, the fish were i.p. injected with 5 mL of sRPMI culture medium [RPMI culture medium (Sigma-Aldrich) supplemented with 2 mM glutamine (Sigma-Aldrich), 100 I.U./mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich) and adjusted with 0.35% NaCl to gilthead seabream serum osmolarity (353.33 mOs)] and tissue-attached cells were dislodged into the sRPMI by massaging the abdomen for 10 min. Incisions were made below of the lateral fin to access the peritoneum and the PE was aspirated and collected into 15 mL Falcon tubes. Then, the specimens were decapitated and the HK were removed and kept on sRPMI or RNAlater (Sigma-Aldrich) for reactive oxygen species (ROS) production assays and gene expression analysis, respectively, as described below. The experiments described were approved by the Consejería de Agua, Agricultura y Medio Ambiente of the Región de Murcia (approval number A13160507).

2.2.Peritoneal exudate and head kidney cell suspensions

The PE (approximately 5 mL) was passed through 70 µL cell strainers to remove large clumps of cells. After centrifugation at 600 x *g* for 5 min, cells were resuspended in 5 mL of sPBS (PBS, Sigma-Aldrich + 0.35% NaCl). The HK cells were disaggregated using a 100 µL cell strainer and resuspended in 5 mL sPBS. After centrifugation at 600 x *g* for 5 min, cells were washed twice and resuspended in 5 mL of sPBS. The total cell number in PE was counted using a Neubauer chamber while the number of total HK cells was analyzed by an automatic cell counter (Biorad).

2.3.Viability assay

Aliquots (0.5×10^6) of PE and HK cell suspensions were diluted in 200 µL of PBS containing 40 µg/mL propidium iodide (PI), which stains dead cells. The mean fluorescence intensity was analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with the FlowJo v10.0.4 software.

2.4.Determination of acidophilic granulocytes and IgM positive cells

To analyze the percentage of acidophilic granulocytes (AGs) and IgM^+ B lymphocytes, 10^5 PE and HK cells were incubated with a monoclonal antibody (mAb) specific to gilthead seabream AGs (G7, 1:10) (Sepulcre et al., 2002) or a commercial mAb against gilthead seabream IgM (Aquatic Diagnostics, 9H8, 1:100) in 100 µL of FACS buffer [PBS supplemented with 2% fetal bovine serum (FBS) (Sigma-Aldrich) and 0.05% sodium azide (Sigma-Aldrich, S8032)], respectively. After 30 min at 4 °C, cells were centrifuged at 600 x *g* for 5 min, and the supernatant was removed by aspiration before washing the cells twice with FACS buffer. Cells were stained for 30 min at 4°C with Alexa Fluor 488 F(ab')2 fragment of goat anti-mouse IgG (H+L) (1:500, ThermoFisher Scientific), washed twice and the fluorescence intensity was measured using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with FlowJo v10.0.4. Appropriate isotype control antibodies were used, as previously described (Sepulcre et al., 2002).

2.5.Determination of mast cells

The percentage of MCs in the PE was analyzed by transmission electron microscopy, according to their ultrastructural features described by Mulero et al. (2007). For this, PE cells (5×10^6) were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and processed for transmission electron microscopy. Cells from 5 fish were examined using a JOEL JEM-1011 transmission electron microscope. At least, 20 pictures in random locations of the samples were taken and the percentage of MCs was determined.

2.6.ROS production assay

ROS production was measured as the dihydrorhodamine 1,2,3 (DHR 123)-dependent fluorescence produced by 0.5×10^6 PE or HK cell suspensions. The ROS activity was determined by incubating the cells with 10 µM DHR 123 (Sigma-Aldrich, D1054) in Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich) for 5 min and 1 µg/mL phorbol myristate acetate (PMA) (Sigma-Aldrich) for 30 min at 21 °C. Two technical replicates were used for all biological samples. Cells were analyzed using a FACSCalibur flow cytometer and data were analyzed with the FlowJo v10.0.4 software.

