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Adaptive immune responses at mucosal surfaces of teleost fish

Jan H.W.M. Rombout [a, b], Guiwen Yang [b, c], Viswanath Kiron [a, *]

[a] Faculty of Biosciences and Aquaculture, University of Nordland, 8049 Bodø, Norway
[b] Cell Biology and Immunology Group, Wageningen University, Wageningen, The Netherlands
[c] Shandong Provincial Key Laboratory of Animal Resistance Biology, School of Life Sciences, Shandong Normal University, Jinan 250014, China

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ABSTRACT

This review describes the extant knowledge on the teleostean mucosal adaptive immune mechanisms, which is relevant for the development of oral or mucosal vaccines. In the last decade, a number of studies have shed light on the presence of new key components of mucosal immunity: a distinct immunoglobulin class (IgT or IgZ) and the polymeric Ig receptor (pIgR). In addition, intestinal T cells and their putative functions, antigen uptake mechanisms at mucosal surfaces and new mucosal vaccination strategies have been reported. New information on pIgR of Atlantic cod and common carp and comparison of natural and specific cell-mediated cytotoxicity in the gut of common carp and European seabass, is also included in this review. Based on the known facts about intestinal immunology and mucosal vaccination, suggestions are made for the advancement of fish vaccines.

1. Introduction

Aquaculture is a fast-growing food producing sector, and health management of the cultured species is critical for the sustainable growth of the industry. In this context, mucosal health of fish should be given prime importance as mucosal surfaces like the skin, the gills, the gut and the urogenital system constitute the first line of defence. The importance of mucosal barriers in aquatic animals is far more than those of their terrestrial counterparts as the aquatic species are continuously interacting with the microbiota in their environment. Over the last decades, efforts have been made to gain a better understanding of mucosal immune system, which in turn helps to develop vaccination strategies aimed at maximizing mucosal and consequently organismal health.

Vaccination is the most-appropriate method for the control of disease-causing pathogens from the economic, environmental and ethical point of view. At present, fish are commonly vaccinated by injection or immersion methods. Injection route is in general very effective, but it is labour-intensive and only practiced for high-value species like Atlantic salmon, *Salmo salar*. All life stages are prone to diseases, especially the early phases during which disease-related mortality frequently occurs. In farms, the young animals are subjected to immersion vaccination since it is not feasible to inject them individually. Novel vaccination methods that are cost-effective, simple, effortless, and less stressful to animals of all stages including young fish should be developed for aquaculture. The ideal technique that fulfils these criteria is oral vaccination (via feed), although this delivery route is not commonly used by the industry [1–4]. Modern tools such as nano-technology, which can be used to manipulate vaccines' size, cell-targeting and amount, may be adopted in aquaculture too [5].

More knowledge on both the antigen delivery and the mucosal immune defence systems, in particular on the mucosal adaptive immune responses in fish, should be generated. Peyer's patches, antigen transporting M cells, IgA- and the IgM-joining J chain – all the essential components of the mammalian mucosal immune system – are not yet reported in teleost fish [2]. The first inferences on local and/or mucosal responses of a variety of fish species were based on the detection of specific antibodies in mucosal secretions after intestinal [6–11] or immersion [12–15] immunisations. Nevertheless, upon systemic immunisation these specific mucosal antibodies were not or hardly detected. This differential generation of specific antibodies and the new information on specific antibody-producing cells at mucosal sites after intestinal [3,11] or immersion [14,15] vaccination inspired many scientists to study mucosal structures in different teleosts. The present review focuses on the mucosal adaptive immune system in fish. In fact, it is rather surprising that after the first publication on successful oral vaccination of rainbow trout, *Oncorhynchus mykiss* in 1942 [16] not much information on mucosal immunology in fish has been

* Corresponding author. Tel.: +47 755 17399.
E-mail address: kvi@uin.no (V. Kiron).

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gathered compared to the knowledge on the mammalian mucosal immune system. For instance, concrete evidence on the existence of a common mucosal immune system and a separate mucosal immunoglobulin class or isotype has not yet been reported.

This review gives an insight into antigen uptake at the mucosal surfaces and subsequent local responses, the transport of immunoglobulins to mucosal surfaces by the polymeric Ig Receptor (pIgR) and its role in immune defence. Further, the possible functions of the abundant number of intraepithelial lymphocytes (mainly T cells) in the mucosal epithelia and the induction of oral tolerance in fish are also described. In addition, the significance of mucosal vaccination is summarized.

2. Mucosal *vs* systemic antigen responses

The most commonly used fish vaccination methods are injection [intraperitoneal (ip) or intramuscular (im)] and immersion (bath or spray). Besides these methods, antigens could be delivered via feeds – oral vaccines. The ip or im injections can be considered as systemic vaccinations since they produce only internal immune responses that are easily detectable in blood. In mammals, ip injection has also been claimed as a suitable priming route prior to oral vaccination [17]. In fish, ip injection can induce a certain degree of mucosal responses [18]. Immersion vaccination of fish, on the other hand, leads to uptake by the skin, the gills and the gut (after drinking) [19], subsequently inducing local responses. It has been reported that a hyperosmotic stressor, applied ahead of the immersion vaccination, brings about better uptake and higher responses, mostly at the mucosal surfaces [13]. Nevertheless, it is necessary to discover appropriate adjuvants that can reduce the amount of antigens required for mucosal vaccination. In fact, although many mucosal adjuvants for fish have been patented (see http://www.patentfish.com/as-mucosal-adjuvants), not many are being used for practical purposes.

