



# A review of the immunological mechanisms following mucosal vaccination of finfish

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Mucosal organs are principle portals of entry for microbial invasion and as such developing protective vaccines against these pathogens can serve as a first line of defense against infections. In general, all mucosal organs in finfish are covered by a layer of mucus whose main function is not only to prevent pathogen attachment by being continuously secreted and sloughing-off but it serves as a vehicle for antimicrobial compounds, complement, and immunoglobulins that degrade, opsonize, and neutralize invading pathogens on mucosal surfaces. In addition, all mucosal organs in finfish possess antigen-presenting cells (APCs) that activate cells of the adaptive immune system to generate long-lasting protective immune responses. The functional activities of APCs are orchestrated by a vast array of proinflammatory cytokines and chemokines found in all mucosal organs. The adaptive immune system in mucosal organs is made of humoral immune responses that are able to neutralize invading pathogens as well as cellular-mediated immune responses whose kinetics are comparable to those induced by parenteral vaccines. In general, finfish mucosal immune system has the capacity to serve as the first-line defense mechanism against microbial invasion as well as being responsive to vaccination.

**Keywords:** gill, gut, IgM, IgT, mucosal, oral, skin, vaccine

## Introduction

Mucosal surfaces are important physical barriers whose main function is to protect the systemic environment of the body against microbial invasion. An ideal mucosal vaccine should have the capacity to produce protective immunity that is able to prevent microbial invasion, colonization, and establishment of infection at portals of entry (1). All mucosal organs are endowed with antigen-presenting cells (APCs) that play a vital role in antigen uptake and processing followed by presentation to naïve B- and T-lymphocytes to induce a long-lasting protective immunity (2). Despite so, our understanding of the immunological basis of mucosal vaccine protection has for a long time lagged behind systemic immunity not only in finfish, but also in higher vertebrates, which has led to a corresponding delay in developing highly protective mucosal vaccines across the vertebrate taxa.

The demand for mucosal vaccines in aquaculture has been exacerbated by different stages of fish production cycles in which administering vaccines by injection might not be feasible thereby rendering the use of mucosal vaccines as an alternative. For Atlantic salmon (*Salmo salar* L), administering vaccines by injection at the freshwater stage is the most commonly applied method while boost vaccination for fish in cages at sea is only applicable by oral vaccination. On the other hand, vaccinating small fish by injection causes stress-related mortalities, which make immersion vaccination a

better alternative. While the mode of vaccine delivery is to a large extent dependent on the fish production cycle, developing highly protective vaccines for oral and immersion vaccination has been a serious challenge for a long time because the process of optimizing vaccine delivery methods and measuring immune responses for mucosal vaccines is more complicated than for injectable vaccines (3). For example, the vaccine dose taken up by oral or immersion vaccination is difficult to accurately quantify. Unlike injectable vaccines whose immune response to vaccination is determined by measuring serum antibody levels, there is no optimized quantitative assay established for measuring antibody levels in the mucus of vaccinated fish. Therefore, it is difficult to optimize mucosal vaccine performance for finfish (3). Despite so, gene expression studies show that mucosal vaccines are able to induce immune responses in vaccinated fish. It is anticipated that generating vaccines that have the capacity to induce a combined effect of highly protective mucosal and systemic immune responses could be more effective at attaining sterile immunity. Mucosal immune responses would serve as gatekeepers at the portals of pathogen entry while systemic immunity would serve as a secondary barrier to block the spread of infection to target organs in infected fish.

Considerable progress has been made in optimizing the performance of injectable vaccines in aquaculture (4, 5), but not for mucosal vaccines. However, recent advances in mucosal immunology show that teleosts fish, like all vertebrates, are endowed with a protective immune system although there has been no comprehensive review that puts together a summation of underlying mechanisms of mucosal vaccine protection in finfish, thereby creating the basis for this review. Therefore, this review puts together a collection of different components of the mucosal immune system of finfish with the view to shed insight on how these elements prevent microbial invasion on mucosal surfaces as a basis for designing highly protective mucosal vaccines for finfish.

## Immunological Mechanisms of Vaccine Protection in Different Mucosal Organs

Mucosal organs in finfish have been classified into four broad categories, namely the gut, gills, skin, and nasal mucosa by different scientists (6–9). Hence, in this review, we discuss the immunological basis of mucosal vaccine protection based on this classification.

### Gut Mucosal Responses to Vaccination

The gut immune system of finfish is made of two components, namely the innate and adaptive immune system whose immune responses to vaccination are discussed below.