2.7.Analysis of gene expression

HK samples were defrosted and RNAlater was removed. Total RNA was extracted from PE cell suspensions and HK tissues with TRIzol Reagent (ThermoFisher Scientific) following the manufacturer's instructions and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA was treated with DNase I (amplification grade, 1 unit/µg RNA, ThermoFisher Scientific) to remove genomic DNA traces that might interfere with the PCR reactions, and SuperScript III RNase H- Reverse Transcriptase (ThermoFisher Scientific) was used to synthesize first strand cDNA with oligo $(dT)_{18}$ from 1 µg of total RNA, at 50 °C for 50 min. The ß-actin (*actb*) gene was analyzed by PCR with an Eppendorf Mastercycle Gradient Instrument (Eppendorf). The reaction mixture was incubated for 3 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at the specific annealing temperature and 1 min at 72 °C, and finally at 72 °C for 10 min. Real-time PCR was performed with an ABI PRISM 7500 (ThermoFisher Scientific) using SYBR Green PCR Core Reagents (ThermoFisher Scientific). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample. The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with three technical replicates. Less than 3% variation in the *rps18* gene expression was observed among samples.

2.8.Determination of IgM specific titer

The *V. anguillarum* specific IgM titer was determined 1 mpb by ELISA in serum of hkVa-vaccinated groups. Ninety-six-well ELISA plates were pre-coated with carbonate/bicarbonate solution and left for 45 min and then coated again and left for 16 h with 10^5 hkVa/well. The following day, the plates were washed three times with low salt buffer (2.42 g TRIS, 22.22 g NaCl and 2.5 ml Tween 20 dissolved in 1 l of distilled water, pH 7.3). Unspecific bindings were blocked by adding 1% BSA (Sigma-Aldrich) in PBS to the plate and shaking for 2 h. The plates were washed once with low salt buffer before adding doubling-dilutions of serum and shaking for 2 h. The plates were then washed three times with high salt buffer (2.42 g TRIS, 29.22 g NaCl and 5 ml Tween 20 dissolved in 1 l of distilled water, pH 7.7) followed by incubating with an mAb specific to gilthead seabream IgM (#F03, Aquatic Diagnostics Ltd). The plates

were rinsed three times with high salt buffer, incubated for 1 h with an anti-mouse IgG (whole molecule)-peroxidase antibody produced in goat (Sigma-Aldrich) and washed again three times with high salt buffer. Finally, the chromogen tetramethylbenzidine (TMB) (Sigma-Aldrich) was added, the reaction was stopped with 2M H_2SO_4 and the absorbance was read at 450 nm using a FLUOstart luminometer (BGM, Lab Technologies).

2.9.Statistical analysis

Data were analyzed by independent-samples T-Tests (control group compared with hkVa group) or by one-way ANOVA and Tukey *post hoc* tests (comparisons among control, histamine and Co 48/80 groups and among the hkVa-treated groups), using the software GraphPad Prism 5.

3. Results

3.1.Cell viability is not modified by histamine or Co 48/80 used alone or combined with hkVa

None of the treatments used in this study affected PE or HK cell viability compared with the control specimens (Fig. 2).

3.2.Histamine and Co 48/80 alters the percentage of acidophilic granulocytes and mast cells and the ight transcript levels of PE and HK

As expected (Chaves-Pozo et al., 2005), hkVa injection resulted in increased total PE cells at 1 dpp (Fig. 3A). However, the presence of histamine decreased the percentage of PE AGs compared with the control group, while the combination of histamine plus hkVa increased this percentage compared with the hkVa group (Fig. 3B). As regards the HK, hkVa-treated fish had a lower percentage of AGs compared with the control group, while the combination of Co 48/80 and hkVa decreased the percentage compared with the hkVa group (Fig. 3C). Of particular note was the fact that the percentage of MCs in PE was significantly lower in hkVa-immunized groups than in the PBS-injected groups (Fig. 3D). However, no changes were observed in the percentage of IgM^+ B lymphocytes in the PE (Fig. 3E) and HK (Fig. 3F) for any of the treatments used in this

study. Finally, *ight* transcript levels decreased in the PE of immunized fish (Fig. 3G), while Co 48/80 slightly decreased them in the HK of immunized fish (Fig. 3H).