In mammals, exposure of mucosal surfaces to antigens results in the secretion of antigen-specific IgA at these locations. Mammals have a common mucosal immune system, in which stimulation of one epithelium can also give rise to specific IgA or IgM responses in other mucosal organs, aided by the so-called systemic and mucosal homing receptors on immune competent cells [20,21]. It is not yet clear if fish possesses a common mucosal system or not. Till now specific homing of mucosal leucocytes has not been clearly detected [2,3], although suggestions on a homing model have been made by Fillatreau et al. [22]. However, evidences indicate induction of specific antibodies in the skin mucus, but not in the serum, following oral vaccination [7,8]. Orally administered antigens are taken up and transported via the end gut (the so-called 2nd segment), and if an adequate amount of antigen reaches this segment, local as well as systemic antibody responses are induced in fish [8]. On the other hand, when antigens are delivered anally they reach the 2nd segment immediately, and, therefore, even a small amount of antigen is sufficient to evoke systemic responses and memory formation [8,9]. Mucosal vaccines can be effective immune stimulators only if the antigens can reach the correct inductive sites and do not induce oral tolerance as suggested by Kim and Jang [23]. In addition, the efficacy of these vaccines in fish needs to be confirmed through pathogen challenge studies.

3. Mucosal antibodies

The spatial and quantitative differences in generation of specific antibodies in fish strongly suggest that differences exist between mucosal- and systemic-derived antibodies. Such differences were first reported in 1981 by Lobb and Clem [24], based on the presence of secretory component bound to dimeric Ig molecules in the skin mucus of sheepshead, *Archosargus probatocephalus*. A decade later, differential binding of monoclonal antibodies (mAb) to mucosal- and serum-derived IgM (mainly tetramers and dimers) was described in common carp, *Cyprinus carpio* [25]. The mAb (WCIM) derived from the skin mucus IgM recognized IgM heavy (H) chain of the skin mucus of common carp, but not that of the serum; strong and specific immunohistochemical reactions were also observed at mucosal Ig-localised sites such as the bile capillaries, ducts and the skin epithelium [25]. On the contrary, another mAb (WCI12), which is derived from serum IgM and that recognizes both H chains could be used for the detection of mucosal responses after intestinal and immersion immunisation, although it had a lower affinity for mucus IgM.

A new type of immunoglobulin H chain class has been reported in fish. In zebrafish, *Danio rerio* [26], common carp [27], mandarin fish, *Siniperca chuatsi* [28] and grass carp, *Ctenopharyngodon idella* [29] it is called IgZ, but in rainbow trout [30], Atlantic salmon [31] fugu, *Takifugu rubripes* [32], three spined stickleback, *Gasterosteus aculeatus* [33] and two Perciform species [cf [34]] it is termed IgT. The IgT in rainbow trout was suggested to have a role in mucosal immunity [34,35]. Among the two IgZ isotypes in carp, IgZ2 has a preference for mucosal tissues, while IgZ1 is associated with systemic organs [36]. IgZ2 appears to be a chimeric form having both μ1 and ζ4 domains, and trout IgT lacks this μ1 domain [22].

In addition to IgM and IgT/Z, IgD has also been described in a variety of teleosts [37–43]. Although it is known that IgD can be secreted [43], its involvement in mucosal responses has not been clarified. Histochemical observations on the digestive tract of rainbow trout [44] have revealed the preference of IgM+ cells in the lamina propria and IgT+ cells in the epithelium. These data indicate that the intraepithelial lymphocytes (IELs) are not exclusively T cells as thought before and hence the intestinal epithelium also seems to be a site where B cells are recruited. In rainbow trout, oral vaccination with an alginate encapsulated DNA vaccine against IPNV resulted in increased IgM+ and IgT+ B cell populations, an indication that both B cells are important for mucosal responses [44]. However, Zhang et al. [34,35] reported that IgT is the main immunoglobulin responsible for mucosal immunity. It has to be noted that the aforementioned studies [35,44], differed in the pathogen examined (parasite *vs* virus) and the timing of the responses measured (late *vs* early). In addition to the already assigned mucosal role of IgT, its involvement in systemic responses cannot be neglected as observed in trout spleen [45]. Accordingly, Castro et al. [45] has described intestinal IgM+ and IgT+ cells in trout as B cells, even though immunocytochemical observations do not provide any evidence on the presence of plasma cells. In a much earlier study on common carp, staining (mAb WCI12) of the gut IELs for membrane and cytoplasmic IgM indicated that the majority of Ig+ IELs were small plasma cells; having a rim of Ig+ cytoplasm and a minor amount of membrane Ig [46]. These findings in trout and carp may be pointing to the fact that teleost gut has a limited number of classical plasma cells and that they are not easily detectable in the mucosal tissues. Further investigations are essential for understanding the existence and role of IgZ2 or IgT plasma cells in the gut of teleosts.

A variety of Ig genes is present in fishes. The evolutionary origin of the mucosa-associated IgT is yet to be clarified, and its appearance in some lineages of bony fishes could be due to selection pressures arising from the necessity to protect the mucosal surfaces [47]. Further, IgT/Z shares many functional similarities with mammalian IgA [22]. Even if IgT/IgZ cannot serve as IgA equivalent in teleosts, we cannot neglect the “power” of alternative splicing of pre-mRNA in fish, recently summarized by Maisey and Imarai [48] and Quiniou et al. [49]. Such splicing may also be responsible for differences in IgM heavy chains that can result in mucosal and

systemic IgM variants [22]. Similar mechanisms can result in organ-dependent differences in mucosal molecules. Even an amino acid difference or a minor carbohydrate change may be responsible for the differential behaviour of molecules in the mucosal immune system.