#### Innate Immune Responses to Mucosal Vaccination in the Gut

A layer of mucus containing antimicrobial peptides, complement factors, immunoglobulins, and other surface defensins covers the mucosal surface of the gut (Table 1). Rombout et al. (6) have recently reviewed the type of immune cells found in the gut of fish. APCs, such as monocytes and macrophages, are found in

**TABLE 1 | Innate immune components of the gut mucosa in finfish.**

| Component                    | Regulatory/effectors cells/genes | Reference |
|------------------------------|----------------------------------|-----------|
| Mucus and surface defensins  | Muc2                             | (13)      |
|                              | Antimicrobial peptides           | (14, 15)  |
|                              | Complement system                | (16–19)   |
| Cell types                   | Goblet cells                     | (14)      |
|                              | Macrophages                      | (6, 7)    |
|                              | Granulocytes                     | (6, 7)    |
|                              | Rodlet cells                     | (20)      |
|                              | M-cells                          | (12)      |
|                              | Enterocytes                      | (21, 22)  |
| Pattern recognition receptor | Toll-like receptors              | (23, 24)  |
|                              | Peptidoglycan PRR                | (25)      |
| Proinflammatory cytokines    | IL-1 $\beta$                     | (26, 27)  |
|                              | TNF $\alpha$                     | (28)      |
|                              | IL-6                             | (27)      |
| Chemokines                   | CCR6                             | (29)      |
|                              | CCR9B                            | (29)      |
|                              | CXCL10                           | (30)      |
|                              | CK12                             | (31)      |

the lamina propria (LP) while the intra epithelial lymphocytes (IELs) mainly comprises of B and T-lymphocytes. Zhang et al. (10) and Li et al. (11) have shown that teleosts B-cells possess phagocytic properties that play an important role as APCs. It has been shown that IgT coats gut commensal bacteria in a similar pattern as IgA in mammals (10). In addition, Fuglem et al. (12) have reported of M-cells and dendritic-like cells in the gut of salmonid intestinal epithelium.

Martin et al. (32) compared the antigen uptake ability of intestinal leukocytes with their head-kidney (HK) and peripheral blood (PBL) counterparts and showed that intestinal phagocytes, when activated, ingested as many yeast cells as their HK counterparts, indicating that gut APCs have the same capacity for antigen uptake as their systemic counterparts. Rombout et al. (21, 22) have shown that there are differences in the distribution of APCs between different gut segments with the second segment having more APCs than the first. Similarly, Fuglem et al. (12) also showed that the uptake of gold-BSA was restricted to dendritic-like cells and other epithelial cells located in mucosal folds found in the second gut segment. In another study, Chen et al. (33) recently showed uptake of inactivated infectious pancreatic necrosis virus (IPNV) antigens in the second gut segment following oral and anal intubation, which were also detected in the HK melanomacrophages of Atlantic salmon (*S. salar* L). Overall, these studies indicate that antigens deposited in the second gut segment were more likely to be taken up by APCs than antigens deposited in other gut segments. Therefore, the challenge is to develop vaccine delivery systems able to deposit antigens in the second gut segment.

Similar to systemic immune responses (5, 34), mucosal antigen uptake is linked to expression of different proinflammatory cytokines, such as IL-1 $\beta$ , TNF $\alpha$ , and IL-8, in the gut (Table 1). In addition, Table 1 shows that chemokines expressed in response to antigen delivery through the gut mucosa are comparable to those induced by parenteral vaccination (35, 36) suggesting that antigen uptake by gut APCs is coordinated by chemokines and cytokines comparable to those induced by systemic antigen

delivery systems (35–37). However, there is need for detailed investigations to elucidate the role of these chemokines and cytokines in enhancing the performance of mucosal vaccines in finfish.

### Adaptive Immune Responses to Mucosal Vaccination in the Gut

All three immunoglobulin (Ig) isotypes characterized in teleosts fish have been detected in the gut (Table 2). Unlike IgM, IgT is specialized in mucosal surfaces in a similar pattern that IgA functions in mammals and it accounts for the largest proportion of the B-cell population found in the gut of finfish (10). Kai et al. (26) have shown that high IgT levels were only expressed by bath or immersion vaccination unlike IgM that had high levels for vaccines administered by injection (4). Vervarcke et al. (38) compared antibody levels induced by anal intubation of vaccines into the second segment of the gut, oral vaccination through feed and intraperitoneal vaccination against *Vibrio anguillarum* and observed high antibody levels in the skin mucus and bile for fish vaccinated by intubation and not in the intraperitoneally vaccinated fish. In addition, they detected high antibody levels for fish vaccinated by intubation than the orally vaccinated fish. They further noted that the antigen dose of vaccines administered by anal intubation correlated with post vaccination antibody levels suggesting that deposition of vaccines in the second segment of the gut by anal intubation produced better correlates of vaccine protection than oral vaccination. In summary, these studies show (i) compartmentalization of mucosal responses in which antigens delivered by intubation into the gut mucosa produced

high antibody levels in other mucosal organs, which was not the case for vaccines delivered by the intraperitoneal route, (ii) the second segment of the gut is the most antigen absorptive site able to induce high antibody responses, and (iii) antibodies generated by mucosal vaccination were protective against microbial invasion in the gut mucosa.