3.3.Co 48/80 and exogenous histamine alters the innate immune response

As expected from previous studies (Chaves-Pozo et al., 2004, 2005), hkVa injection significantly increased ROS production (Fig. 4A and C) and the percentage of ROS-producing cells (Fig. 4B, D) in PE and HK, respectively, at 1 dpp.

Moreover, Co 48/80 but not histamine increased ROS production (Fig. 4A) and the percentage of ROS positive cells (Fig. 4B) in PE from 1 dpi specimens compared with the control group, while neither histamine nor Co 48/80 had any effect on the same parameters at this time in HK (Fig. 4C-D).

hkVa injection significantly increased the transcript levels of *il1b* (Fig. 5A and D) and *il10* (Fig. 5B and E) expression in PE and HK, respectively, at 1 dpp. Although neither histamine nor Co 48/80 promoted any alteration in the mRNA levels of *il1b*, *il10* and *il4/il13* in the PE and HK of hkVa-injected fish, both compounds decreased the transcript levels of *il4/il13* in the PE of control animals (Fig. 5C).

3.4.Histamine, but not Co 48/80, alters the humoral adaptive immune response

All groups of fish immunized with hkVa showed increased specific IgM titers at 1 mpb (Fig. 6). Although Co 48/80 was found to have no effect on this response, histamine slightly impaired the production of specific IgM (Fig. 6). However, neither histamine nor Co 48/80 altered the specific IgM titers at 3 mpb (data not shown).

3.5.Histamine, Co 48/80 and hkVa all inhibit hrh2 expression in PE cells

PE and HK leukocytes constitutively expressed *hrh1*, *hrh2* and *hrh3* (Fig. 7). Histamine, Co 48/80 and hkVa treatments all decreased the transcript levels of *hr2* in PE cells at 1 dpp (Fig. 7B) but not those of *hrh1* or *hrh3* (Fig. 7A and C). However, no changes in the mRNA levels of *hrh1*, *hrh2* and *hrh3* were found in HK leukocytes (Fig. 7D, E and F).

4. Discussion

The role of histamine in the immune system of fish and the mechanisms responsible for histamine release from MCs are largely unknown. Previous studies demonstrated that the most evolutionarily advanced fish, the Order Perciformes, were able to produce and store histamine in the granules of MCs (Gómez-González et al., 2014; Mulero et al., 2007). In addition, histamine is used as inflammation mediator in gilthead seabream, since it induces fish death when injected i.p., and promotes contraction of the intestinal smooth muscle *ex vivo* and regulates the respiratory burst of AGs (Mulero et al., 2007). Notably, Co 48/80, which has been successfully used to promote MC degranulation in phylogenetically distant fish species (Manera et al., 2011, 2014; Vallejo and Ellis, 1989) induces histamine release from intestinal, gill and peritoneal MCs of gilthead seabream both *in vivo* and *in vitro* (Gómez-González et al., 2014; Mulero et al., 2007).

In the current study, we used hkVa as priming/immunization model in gilthead seabream to further investigate the role of exogenous and endogenous histamine in the immune response of perciform fish. To achieve this, non lethal doses of histamine and Co 48/80 alone or combined with hkVa were used. As expected, hkVa immunization induced the recruitment of leukocytes to the peritoneal cavity. Surprisingly, however, hkVa injection resulted in a pronounced decrease of MCs in the peritoneal cavity, whereas the percentage of AGs, which are functionally equivalent to mammalian neutrophils (Sepulcre et al., 2002), and of IgM^+ B lymphocytes were unaltered. The increased number of PE cells coincided with the depletion of AGs in the HK, suggesting that these cells are mobilized from the HK (the hematopoietic compartment) to the inflammatory site, as previously reported in fish injected i.p. with live *V. anguillarum* (Chaves-Pozo et al., 2005). Furthermore, hkVa injection increased ROS production by PE leukocytes, induced IL-1β and IL-10 expression in both PE and HK leukocytes, and led to the high production of serum IgM antibodies specific to *V. anguillarum*, indicating that hkVa promotes both local and systemic immune responses.