4. Mucosal antibody transport – pIgR and its functions

Polymeric immunoglobulins are considered as the main players of mucosal defence, and polymeric Immunoglobulin Receptor (pIgR) has an important role in the transport of the immunoglobulin molecules. The pIgR is a type 1 membrane glycoprotein that contains a cytoplasmic region, a transmembrane region and an extracellular region with five Ig-like domains (ILD1-5). In birds [50] and amphibians [51] only four ILDs of pIgR are reported. The highly conserved D1 region with three Complementarity-Determining Region-like loops (CDR1-3) is necessary for the initial ligand interaction [52]. However, binding of pIgR ILD1 to polymeric IgA and IgM depends on the CDR types, J chain and a heavy chain [52]. In mammals, the 15 kDa polypeptide termed J-chain is not required for the polymerization of IgA and IgM, but this peptide imparts the polymer's structural and functional characteristics [53]. The J-chain of mammals, birds and amphibians are all able to polymerize human IgA and IgM intracellularly while the J-chain of nurse shark, *Ginglymostoma cirratum*, cannot [51,54]. Till now a J chain has not been reported in any of the teleost species studied [55,56].

In mammals, pIgR is expressed by the mucosal epithelia and hepatocytes, and at these locations, it can bind polymeric IgA and IgM and transcytose them to the luminal sides and bile, respectively [57]. A study on pIgR-deficient mice has shown that this is the only receptor responsible for epithelial transport of the two Ig molecules [58]. Upon release to the apical plasma membrane domain, the extracellular part of the receptor is cleaved off by a proteinase and co-secreted with the IgA or IgM as a protective secretory component (SC) [20,21]. The pIgR amino acid sequences of seven teleosts were published in the past decade: fugu [59], carp [60], orange-spotted grouper *Epinephelus coioides* [61], rainbow trout [35], zebrafish [62], Atlantic salmon [63] and olive flounder *Paralichthys olivaceus* [55]. The seven pIgR sequences were aligned along with the sequence of the Atlantic cod *Gadus morhua* pIgR. The pIgRs of all 8 teleost species (Fig. 1) consist of only two ILDs, which correspond to the ILD1 and ILD5 of mammals [3,50,51,55,59–61,63]. It is obvious that all the three CDRs on ILD1 are absent in teleosts [2]. However, IgM binding studies showed that this small molecular weight pIgR can bind to teleost IgM [35,61] and IgT [35]. In addition, the skin epithelial cells, enterocytes and hepatocytes express pIgR cDNA [55,59–61,63], and pIgR could bind to IgM at these sites [59,60]. Therefore, the lack of a J chain and CDR1-3 in teleosts seems not to impede the binding of Ig to pIgR.

Zhang et al. has described a secretory component of 38 kDa, for the trout gut mucus (tSC), but not for the trout serum [35]. According to the authors, the molecular mass of this tSC was near to the theoretical molecular mass obtained from the sequence of pIgR. In addition, it was shown that this tSC was associated with the gut mucus IgT and IgM. In olive flounder, a recombinant pIgR could interact with both mucus and serum IgM, and a flounder secretory component (fSC) could be detected in the skin mucus and not in the serum [55]. The molecular mass of fSC is around 37 kDa, which is also reported to be near the theoretical mass of the sequence of olive flounder pIgR [55]. In fugu, an SC with a molecular mass of 60 kDa has been reported based on a Western blot analysis with a pIgR specific antibody [59]. However, our molecular weight calculations using ExPASy and protein calculator (http://protcalc.sourceforge.net/) revealed that most teleost SC can be around 30 kDa, at least when the signal peptide (SP), the transmembrane domain (TM) and the cytoplasmic region (CYT) are excluded from the sequence. Therefore, the 60 kDa SC reported in fugu [59] could be the product of post-translational modifications. Even the estimated sizes of 38 kDa [35] and 37 kDa [55] are overestimated, but that may be due to the inclusion of SP, TM and CYT, which are not included in the functional SC.

In fish, a number of *pigr* genes are discriminated, and they may have different putative functions in mucosal defence. Ten *pigr*-like genes are present on chromosome 2 of zebrafish, and they encode secreted and putative inhibitory membrane-bound receptors. Immune tissues express *pigr*-like genes as well as *pigr* transcripts, while lymphoid and myeloid cells have only *pigr*-like gene transcripts [62]. The *pigr* gene expression was significantly up-regulated in the mucosa of infected fish; after an ectoparasite (*Lepeophtheirus salmonis*) infection on the skin of Atlantic salmon [63] or a bacterial (*Vibrio anguillarum*) infection in the gut of carp (G. Yang, unpublished). In zebrafish, *pigr*-like gene expression was elevated during a bacterial (*Streptococcus iniae*) infection while the transcripts were down-regulated after viral (Snakehead rhabdovirus) infection [62]. Up-regulation of pIgR expression is an accepted phenomenon in mammals and seems to be infection-, inflammation- or cytokine-driven [64,65], although it also can be down-regulated, for instance, in the case of inflammatory bowel disease [64].

The pIgR may have a key role in maintaining the normal cross-talk between the commensal microbiota and the intestinal epithelial cells. In pIgR knock-out mice, the stability of the commensal microbiota was disturbed, and gut homeostasis was affected [66]. Further, lack of secretory-Ig increased the access of antigens to gastrointestinal immune system in mice [67]. In fish, very little is known on the role of pIgR in intestinal homeostasis. The pIgR sequence in Atlantic cod reported here (Fig. 1), could be useful in functional studies on this molecule. This fish is unique for its reliance on its innate immune system; it lacks antigen-transporting 2nd gut segment, produces very large amounts of mucus and IgM in its gut, and most of the IgMs can be considered as (natural) non-specific antibodies [68–70].