Several scientists (32, 57, 58) have shown that CD8 $\alpha$ , which is a marker for activated cytotoxic T-lymphocytes (CTLs) in fish, accounts for the largest proportion of T-lymphocytes in the gut of teleosts fish. It is interesting to note that upregulation of CD8 $\alpha$  cells has been linked to the corresponding upregulation of MHC-I, suggesting that presentation of antigens via the MHC-I pathway could be linked to activation of naïve CD8 cells into effector CTLs in the gut mucosa of finfish. For example, Kai et al. (26) showed upregulation of MHC-I that corresponded with upregulation of CD8 $\alpha$  in the gut of grouper larvae (*Epinephelus coioides*) exposed to nervous necrosis virus (NNV) by oral vaccination, suggesting that activation of CD8 $\alpha$  was via the MHC-I pathway. Furthermore, upregulation of T-cell receptor (TCR) and CD3 genes has also been shown to correspond with upregulation of CD8 $\alpha$  and MHC-I genes (57, 59, 60). Martin et al. (32) showed a cytotoxic activity that was twice higher in the intestine than in head-kidney leukocytes in rainbow trout while Picchiatti et al. (57) showed high cytotoxic activity in lymphocytes purified from the intestinal mucosa, providing further insight into the cellular-mediated activity of activated CTLs in the gut mucosa of finfish. Put together, these studies show that the gut mucosa is endowed with different components of CD8 T-cell responses whose kinetics corresponds with CTL responses shown to eliminate cells infected with intracellular pathogens in higher vertebrates. As for CD4 responses, transcription factors for their specification into Th1 and Th2 subtypes have been characterized in fish and these include T-bet, GATA-3, and FoxP3 (Table 1). Recently, Wang et al. (61) showed that grass carp virus (GCRV) induced upregulation of T-bet when GATA-3 was downregulated in which upregulation of IFN $\gamma$  correlated with upregulation of T-bet being similar to kinetics induced by parenteral vaccines in finfish (5, 47). Overall, these studies show that the gut mucosa of finfish is endowed with a cellular-mediated immune response orchestrated by CD4 and CD8 genes expressed in response to mucosal vaccination. However, there still remains the challenge of demonstrating the functional role of cellular-mediated immunity in finfish vaccinated by the mucosal route.

**TABLE 2 | Adaptive immune components of the gut mucosa in finfish.**

| Component                   | Regulatory/effectors cells/genes | Abbreviation                 | Reference                |  |
|-----------------------------|----------------------------------|------------------------------|--------------------------|--|
| Humoral responses           | Cell types                       | B-lymphocytes                | (6, 39)                  |  |
|                             |                                  | IgT/IgZ                      | (26, 40–42)              |  |
|                             | Immunoglobulins (Ig)             | IgM                          | (26, 41)                 |  |
|                             |                                  | IgD                          | (41)                     |  |
|                             |                                  | B-cell transcription factors | Blimp1<br>Pax5           | (41)<br>(41)                               |
| Cellular responses          | Major histocompatibility         | MHC-I                        | (26)                     |  |
|                             |                                  | MHC-II                       | (26)                     |  |
|                             | T-helper cells                   | Th1                          | (43)                     |  |
|                             |                                  | Th2                          | (43)                     |  |
|                             |                                  | Treg                         | (33)                     |  |
|                             |                                  | Transcription                | GATA-3<br>T-bet<br>FoxP3 | (27, 44–46)<br>(46–49)<br>(33, 45, 49, 50) |
|                             | CD4-Regulatory cytokines         |                              | IL-2                     | (27)                                       |
|                             |                                  |                              | IL-4/13                  | (27)                                       |
|                             |                                  |                              | IL-10                    | (51)                                       |
|                             |                                  |                              | IL-12                    | (52, 53)                                   |
|                             |                                  |                              | IFN $\gamma$             | (52, 53)                                   |
|                             |                                  |                              | TNF                      | (28)                                       |
|                             |                                  |                              | PD-1                     | (54)                                       |
|                             |                                  |                              | TGF- $\beta$             | (54)                                       |
|                             | Cell type                        |                              | T-lymphocytes            | (54, 55)                                   |
| CD8 T-cells                 |                                  |                              | (26, 32, 54)             |  |
| T-cell receptor             |                                  |                              | (55)                     |  |
| CD8 transcription factor(s) |                                  |                              | Eomesodermin             | (47, 48, 56)                               |
| CD8 regulatory cytokines    |                                  |                              |                          |  |

### Skin Immune Responses to Vaccination

The skin of teleost fish is endowed with different components of the innate and adaptive immune system, which are responsive to vaccination as shown below.