The injection of histamine modulated the innate and adaptive immune responses to hkVa. Furthermore, the injection of histamine alone decreased the number of AGs in the PE, but, when combined with hkVa, it increased the number. These results suggest that exogenous histamine increases the migration of AGs from the HK to the peritoneal cavity in response to hkVa. However, exogenous histamine failed to further enhance the

innate immune response to hkVa, i.e. ROS production and cytokine gene expression. As AGs are the main source of ROS and IL-1β in gilthead seabream (Mulero et al., 2008; Sepulcre et al., 2007) and these cells increased in the PE of the fish treated with histamine and hkVa, histamine seems to inhibit the production of ROS and IL-1β in response to hkVa. These results may also explain the slight impairment of adaptive immune responses, i.e. IgM production to hkVa, observed in fish injected with histamine and hkVa. This effect of histamine agrees with previous studies in rabbits, in which histamine reduced IgM titers specific to sheep red blood cells (Tripathi et al., 2010). In sharp contrast, however, it was found that histamine enhances the *in vitro* proliferative responses of anti-IgM-stimulated B cells via HRH1 signaling (Banu and Watanabe, 1999), which induces IgM secretion in the human B lymphoma cell line BMNH but not in the lymphoblastoid B cell line CESS or human peripheral monocytes (Falus, 1993). Histamine was also found to selectively enhance IgE and IgG4, but not IgG1, IgG2, IgG3, IgM, IgA1 or IgA2, production induced by anti-CD58 monoclonal antibody in primary human B cells (Kimata et al., 1996).

Since we previously demonstrated that Co 48/80 is also able to induce MC activation and histamine release in gilthead seabream (Gómez-González et al., 2014; Mulero et al., 2007), while the same compound has been shown to act as a powerful vaccine adjuvant in mammals (McLachlan et al., 2008), we evaluated the impact of Co 48/80 in hkVa-immunized fish. Although Co 48/80 reduced the number of AGs in the HK, a concomitant increase of the same was not observed in the peritoneal cavity. This result suggest that endogenous histamine release promotes AG mobilization from the HK to other organs, as has been demonstrated in mice, in which the subcutaneous or nasal administration of small-molecule MC activators, including Co 48/80, with vaccine antigens increased dendritic cell and lymphocyte recruitment to draining lymph nodes (McLachlan et al., 2008). This result deserves further investigation.

In contrast to exogenous histamine, Co 48/80 treatment increased ROS production by PE leukocytes, while the percentage of AGs remained unaffected, indicating that the release of endogenous histamine from MCs increases ROS production of AGs, as has been shown *in vitro* with the Hrh2 agonist dimaprit (Mulero et al., 2007). Unexpectedly, however, Co 48/80 failed to enhance the expression of genes encoding major cytokines and the humoral adaptive immune response to hkVa, unlike exogenous histamine did.

Another interesting finding of this study is the identification of HRs in leukocytes and their corresponding expression profile. We previously demonstrated that gilthead seabream HK leukocytes express *hrh1* (García-García et al., 2016). In this study, we extend these observations by demonstrating that gilthead seabream PE and HK leukocytes express *hrh1*, *hrh2* and *hrh3*. More interestingly, the injection of histamine, Co 48/80 or hkVa resulted in the drastically reduced expression of *hrh2* in PE leukocytes, while that of *hrh1* and *hrh3* was unaffected. These results suggest that immune challenge by both exogenous and endogenous MC-derived histamine would potentiate the inhibitory effects of histamine on PE leukocytes by impairing Hrh2 signaling (Mulero et al., 2007). Further studies are required to better understand the role of the three histamine receptors in the immune response of fish.

To summarize, this is the first report showing that exogenous and endogenous MC-derived histamine is able to modulate *in vivo* the immune response of fish. Unfortunately, although systemic administration of histamine and Co 48/80 is safe, neither compound can be regarded as an efficient adjuvant for seabream vaccination.