5. Mucosal T cells

An efficient immune system depends on self-referential T and B lymphocytes, which are part of the adaptive immune system [71]. In mammals, T cells are predominant in the intestinal epithelium, while B cells are mainly present in the intestinal mucosa [72]. Most of the lamina propria T cells express αβ-TCR with CD4 or CD8αβ. IELs are mainly CD8+ T cells, and they mediate cytolytic activity and express CD8αβ or CD8αα. These CD8αα-positive IELs also include the γδ-TCR+ T cells, and they express NK-cell receptors and mucosal integrin [72]. In addition, all mature T cells have CD3 consisting of ε, γ, δ, ζ polypeptide chains that assemble and form εγ, εδ or ζζ dimers. T- as well as B-cell receptors have variable (V), diversity (D) and joining (J) gene segments, and the assembly of antigen receptor variable gene causes the development of the final B- and T-cell repertoire [73,74]. V(D)J recombination is initiated by the recombination activating genes *RAG1* and *RAG2*, finally resulting in the production of T and also B cells with receptors (TCR and Ig, respectively) specific for particular antigens [74,75]. VDJ recombination by *rag* genes also occurs in fish [76–78]. In mammalian thymus, T lymphocytes are selected and strongly self-reacting T cells are deleted via the interaction between self-peptide and self-MHC molecules [71]. For the recognition of antigens, most T cells are dependent on MHC-I or MHC-II molecules that bind and present antigens to T cells. However, many IELs have the γδ TCR that

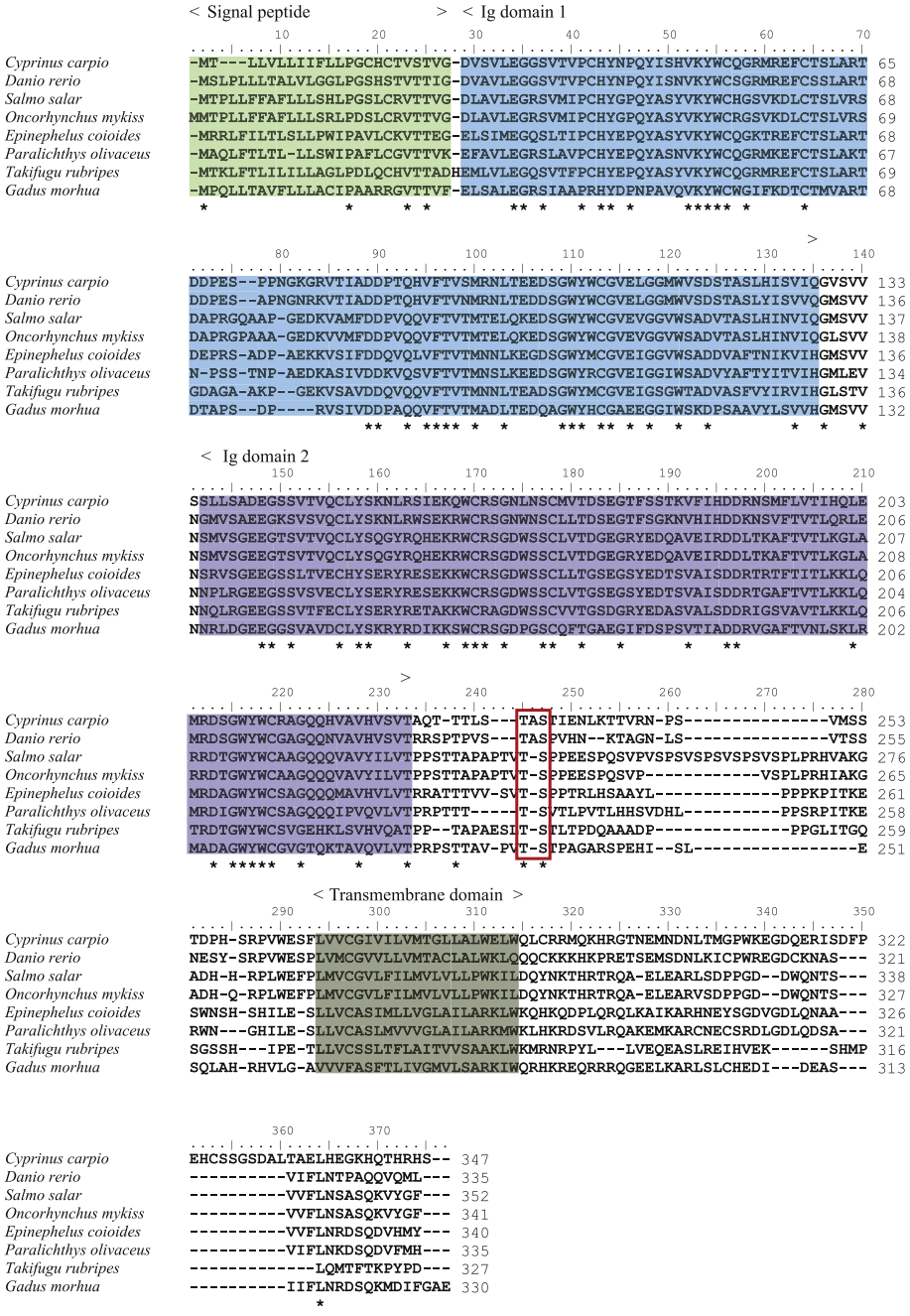

Fig. 1. Alignment of deduced polymeric Ig Receptor (pIgR) protein sequences of 8 teleost species: *Cyprinus carpio* (common carp; accession nr: ADB97624), *Danio rerio* (zebrafish; accession nr: XP694833), *Salmo salar* (Atlantic salmon; accession nr: ACX44838), *Oncorhynchus mykiss* (rainbow trout; accession nr: ADB81776), *Epinephelus coioides* (orange spotted grouper; accession nr: ACV91878), *Paralichthys olivaceus* (olive flounder; accession nr: HM536144), *Takifugu rubripes* (fugu; accession nr: BAF56575) and *Gadus morhua* (Atlantic cod; accession nr: KJ460333). In the putative cleavage domain of the pIgR, T(A)S is shown in a red box. This alignment is done manually. Preliminary results in carp indicated specimen- and organ-dependent absence of the amino acid A. The signal peptide is shaded green, the Ig domain 1 is shaded blue, the Ig domain 2 is shaded purple and the transmembrane domain is shaded olive green. Asterisks indicate fully conserved residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

can function without interference of MHC class I or II and hence they form a bridge between innate and adaptive immune systems [79,80]. It has been suggested that the γδ TCR in seabass acts more as a pattern recognition receptor in contrast to the more specific αβ TCR [80]. It has also been reported that memory γδ T cells of intestinal tissues are multifunctional and provide protection against pathogens [81]. These T cells play an active and regulatory role in maintaining the integrity of epithelial tissues, induce cytolysis of infected cells, support mucosal IgA production, maintain epithelium homeostasis, and have a role in oral tolerance induction (cf [2]).