### Innate Immune Responses to Mucosal Vaccination in the Skin

Mucus overlying the epidermis is the first line of defense against microbial invasion on the skin surface. It exerts its protective role by being continuously produced and sloughing off to prevent pathogen adherence. Second, it serves as a vehicle for several immune factors that include lysozymes, proteases, alkaline phosphatases, complement, immunoglobulins, lectins,

and C-reactive proteins that prevent pathogen invasion into the skin (14, 15, 62–64). Despite so, bath vaccination studies have shown that the primary site of attachment of *V. anguillarum* is the skin in which the bacteria cause local inflammatory responses upon attachment (65, 66). This causes the skin epithelial cells to phagocytose pathogens that attach to the skin surface (7, 65). And as such, different phagocytic cells have been characterized that include macrophages, granulocytes, goblet cells, sacciform cells, club cells, and malpighian cells in finfish (Table 3). In addition, dendritic-like cells have also been characterized from the skin mucosa of different fish species (67–69). The presence of monocytes, macrophages, and dendritic-like cells (67–71) indicates that the skin mucosa is endowed with APCs like other mucosal organs found in finfish. Upregulation of different toll-like receptors (TLRs) after pathogen invasions suggests that cells of the skin mucosa have sensors that are able to detect and bind to pathogen-associated molecular patterns (PAMPs) expressed by invading pathogens (72, 73). Li et al. (74) showed upregulation of TLR2 in orange-spotted grouper (*E. coioides*) exposed to *Cryptocaryon irritans* infection while Zhao et al. (75) showed increased levels of TLR1, 2, and 19 in response to *Ichthyophthirius multifiliis* infection in channel catfish (*Ictalurus punctatus*). It is interesting to note that upregulation of TLRs genes has been shown to correspond with upregulation of proinflammatory genes. For example, Li et al. (74) showed upregulation of TLR2 that coincided with upregulation of IL-1 $\beta$  in orange spotter grouper exposed to *C. irritans* pointing to antigen recognition by APCs orchestrated by proinflammatory genes.

In summary, the skin mucosa of finfish exert its protective mechanisms by (i) having mucus, which prevents pathogen attachment by continuously being produced and sloughing off, (ii) the presence of different host defensins that degrade, opsonize, and neutralize invading pathogens, (iii) carrying out phagocytosis of pathogens that attach onto the skin surface, and (iv) carrying out antigen uptake and processing by APCs that activate the adaptive immune system thereby creating the basis for mucosal vaccination via the skin.

**TABLE 3 | Innate immune components of the skin mucosa of finfish.**

| Component                    | Regulatory/effectors cells/genes | Reference    |
|------------------------------|----------------------------------|--------------|
| Mucus and surface defensins  | Muc5B                            | (13)         |
|                              | Antimicrobial peptides           | (14, 27)     |
|                              | Complement system                | (76)         |
| Cell types                   | Macrophages                      | (71, 72)     |
|                              | Granulocytes                     | (71, 72)     |
|                              | Goblet cells                     | (14, 77, 78) |
|                              | Sacciform cells                  | (78)         |
|                              | Club cells                       | (78)         |
|                              | Malpighian cells                 | (78, 79)     |
| Pattern recognition receptor | Toll-like receptors              | (72, 73)     |
| Proinflammatory cytokines    | IL-1 $\beta$                     | (27, 80)     |
|                              | TNF $\alpha$                     | (80, 81)     |
|                              | IL-6                             | (27, 82)     |
|                              | IL-8                             | (27, 80, 81) |
| Chemokines                   | CXCR4                            | (83)         |
|                              | CXCL10                           | (30)         |
|                              | CK1, 3, 9, and 11                | (84)         |

## Adaptive Immune Responses to Mucosal Vaccination in the Skin

All Ig isotypes characterized in fish have been detected in the skin mucosa (Table 4) and as pointed out by Zhang et al. (10), IgT is the major Ig isotype found in the skin mucosa although IgM is also present but in low quantities. In general, fish antibodies are protective against invading pathogens on mucosal surfaces as shown by Wang et al. (85, 86) and Dickerson and Clark (87) that naive fish exposed to sub-lethal infection of *I. multifiliis* become resistant to subsequent challenge. In their findings, they observed that resistance correlated with antibody levels in sera and skin mucus of immune fish. They further noted that antibodies from resistant fish easily bound to the immobilization antigens (i-antigens) found on the parasite cell and ciliary membranes. Antibody-mediated cross-linking with i-antigens resulted in expulsion of the parasite from exposed fish indicating that mucosal antibodies have the capacity to eliminate pathogens from mucosal surfaces in finfish. Recovered fish were protected from subsequent exposure to the parasite for a long time indicating that sub-lethal infection of *I. multifiliis* could serve as a live vaccine. These studies showed that exposure to *I. multifiliis* infection through the skin mucosa induced both mucosal and systemic antibody responses. In addition, they noted that vaccinating fish using purified i-antigens, provided long-lasting protective immunity against *I. multifiliis*, which makes the i-antigens to be the most promising candidate for subunit vaccine production.