Acknowledgments

We thank I. Fuentes for her excellent technical assistance and the "Servicio de Apoyo a la Investigación" of the University of Murcia for their assistance with cell culture, flow cytometry, microscopy and real-time PCR. This work has been funded by the Fundación Séneca (Coordination Centre for Research, CARM, 19883/GERM/15 to AGA and PhD fellowship to NEGG) and the Spanish Ministerio de Economía y Competitividad and FEDER funds (AGL2014-53167-C3-1-R to AGAy, AGL2014-53167-C3-2-R to AGAl).

Conflict of interest

The authors declare no conflict of interest.

Footnotes

[1]Abbreviations: MCs: mast cells; Co 48/80: compound 48/80; HK: head kidney; PE: peritoneal exudate; hkVa, heat-killed *Vibrio anguillarum*; MFI: mean fluorescence

intensity; ROS: reactive oxygen species; PI: propidium iodide; AGs: acidophilic granulocytes

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Figure legends

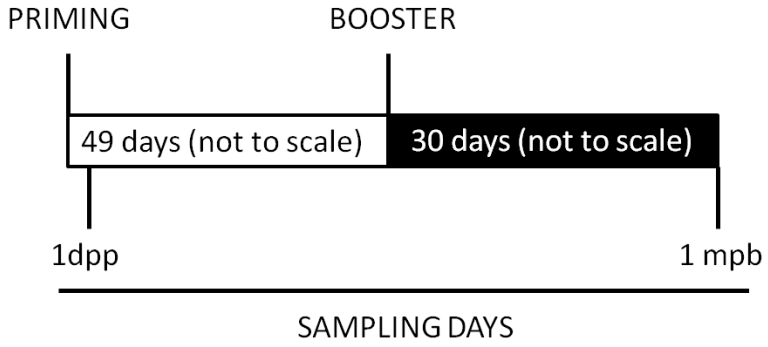

Figure 1. Experimental design and sampling schedule. Fish were injected with PBS, histamine (H) or compound 48/80 (Co) alone or in the presence of heat-killed *V. anguillarum* (hkVa) at day 0 (priming) and 49 (booster). Samples were collected 1 day post-priming (dpp) and 1 month post-booster (mpb). The six experimental groups were: C: PBS; H: histamine; Co: Co 48/80; Va: hkVa; H+Va: histamine plus hkVa; Co+Va: Co 48/80 plus hkVa.

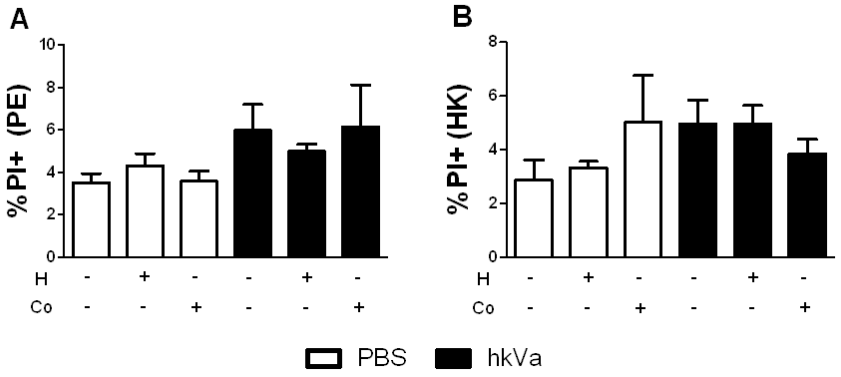

Figure 2. Histamine and Co 48/80 did not affect cell viability. Specimens were injected i.p. with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or combined with hkVa (solid bars). PE (A) and HK (B) non-viable cells were assayed at 1 dpp by flow cytometry as the percentage of PI^+ cells. Data are shown as the mean ± SEM of 5 individuals. Statistical analysis was performed among groups according to one-way ANOVA and Tukey post-hoc test.