As in mammals, teleost fish also have thymus-derived T cells that can be subdivided into distinct subpopulations, such as cytotoxic T cells, helper T cells, regulatory T cells, γδ T cells and non-specific cytotoxic cells (NCC). Although many fish T cell specific antibodies have been available, those that recognize the well-defined T cell molecules were unavailable. In the last decade, genes encoding a number of cell marker molecules including *Cd3*,

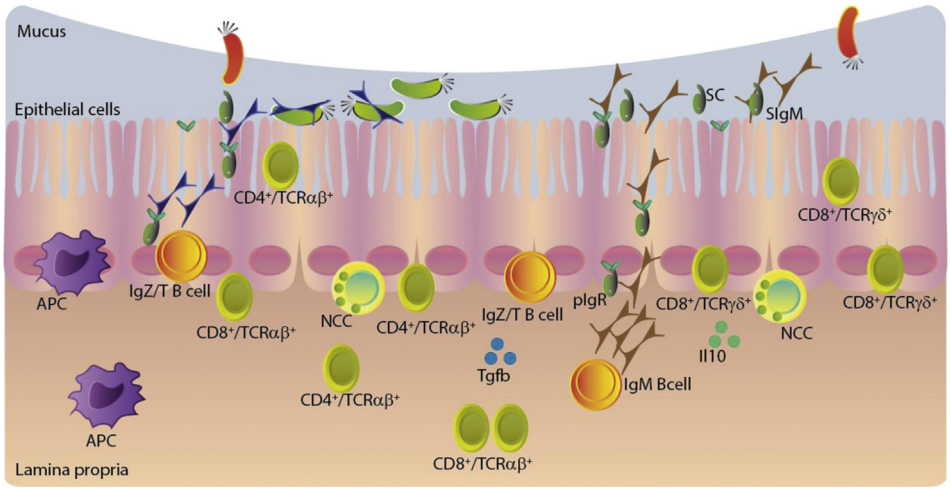

Fig. 2. Schematic representation of different immune cells in the teleost intestine, based on the extant knowledge. CD8α+ TCRαβ T cells dominate the CD4+ subset. Most TCRγδ T cells are probably CD8α+. The majority of B cells among IEL is IgT/Z+, while IgM+ B cells are merely present in the connective tissue. A part of the IEL may be non-specific cytotoxic cells (NCC), indicated as small granular lymphocytes. Antigen presenting cells (APC) are also shown. Commensal microbes (green) are coated with Ig. Pathogenic microbes are shown in red. In addition to immune cells, cytokines Il10 and Tgfb are included as they are the main effectors in oral tolerance induction. The transport of immunoglobulins by pIgR towards the lumen, the cleavage of pIgR extracellular component and delivery to the mucus as pIg–SC complex or as SC alone are also illustrated. The existence of dendritic cells in fish gut is debatable. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cd4, *Cd8*, *Mhc I* and *Mhc II* were described in a variety of fish species, and the increasing availability of the relevant antibodies will improve our understanding of the fish immune system [82]. T cells are abundant in mucosal tissues (the gut, the gills and the skin) of teleosts, and it is already known that teleost gut contains abundant numbers of T cells [2,82–87]. However, only recently the presence of CD3ε+ T cells in interbranchial lymphoid tissue of salmon gills was reported [85,86] and the authors are convinced that this type of tissue will be discovered in other teleost species too. The best-studied mucosal T cells in fish are IELs, but there is not much information on their functional relevance [83,84,86,87]. In carp, a specific T cell mAb (WCL38; [88]) has been found to react with around 50% of the mucosal T cells, but seldom with peripheral and thymic T cells. This antibody revealed positive IELs at 3 days post fertilization, one day before the thymus starts to populate with lymphoid cells. In European seabass, *Dicentrarchus labrax* comparable results were obtained using the "pan" T cell mAb (DLT15; [76,78]). In mammals, local intestinal T cells originate from the intestinal immune compartment [89]. These so-called cryptopatch T cells have CD8αα rather than thymus-derived mature T cells having CD8αβ [75]. However, the claim that intestinal intra-epithelial αβ T cells are largely derived from thymus, rather than from cryptopatch cells [90,91] is presently debated [92]. In species such as carp and seabass an extra-thymic origin has also been speculated for at least a subpopulation of mucosal T cells [2,76,78]. The *rag1* expression in the thymus as well as in the intestinal epithelium indicates that the recombination of immune receptors (probably TCR) can occur in both organs [77,93]. As mentioned above, an extra-thymic origin of IELs has been suggested in mammals too [89,94–96]. In addition, decades ago it was shown in mammals that TCRγδ/CD8αα IEL can develop in the absence of a functional thymus [97] and more recently the role of the gut as a primary lymphoid organ has been postulated [98]. Nonetheless, thymus and intestine appear to be the first organs to be populated with T cells in carp as well as in seabass, and later on systemic lymphoid organs like the head kidney and the spleen get invaded by T cells [76]. The early presence of T cells during the ontogeny of the immune system in fish seems to be more related to self/non-self recognition and selection, rather than to functional reactions of T cells as they take place at the later stages of development [76]. It has been shown that the majority of seabass, trout and salmon IELs are CD3/CD8+ [84,87,99,100]. The aforementioned studies and Fig. 2 (schematic presentation of immune cells in the gut of fish) clearly indicate that a considerable number of IELs represent T cells. Four TCR chains (α, β, γ, δ) are already reported for Japanese olive flounder [101], but because of the lack of suitable markers for the γδ TCR, not much is known on the γδ T cells in fish. In seabass, the intestine contains clearly more CD8α than CD4 T cells and the number of such cells increases from the foregut to the hindgut [87]. Recently, it has been reported that seabass IELs express γTCR [102]. Moreover, it has been suggested that in seabass *rag1*-driven somatic recombination may generate TCRγ/CD8α genotype in the intestinal T cell population. In addition, some functional aspects of the seabass TCRγ have been published: their diversity (by CDR3-length spectratyping) and regulation of gene expression after *in vitro* stimulation with poly I:C and *in vivo* viral infection [80].