In another study, Zhao et al. (92) showed that antibody-secreting cells (ASC), which include B-cells, plasmablasts, and non-replicating plasma cells, were found in low numbers in the skin of channel catfish. However, after immunization against *I. multifiliis* these antibodies increased by 20-fold. Thereafter, the number of ASC in the skin remained high for a long time and they were shown to confer long-term protective immunity against reinfection. However, it is vital to point out that although antibody responses were protective against parasitic infections such as *I. multifiliis*, there are limited studies that show protective immunity against viral and bacterial infections in the skin (93, 94). Hence, there is need for more studies to determine the protective mechanisms of skin antibodies for other pathogens. However, based on studies carried out this far (85–87, 92, 95),

**TABLE 4 | Adaptive immune components of the skin mucosa of finfish.**

| Component          | Regulatory/effectors cells/genes | Abbreviation   | Reference    |          |
|--------------------|----------------------------------|----------------|--------------|----------|
| Humoral responses  | Cell types                       | B-lymphocytes  | (7, 42, 88)  |          |
|                    | Immunoglobulins (Ig)             | IgT/IgZ        | (7, 40, 42)  |          |
|                    |                                  | IgM            | (27, 76)     |          |
|                    |                                  | IgD            | (7, 8, 10)   |          |
| Cellular responses | Major histocompatibility         | MHC-II         | (76, 81)     |          |
|                    |                                  | Th17           | (89)         |          |
|                    |                                  | Th2            | (90)         |          |
|                    |                                  | T-bet          | (46)         |          |
|                    | Transcription                    | GATA-3         | (27, 46, 90) |          |
|                    |                                  | CD4-Regulatory | IL-4/13      | (27, 90) |
|                    |                                  | cytokines      | IL-10        | (27)     |
|                    |                                  |                | IL-12        | (91)     |
|                    |                                  | PD-1           | (54)         |          |
|                    |                                  | TGF- $\beta$   | (81, 82)     |          |

indications are that vaccination via the skin mucosa can induce protective antibody responses thereby creating the basis for vaccination by immersion or bath exposure as shown that fish exposed to *I. multifiliis* via the skin were protected by mucosal antibodies against subsequent exposure.

As for cellular-mediated immunity, there are no studies that categorically show the ability of vaccine-induced CD8 T-cell responses to prevent microbial invasion into the skin mucosa of vaccinated fish. In general, only a few studies have been carried out on cellular-mediated immunity found in the skin mucosal of finfish (27, 96). However, it is interesting to note that induction of humoral immune responses due to parasitic flagellate *Ichthyobodo necator* infection correlated with increased CD4<sup>+</sup> T-cell expression when CD8 $\alpha$  was downregulated in the skin of rainbow trout (27). In their study, Chettri et al. (27) observed upregulation of the transcription factor GATA-3 and IL-4/13A, which correlated with upregulation of IgM<sup>+</sup>, suggesting that exposure to *I. necator* induced CD4<sup>+</sup> responses in the skin mucosa of infected rainbow trout whose kinetics are similar to those induced by inactivated IPNV vaccines in Atlantic salmon (47). In general, these findings demonstrate that the cellular-mediated immune system of the skin mucosa in finfish is responsive to antigen stimulation and that increased CD4<sup>+</sup> and IL-4/13A levels could play an important role in enhancing antibody functions as shown in mammals (97–99).

### Gill Mucosal Responses to Vaccination

Similar to other mucosal organs in finfish, immune responses to vaccination in the gills are coordinated by different innate and adaptive immune responses as shown below.