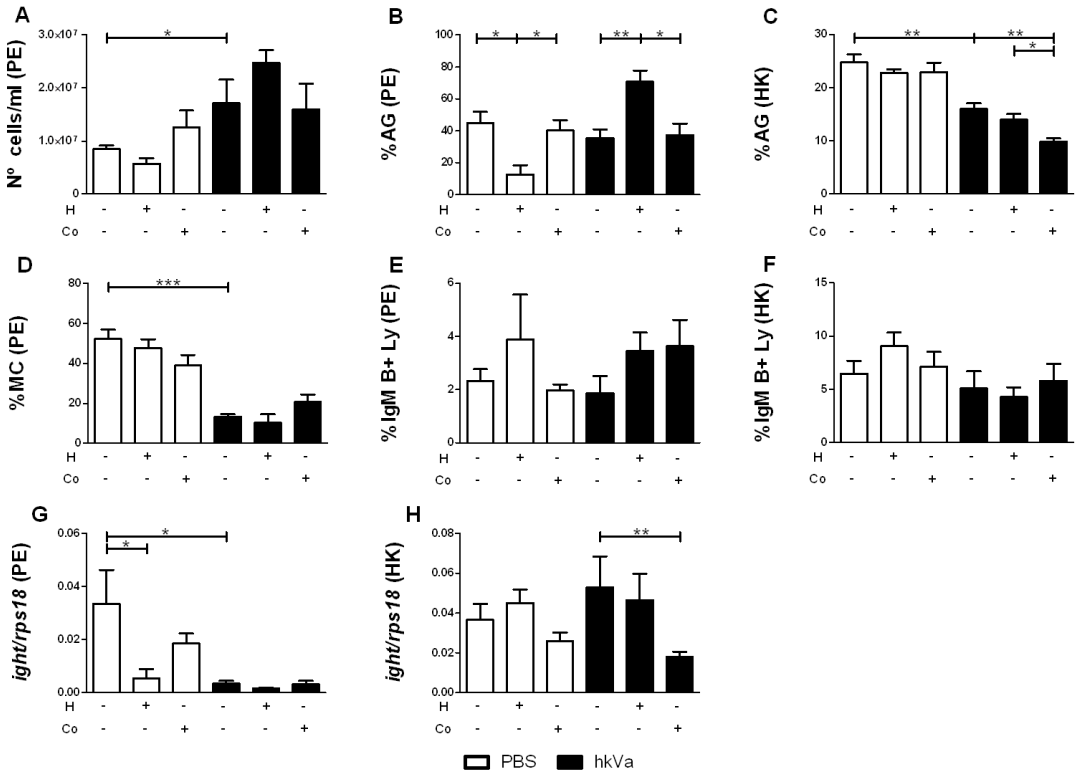

Figure 3. Histamine and Co 48/80 modulate the percentages of AGs and MCs and the transcript levels of *ight* in PE and HK. (A) Total PE cells in fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or combined with hkVa (solid bars) 1 dpp. (B- H) The percentages of AGs [$G7^+$ cells (B) and FSC^{high}/SSC^{high} (C)], MCs (D) and IgM^+ B lymphocytes ($9H8^+$) (E and F), and the mRNA expression of *ight* (G and H) were analyzed in PE (B, D, E and G) and HK (C, F, H) cell suspensions at 1 dpp. Data are mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to a Student *t*-test (control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *p < 0.05, **p<0.01 and ***p<0.001.