Lymphocytes of the mucosal tissues with non-specific and cell-mediated cytotoxicity are also essential for the proper functioning of the immune system of mammals [103]. In fish, lymphoid organs such as the thymus, the kidney and the spleen have NCCs, and the non-parenchymal cells in the liver also have NCC-like cells, although with a minimum cytolytic activity [104]. The NCCs can eliminate xenogeneic targets and such cells in fish anterior kidney and spleen are small a-granular lymphocytes and have functions similar to those of mammalian large granular lymphocytes [105,106]. NCC activity against a human NK-sensitive cell line (K562) in different lymphoid organs of seabass and common carp is shown in Fig. 3A. In both species, the head kidney, the spleen and blood had high NCC activity, while the thymus showed negligible activity. The mucosal organs such as the gut and the gills of seabass had considerable NCC activity, while those of the carp did not exhibit such activity. This lack of NCC activity among the gut cells corresponds to an earlier observation in carp [88] – the anti-catfish NCC marker (5C6 – reacting with NCC/NK cells in a variety of vertebrate species [107]) did not react with IEL of carp [88] while it was immune-reactive with cells in other lymphoid organs. Although not included, our preliminary results on cod IEL also

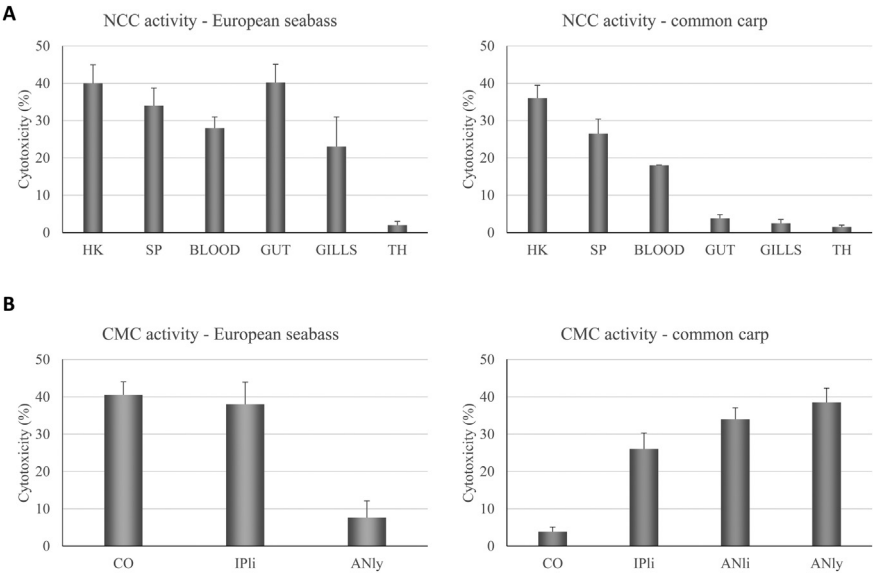

Fig. 3. A. Non-specific cell mediated cytotoxicity (NCC) against xenogeneic target cells (K562; a human myelogenous leukemia cell line) in European seabass and common carp lymphoid organs. Each bar shows the mean + SEM obtained from six animals at an effector/target ratio of 50:1. Both species are studied under the same experimental design as described earlier [87]. Note the high non-specific cytotoxicity in the head kidney (HK), the spleen (SP) and blood and low activity in the thymus (TH). Moreover there is also high NCC activity in the gut and the gills of European seabass while this activity is not present in common carp. B. Cell mediated cytotoxicity (CMC) of European seabass and common carp against xenogeneic K562 cells after two anal immunisations (at 0 and 2 weeks) with PBS (CO), intraperitoneal immunisation with living cells (IPli), anal immunisation with lysed cells (ANly) and anal immunisation with living cells (ANli). At 3 weeks post immunisation, the cytotoxic assay was carried out according to an earlier description [87]. Each bar is the mean + SEM obtained from three animals at an effector/target ratio of 50:1. Note the specific cytotoxicity in carp IEL which is the highest when anally immunised with lysed cells. In contrast, the NCC-activity in seabass is down-regulated especially when anally immunised with lysed cells. It is not clear whether the cytotoxicity in fish intraperitoneally immunised with live cells is due to specific and/or non-specific cytotoxicity.

showed NCC activity, which is not unexpected as the fish relies strongly on non-specific immunity.