#### Innate Immune Responses to Mucosal Vaccination in the Gills of Finfish

Similar to other mucosal organs of finfish, the gill surface is covered by a mucus layer that contains complement, antimicrobial compounds, antibodies, and other surface defensins (Table 5). Different phagocytic cells have been characterized in the gills of different fish species and these include monocytes, macrophages, rodlet cells, eosinophilic granular cells, and neutrophils (Table 5). In addition, dendritic-like cells have also been reported from the gills (100). The expression pattern of CD83, a known surface marker of activated dendritic-like cells in fish (101–103), was shown to correlate with increased uptake of poly(I:C) in the gill mucosa of turbot (*Scophthalmus maximus*) (104), suggesting that dendritic-like cells in finfish express surface markers characteristic of activated APCs after antigen uptake. In addition, upregulation of IL-1 $\beta$ , IL-8, and TNF $\alpha$  has been shown to correspond with upregulation of TLR genes after vaccination in gills of different fish species (23, 25, 74, 105). Furthermore, some studies have also reported of upregulation of chemokines such as CXCL10, CK3, CK9, CK11, and CK12 that correspond with the homing of APCs to antigen deposition sites in the gills (35, 106–108). Put together, these observations show that the gill mucosa in finfish is bestowed with (i) a mucus layer that serves as a vehicle for host defensins, (ii) APCs comprising monocytes, macrophages, and dendritic-like cells, and (iii) regulatory cytokines and chemokines able to coordinate the functional activities of APCs. Therefore, it can

**TABLE 5 | Innate immune components of the gill mucosa of finfish.**

| Component                    | Regulatory/effector cells/genes | Reference                  |
|------------------------------|---------------------------------|----------------------------|
| Mucus surface defensins      | Muc5B                           | (13)                       |
|                              | Complement system               | (16, 18, 109)              |
|                              | Antimicrobial peptides          | (15, 109, 110)             |
| Cell types                   | Macrophages                     | (111, 112)                 |
|                              | Rodlet cells                    | (20)                       |
|                              | Eosinophilic granulocytes       | (111, 113, 114)            |
|                              | Neutrophils                     | (111)                      |
| Pattern recognition receptor | Toll-like receptors             | (23, 72, 92, 111, 114–116) |
|                              | Peptidoglycan PR                | (25)                       |
| Proinflammatory cytokines    | IL-1 $\beta$                    | (117–121)                  |
|                              | IL-8                            | (121)                      |
|                              | TNF $\alpha$                    | (119–121)                  |

be concluded that the gill mucosa is endowed with a functional innate immune system responsive to vaccination able to activate the adaptive immune system.

#### Adaptive Immune Responses to Mucosal Vaccination in the Gills of Finfish

Adaptive immune components of the gut identified in finfish are shown in Table 6. Olsen et al. (105) have shown that IgT<sup>+</sup> B-cells are predominantly located in the epithelial lining of the gill lamellae, suggesting a primary role of this Ig isotype in mucosal defense against pathogen invasion, whereas IgM positive cells are located in gill arterioles and lamellar capillaries. Kai et al. (26) showed that IgM and IgT levels in the gill mucosa were only upregulated by bath or immersion vaccination, suggesting that the route of vaccine delivery had an influence on the induction of humoral immune responses in the gills. Jorgensen et al. (108) showed that IgT and IgM were able to bind to the surface structures of *I. multifiliis* in the gills of immune rainbow trout shortly after invasion. In their studies, they noted that IgT<sup>+</sup> B-cells were predominantly located in the gill epithelia in the secondary lamellae corresponding to observations made by Olsen et al. (105). Parasites in immune fish were stained with IgT and yet no stain was detected from parasites from non-immune fish. They also observed that IgM<sup>+</sup> B-cells were only found inside the capillaries of the secondary gill lamellae and yet the parasites located on the gill surface were stained with IgM, suggesting that there is possible diffusion of serum IgM from the systemic compartment to the exterior surface for it to stain the parasites at the mucosal surface within hours after penetration. In summary, these studies show that (i) there is compartmentalization in the distribution of IgT and IgM with the former being on the outer surface of the epithelia lining while the latter being in the capillaries and (ii) both IgM and IgT have the capacity to bind the exterior surfaces of invading pathogens.

Several scientists have shown the activation of naïve CD4 and CD8 T-cells in the gill mucosa of different fish species (58, 126, 127). Takazawa et al. (58) showed that CD8 $\alpha$  cells account for a large proportion of the total lymphocytes found in the gills of salmonids. It is interesting to note that induction of MHC-I and CD8<sup>+</sup> responses in the gills is mostly by bath or immersion