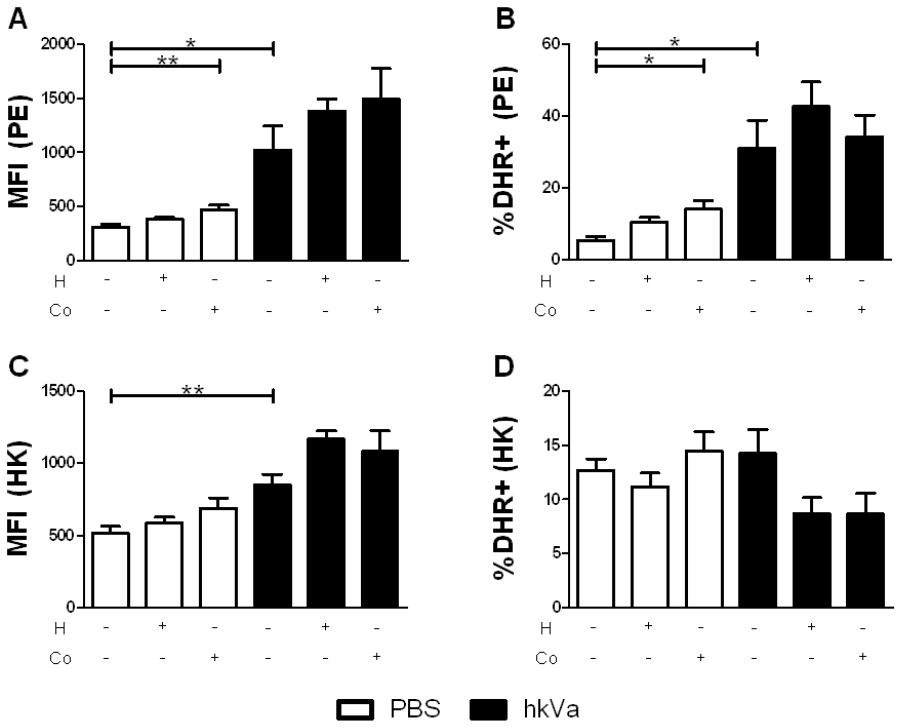

Figure 4. Compound 48/80 increases the ROS production and ROS-producing cells in PE. PE (A and B) and HK (C and D) cells collected at 1 dpp from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or in combination with hkVa (solid bars) were labeled with DHR 123 and stimulated for 30 min. The mean fluorescent intensity (MFI) (A and C) and the percentage of DHR+ cells (B and D) were then analyzed by flow cytometry. Data are mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to a Student *t*-test

(control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *p < 0.05 and **p<0.01. (DHR+: positive DHR 1, 2, 3 cells).

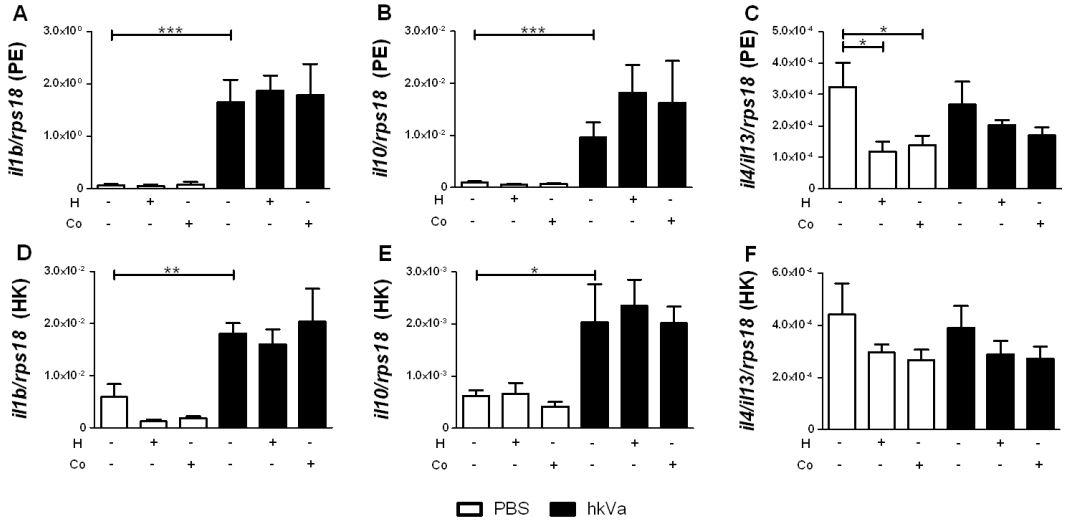

Figure 5. hkVa injection increases the transcript levels of *il1b*, *il10* in PE and HK while histamine and compound 48/80 diminish the mRNA expression of *il4/il13* in PE. The transcript levels of *il1b* (A and D), *il10* (B and E) and *il4/il13* (C and F) were determined by RT-qPCR in PE (A-C) and HK (D-F) cells collected at 1dpp from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or in combination with hkVa (solid bars). The *rps18* was used as an endogenous control. Data represent gene expression *vs.* the endogenous control shown as the mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to Student *t*-test (control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *p < 0.05, **p<0.01 and ***p <0.001.