Anal immunisation of carp with xenogeneic K562 cells (live or lysed) can induce specific cytotoxicity in IEL, and the cytotoxicity values are apparently higher than that after ip injection with live cells (Fig. 3B). In carp, these conclusions can easily be drawn as NCC activity appears to be nil in IEL, while the inferences are less clear in seabass as they have a high NCC activity in their gut. IP injection with living K562 cells did not influence the cell-mediated cytotoxicity, but anal immunisation with lysed cells can suppress the cytotoxicity, and perhaps even the NCC activity. The data presented in Fig. 3 and those of two earlier reports in ginbuna crucian carp *Carassius auratus langsdorfii* [108] and common carp [109] clearly indicate that cellular antigens can be taken up by the gut to induce specific cytotoxicity in peripheral blood lymphocytes (PBL) [108,109] as well as in IEL. It has also been shown that repeated intestinal immunisation can suppress the cytotoxicity induced in carp PBL [110]; a phenomenon well known as oral tolerance.

6. Evidence of oral tolerance in fish

The concept of oral tolerance in fish was first reported in the nineties, following recurrent intestinal administration of proteins or bacterial antigens in common carp [9,111], rainbow trout [112] and Atlantic salmon [113,114]. None of these studies has paid attention to the mechanisms behind oral tolerance, and hence, the interpretation is dependent on what is known in mammals. According to Pabst and Mowat [115] "oral tolerance is the state of local and systemic unresponsiveness that is induced by oral administration of innocuous antigens such as food proteins." At present, oral tolerance is considered as a multifaceted process in which multiple cellular and molecular processes are needed to ensure durable tolerance to innocent gut-derived antigens, both in mucosal and systemic immune system. In humans, not only cells such as M cells, dendritic cells (DCs), Tr1, Th3, Th17, Foxp3+ Treg, LAP+ cells, but also cytokines viz. TGFβ, IL10, IFNγ and pathways like Cox2, retinoic acid and Foxp3 are involved in the induction of oral tolerance [116]. Further, CD8+ T cells or IELs that express αβ/γδ are necessary for oral tolerance and it has been reported that induction and maintenance of oral tolerance is mediated by γδ IELs [117]. Low dose antigen feeding causes Treg induction and gut homing receptor expression. In this case, anti-inflammatory cytokines (IL4, IL10, TGFβ) cause anergic T cells to act as suppressor cells to finally evoke tolerance. High dose of antigen feeding causes induction of T cell anergy and susceptibility to apoptosis that result in secretion and up-regulation of TGFβ. The gut DCs, CD4+, CD8+ T cells, Th3 cells, macrophages, enterocytes and antigen-pulsed intestinal epithelial cells can all secrete TGFβ. The Foxp3+ Treg cells (mainly CD4+ and CD25+ T cells) are the most-important subpopulation to induce oral tolerance [115], and the secretion of IL10 and TGFβ mediates the whole immunosuppression process. In teleosts, Il10 and Tgfb are produced in mucosal tissues [118–120]. In addition, CD4+ cells exist in fish mucosal tissues [87], suggesting that the main players in mucosal immune-suppression are present in the teleost gut epithelium also. However, many other mucosal components mentioned above in the mammalian oral tolerance process are not yet reported in fish. Although not clearly highlighted in the recent review of Pabst and Mowat [115], there is some older evidence that γδ T cells can also play a significant role in oral tolerance of mammals, as depletion of these cells inhibits or prevents the immunosuppression [117,121–124]. In addition, the mammalian γδ T cells appear to be potent producers of IL10 and TGFβ. Further, M cells and the underlying lymphoid follicles of Peyers patches have a subordinate role in oral tolerance induction, especially against bacteria [115], while CD103+ DC in the lamina propria may be crucial for the tolerance against soluble antigens, probably via inducing the generation of Foxp3+ Treg cells.

As mentioned earlier, γδ T cells seem to be abundant in the intestine of teleost fish, and their ability to recognise antigens without interference of MHC may be an advantage in the

recognition of intestinal antigens. In common carp IEL, the expression of *il1b*, *tnfa*, *il10* and *tgfb* genes has been monitored [119] in healthy and soy-induced inflamed gut tissues; all four genes were up-regulated, although not simultaneously [119]. In rainbow trout, *il1b*, *tnfa*, *ifng*, *il8* and *tgfb* genes were up-regulated in the proximal gut, while *tgfb* was down-regulated in the distal gut, after *Aeromonas salmonicida* immersion infection [120]. Based on these results in carp and trout, it could be speculated that at least part of the IELs have T cell regulatory functions, although it is too early to state that the mentioned IEL types are the main Treg cells in teleost fish.

7. Mucosal vaccinations

The last decades have witnessed a substantial increase in the number of commercially available fish vaccines as described in different publications [1,3,4,125–128]. The ip vaccination is very effective and useful for older fish, but it is labour-intensive and expensive. Immersion or bath vaccination causes uptake at the skin, the gills and the gut (via drinking), and is the most frequently adopted method, particularly in the case of younger animals. However, this method needs larger amounts of vaccine and does not result in an optimal protection when compared with injection. Bath vaccination using live attenuated *V. anguillarum* was found to be effective in eliciting Th-like immune responses in zebrafish and turbot mucosal tissues, indicating the protection efficacy of this vaccination method [129]. Mucosal vaccination increases specific antibodies and antibody-secreting cells [11–14,126] in the mucosal tissues, pointing to the potential to induce local or mucosal immunity. Accurate measurement of antibodies in mucosal secretions and functional assays on mucosal T cells are still difficult in fish [130]. Further, oral vaccines need special treatments to make them insusceptible to degradation and guide them along the epithelia to reach the local immune system [130]. Moreover, orally delivered antigens may make the immune cells at both mucosal and systemic compartments of the immune system non-responders [23]. All these indicate the need for gathering information on the mechanisms by which vaccines trigger diverse responses [131].