**TABLE 6 | Adaptive immune components of the gill mucosa of finfish.**

| Component                   | Regulatory/effector cells/genes | Abbreviation              | Reference                  |
|-----------------------------|---------------------------------|---------------------------|----------------------------|
| Humoral responses           | Cell types                      | B-lymphocytes             | (7)                        |
|                             |                                 | Immunoglobulins (Ig)      | IgT/IgZ (40, 42, 109, 110) |
|                             |                                 | IgM                       | (121–123)                  |
|                             |                                 | IgD                       | (35)                       |
| Cellular responses          | Major histocompatibility        | MHC-I                     | (119)                      |
|                             |                                 | MHC-II                    | (109, 110, 119)            |
|                             | T-helper cells                  | Th1                       | (46, 124)                  |
|                             |                                 | Th2                       | (90)                       |
|                             |                                 | Treg                      | (45, 49)                   |
|                             |                                 | CD4 Transcription factors | GATA-3 (46, 90)            |
|                             | Chemokines                      | T-bet                     | (46, 49, 125)              |
|                             |                                 | FoxP3                     | (45, 47, 50)               |
|                             |                                 | CCR6                      | (29)                       |
|                             |                                 | CCR7                      | (35, 107)                  |
|                             |                                 | CCR9B                     | (29)                       |
|                             | CD4-Regulatory cytokines        | CXCL10                    | (30)                       |
|                             |                                 | CK1, 3, 9, 11 and 12      | (29)                       |
|                             |                                 | IL-2                      | (125)                      |
|                             |                                 | IL-4/13                   | (90)                       |
|                             |                                 | IL-10                     | (124)                      |
|                             |                                 | IL-22                     | (125)                      |
|                             |                                 | IFN $\gamma$              | (125)                      |
|                             | Cell type                       | TGF- $\beta$              | (119)                      |
|                             |                                 | T-lymphocytes             | (124)                      |
| CD8 T-cells                 |                                 | CD8 $\alpha$ (124)        |                            |
| T-cell receptor             |                                 | TCR genes (124)           |                            |
| CD8 transcription factor(s) |                                 | Eomesodermin (48, 56)     |                            |
| CD45                        |                                 | CD45 (124)                |                            |
| CD8 regulatory cytokines    |                                 |                           |                            |

vaccination (26, 52). Kai et al. (26) and Overgard et al. (52) observed that upregulation of CD8 $\alpha$  cells coincided with upregulation of MHC-I after vaccination using viral antigens pointing to possible activation of CD8 cells via the MHC-I pathway. Overgard et al. (53) also showed upregulation of TCR genes alongside upregulation of MHC-I and CD8 $\alpha$  pointing to possible binding of TCRs to MHC-I molecules leading to activation of CD8 $\alpha$  cells. Moreover, Aquilino et al. (128) showed upregulation of CD3 genes and perforins alongside increased levels of CD8 $\alpha$  and MHC-I in rainbow trout exposed to viral hemorrhagic septicemia virus (VHSV). Put together, these studies show that the gill mucosa is endowed with different components of CD8 T-cell mediated immune genes ranging from TCRs that bind to antigens presented on MHC-I molecules to CD3 molecules that activate naïve CD8 T-cells to effector CTLs and perforins that carry out cytotoxicity killing of cells infected with intracellular pathogens. However, there is need for detailed investigations to elucidate the role of CTL responses in mucosal vaccine protection in finfish.

As for CD4 T-cell responses, activation of naïve CD4 T-cells into effector subtypes is via the MHC-II pathway (34). Olsen et al. (105) showed that MHC-II cells were distributed across the gill filaments where they accumulated in the hyperplastic tissue in rainbow trout. As shown in **Table 6**, different transcription factors that specify the differentiation of naïve CD4 cells into different T-helper (Th) subtypes have been identified and characterized in the gills of different fish species. In addition, different cytokines

and chemokines that regulate the functional activities of different Th-cells have also been characterized in different fish species. Studies carried out by Takizawa et al. (90) have shown that the constitutive expression of IL-4/13 and GATA-3 skewed toward a Th2 response in gill cells of isogenic rainbow trout exposed to poly(I:C). In their studies (90), they showed that the kinetics of IL-4/13A and IFN $\gamma$  in the gill cells were consistent with expression patterns observed from fish vaccinated by injection (47). In general, these studies suggest that CD4 T-cells are responsive to antigen stimulation in the gill mucosa of finfish. Therefore, future studies should seek to identify the helper roles of different Th-subtypes in conferring protective immunity in fish vaccinated via the gill mucosa.

### Nasal Mucosal Responses to Vaccination

Recent advances have shown that the nasal mucosa is endowed with an innate and adaptive immune system comparable to other mucosal organs in finfish (9). Studies carried out by Tacchi et al. (9) have shown that the nasal mucosa expressed different cytokines, chemokines, antimicrobial peptides, complement factors, and TLR genes in rainbow trout vaccinated against *Yersinia ruckeri*. In addition, it also expressed MHC-II genes as well as different B-cell receptor and TCR genes in response to vaccination against *Y. ruckeri*. All three Igs characterized in fish were detected in the nasal mucosa with IgT being the most abundant followed by IgM (9). Tacchi et al. (9) and La Patra et al. (129) showed significant protection against infectious hematopoietic virus infection (IHNV) and *Y. ruckeri* in rainbow trout vaccinated via the nasal mucosa after challenge. In general, indications are that the nasal mucosa uses similar protective mechanisms with those used by other mucosal organs in finfish. However, given that the nasal mucosa is a recently discovered mucosal organ whose protective mechanisms have only been reported in rainbow trout, there is need for follow-up studies in order to consolidate these findings in other fish species.