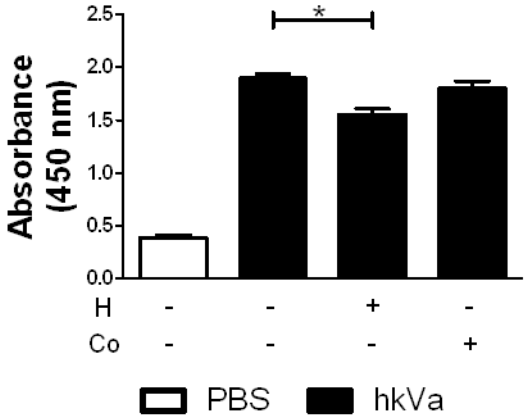

Figure 6. Histamine modulates adaptive immunity. The titers of specific IgM to Va were determined by ELISA in serum collected at 1 mpb from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bar) or in combination with hkVa (solid bars). Data are mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *$p < 0.05$.

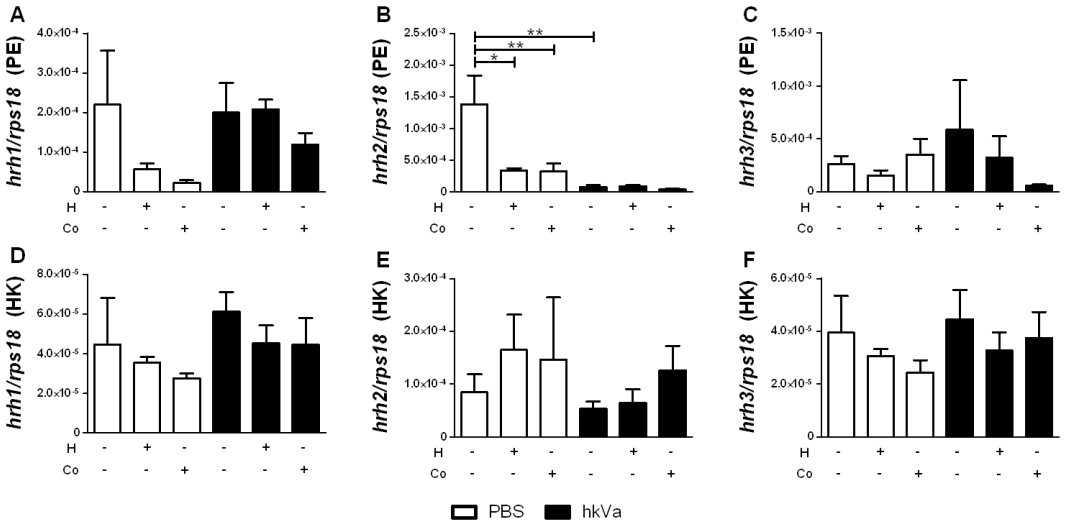

Figure 7. Histamine, Co 48/80 and hkVa inhibit *hrh2* expression in PE leukocytes. The mRNA levels of *hrh1* (A and D), *hrh2* (B and E) and *hrh3* (C and F) were determined by RT-qPCR in PE (A-C) and HK (D-F) leukocytes obtained at 1 dpp from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or in combination with hkVa (solid bars). The *rps18* was used as an endogenous control.

Data represent gene expression *vs.* the endogenous control and are shown as the mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to Student t-test (control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 38/80; hkVa vs. h plus hkVa and Co48/80 plus hkVa). *$p < 0.05$ and **$p<0.01$.