Oral vaccination (via feed) is an ideal method for the aquaculture sector, but not many vaccinations are presently based on this delivery route [1–4], although the first successful attempt was reported as early as in 1942 [16]. In the aforementioned study, *Aeromonas salmonicida* vaccine-fed trout was subjected to immersion challenge, and a reduction in mortality (from 75% to 25%) has been correlated to antibody production. The long-term (64–70 days) vaccine feeding is probably not a realistic approach for fish. However, the prolonged feeding-induced oral tolerance did not result in negative memory formation, possibly due to the type of antigen or fish species used; tolerance induction appears to be a genetic-dependent process [111]. Three decades after the first report on vaccination, the *Yersinia ruckeri* vaccine was licensed for oral administration in the US, followed shortly by acceptance of a *Vibrio anguillarum/ordalii* vaccine for immersion application [1,132]. Many studies have reported the potential of encapsulated oral vaccines [e.g. bioencapsulated in rotifers, brine shrimp or water fleas; microencapsulation in alginate, PLGA, chitosan microparticles or liposomes (cf [1–3])], but none of them have been licensed for vaccination in fish. These vaccines are protected from degradation and possess adjuvant effects such as the ability to adhere to mucosal epithelium and/or induction of antigen uptake. The development of efficient mucosal adjuvants that can be applied – singly or in combination – via encapsulation is necessary to reduce the amount of required antigens for oral or immersion vaccination. In this context, biofilm vaccines or genetically modified plants, algae or fungi (cf [1,3]), allowing the combination of a vaccine component (i.e. a peptide) with adjuvant or immune-stimulatory molecule, should be considered. One such example is a viral G protein produced in the gut surface binding LTB in potato tubers [133,134]. Upon escaping degradation in the proximal part of the gut, this vaccine releases the necessary antigens in the hindgut to cause effective stimulation of the local mucosal lymphoid tissues.

The effect of oral vaccines, including those against viral diseases, has been reported in farmed aquatic animals. Rainbow trout orally vaccinated with polyethylene glycol (PEG) coated lyophilised viral hemorrhagic septicaemia virus (VHSV; incorporated at a special low temperature) in extruded feed particles caused increased expression of *mhc II* and *cd4* mRNAs, VHSV specific antibody levels in the blood and clear protection against the viral infection [135]. Plasmid DNA coding for lymphocystis disease virus (LCDV) incorporated in alginate microspheres [28] or PLGA microcapsules [136] were used for oral vaccination of Japanese olive flounder. Both the carriers loaded with the plasmid can be transported through the gut without being degraded, and once the plasmids are expressed in the lymphoid tissues, specific antibodies are produced. Further, compared to alginate particles, PLGA particles were slightly more effective in the induction of protection [137]. Although, the method seems suitable for oral DNA-vaccination, the exact transport mechanism in the hindgut epithelium is not yet clear. Till now it has been assumed that antigen transport in the hindgut (2nd segment) of fish is mainly based on endocytosis. This part of the gut has a very high endocytotic capacity and can sort molecules in the endolysosomal compartment, for the eventual formation of large supranuclear vacuoles, a well-known characteristic of these enterocytes [2,9,138]. However, recently an antigen-sampling cell type in the second segment of trout was reported to be similar to immature mammalian M cells based on their uptake of 10 nm gold-BSA and lectin-binding features [139]. Since mammalian M cells have a strong phagocytic capability, and epithelial transport takes place without the interference of degrading lysosomes, the uptake and transport of particles of different sizes should be studied to confirm the similarity of this trout cell type to mammalian M cells. Further, the uptake of PLGA particles by intestinal epithelium [135,136,140] and local cytotoxicity induced by anally intubated target cells [108,109] indicate the induction of phagocytosis, which may allow cellular antigens to pass the barrier. However, it is not known if this antigen transport occurs through specialized cells or regular enterocytes. For devising better vaccination strategies, it would be worthwhile to study the phagocytic mechanisms and the participating molecules in more detail – especially the uptake and transport of PLGA particles, as they seem to be suitable vectors for antigen-transport and hence mucosal vaccination.

8. Concluding remarks

The recent knowledge in fish mucosal immunology could be used to develop effective mucosal vaccines. The discovered IgT/Z can be helpful to monitor mucosal responses and to perform pathogen neutralization studies. The revelation of the function of pIgR in fish, including its up-regulation upon infection or vaccination and probably the differential secretory pathway can be used to unravel the role of secretory IgM and IgT/Z after mucosal vaccination. More attention has to be paid to the role of pIgR-mediated binding to the skin epithelial cells (instead of or in combination with secretion) as this mechanism can result in a powerful local immune barrier at the surface of fish. Further, as CD8α+ TCRαβ T cells dominate the CD4+ subset in the intestine, vaccines could be developed to target these cells so as to increase their efficacy. Based on the information on NCCs and CMCs, it is clear that vaccines inducing cytotoxic T-lymphocytes could protect the host.

Continuous efforts are needed to contain most of the diseases among farmed fishes. Vaccines, which can enter the host through the mucosal membranes and impart its immunogenic properties, should be developed to ward off diseases. Information on the inductive sites, immune effector sites and humoral and cell-mediated immune responses are necessary to understand the immune system programming efficiency of vaccines. Further, their detection, uptake and processing, ability to stimulate secretory antibodies and effector T and B cells migration, their differentiation and maturation to strengthen the mucosal barrier, rather than evoking Treg cells of oral tolerance, have to be delineated. Moreover, in-depth studies have to be conducted to uncover the ability of successful vaccines to elicit strong, long-term memory and effector immune cells at the mucosal surfaces. Thus, vaccine recognition by the innate immune system of the host and the appropriate stimulation of adaptive immune response of high quality is essential for long-term protection from a particular disease. Further, this knowledge is important for the acceptance of the vaccine as well as for the development of vaccines against emerging diseases. Comprehensive evidence on the complete and long-term protection against reinfection should be gathered, giving due consideration to evolution and the adaptive pressures that shape the organisms.

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