### General Discussion and Conclusion

In general, teleosts fish are endowed with different mucosal organs that include the gills, gut, skin, and nasal mucosa (6, 7, 9, 10). It is interesting to note that all mucosal organs have an innate immune systems, which is made of three important elements, namely (i) mucus whose function is not only to prevent microbial attachment to mucosal surfaces by continuously being excreted and sloughed-off but it serves as a vehicle for antimicrobial peptides, complement, and Igs that degrade, opsonize, and neutralize invading pathogens on mucosal surfaces; (ii) APCs that carry out antigen uptake, processing, and presentation to cells of the adaptive immune systems (130); and finally (iii) proinflammatory cytokines and chemokines that coordinate the functional activities of APCs. Overall, this review shows that the innate immune systems in different mucosal organs is responsive to mucosal vaccination and that it has the capacity to activate cells of the adaptive immune systems in finfish.

It is interesting to note that all three Ig isotypes characterized in finfish have been detected in all mucosal organs (6, 7, 9, 10). Based on studies carried out this far, it is evident that mucosal antibody

responses are the only component of the adaptive immune system that has been shown to confer protective immunity in vaccinated fish (85, 86, 131) although the majority of studies that support this observation are based on the neutralizing ability of mucosal antibodies on parasitic infections (85, 86, 131) with only a few studies showing protection against viral and bacterial infections (93, 94). Nevertheless, it is interesting to note that there is compartmentalization in the expression of different Ig isotypes with IgT being the major isotype in mucosal organs while IgM is mostly found in systemic circulation (7, 10, 40). In addition, there is compartmentalization in terms of the physical distribution of IgT and IgM in some mucosal organs as shown that in the gills IgT is mainly found on exterior surfaces of the gill lamellae (105, 131), suggesting that this isotypes functions as a gatekeeper at the portals of entry whereas IgM is mostly found in the arterioles (105, 131), suggesting that its role is to prevent systemic dissemination of pathogens as a secondary defense strategy in situation where IgT on mucosal surfaces fails to prevent the penetration of pathogens into the systemic environment.

Although cellular-mediated immunity is present in all mucosal organs, its existence is mostly demonstrated by gene expression. The ability of these genes to prevent microbial invasion in vaccinated fish has not been clearly elucidated although the kinetics of CD4<sup>+</sup> and CD8<sup>+</sup> gene expression in different mucosal organs suggests that cellular-mediated immunity is responsive to vaccination in finfish. And as pointed out in our previous studies (5, 34, 35), the route of antigen delivery into APCs is deterministic of the type of cellular-mediated immune response induced by vaccination. Antigens delivered via the intracellular route evoke both CD4<sup>+</sup> and CD8<sup>+</sup> responses whereas antigens delivered by the extracellular route only induce CD4<sup>+</sup> responses. And as pointed out by Howarth and Elliot (132), the most protective vaccines are those that stimulate both CD4<sup>+</sup> and CD8<sup>+</sup> responses. Therefore,

live virus and DNA vaccines that evoke both CD4<sup>+</sup> and CD8<sup>+</sup> responses are likely to produce better protection than inactivated vaccines. Although live vaccines produce long-lasting protective immunity given that they are replicative and are more immunogenic than inactivated vaccines (133, 134), they are less used in aquaculture because of the fear of reversion to virulence as shown in the case of IPNV that avirulent strains can revert to virulence under stress conditions (135). On the other hand, DNA vaccines that are also delivered via the intracellular route are not widely used in aquaculture for ethical reasons because of their genetic modified nature (34). This far, only the IHNV-DNA vaccine has been licensed for commercial use in Canada. Hence, the majority of commercial vaccines currently used in aquaculture are inactivated vaccines, which are limited to induction of CD4<sup>+</sup> and humoral immune responses. Hence, there still remains the challenge of developing intracellular antigens delivery systems able to evoke of CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses that do not pose the danger of reversion to virulence.

This review summarizes the different components of the innate and adaptive immune systems in mucosal tissues of fish, which are activated following mucosal infection and/or vaccination studies. Based on the synopsis put forth in this review, mucosal antibodies appear to play a key role in conferring protective immunity in vaccinated fish (85, 86, 92, 93, 95). On the other hand, there still remains the challenge of elucidating the ability of mucosal vaccine-induced cellular-mediated immune responses to protect fish against microbial invasion in mucosal organs.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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