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Recent findings on the structure and function of teleost IgT

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Abstract

As key effector molecules of jawed vertebrate's adaptive immune system, immunoglobulins are produced by B lymphocytes, either as a secretory form (antibody) or as a membrane form (B cell receptor). Until recently, teleost fish B cells were thought to express only two classes of immunoglobulins, IgM and IgD. In addition, IgM in these species was thought to be the only immunoglobulin isotype responding to pathogens both in systemic or mucosal compartments. However, the unexpected discovery of IgT, a new teleost immunoglobulin unearthed in 2005, has provided for new opportunities to analyze further roles of teleost immunoglobulins in these two physiologically distinct compartments. The smoke about the potential function of IgT has cleared recently with the finding that this immunoglobulin appears to be specialized in gut mucosal immunity. Significantly, the new capability of measuring not only IgM but also IgT responses will greatly facilitate the evaluation and understanding of fish immune responses as well as the protective effects of fish vaccines. The purpose of this review is to summarize the molecular characterization of new IgT orthologs and subtypes in teleosts, as well as to describe the new findings concerning the protein structure of IgT, the B cells producing it, and its role in mucosal immunity.

Keywords

IgT; mucosal immunoglobulin; fish

1. Introduction

The adaptive immune system (AIS) appears to have emerged in the common ancestor of all vertebrates, more than 500 million years ago [1,2], since a parallel version of jawed vertebrate AIS was discovered recently in jawless vertebrates [3–5]. Antigen recognition in jawless vertebrates is mediated by variable lymphocyte receptors (VLRs) [6] whereas in jawed vertebrates the key molecules involved in antigen recognition are the B and T cell receptors (BCR and TCR respectively).

A typical immunoglobulin (Ig) molecule consists of two heavy (H) and two light (L) chains (H_2L_2 unit), each of which containing one amino-terminal variable (V) immunoglobulin superfamily (IgSF) domain and one (in the L chain) or more (in the H chain) carboxyl-terminal constant (C) IgSF domain. Generally, the paired V domains provided by each of the H and L chains, are associated non-covalently and confer antigenic specificity, while the H

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chain C domains define effector functions of the immunoglobulins through binding to their receptors on effector cells or activating other immune mechanisms, such as complement [7]. Immunoglobulins can be classified into different isotypes (classes) and subtypes (subclasses) based on the nature of the C domains of their H chains. In mammals, five Ig isotypes, IgM, IgD, IgG, IgE, and IgA, have been identified, which possess specific effector functions in humoral and cellular immune responses (see reference [2] for a recent review on structural, functional and evolutionary aspects of vertebrate immunoglobulins). Among the antibody isotypes, IgM is the most ancient and the only isotype functionally conserved in all gnathostomes (jawed vertebrates) [8]. IgD has been found in all gnathostomes, except birds, indicating that it is also a primordial antibody class despite its highly plastic structure and unclear function in evolution (see reference [2]).

Immunoglobulin isotypes have evolved to play specialized roles either within mucosal or systemic compartments. In mammals and birds, IgM, IgG and IgY isotypes have major roles in systemic responses, while IgA is the main player in mucosal areas. In amphibians, IgM and IgY play a prevalent role in systemic immunity whereas IgX is an isotype chiefly expressed in the gut [9]. Until recently, teleost fish were thought to contain only two classes of immunoglobulins, IgM and IgD. It was also generally accepted that IgM was the only immunoglobulin class responding to antigenic challenge both in systemic and mucosal compartments, and thus, teleost were believed to be devoid of an immunoglobulin specialized in mucosal surfaces. A new teleost Ig H chain gene, named *ighτ* in rainbow trout [10] or *ighζ* in zebrafish [11], was reported in 2005. The assembled immunoglobulin containing the *ighτ* product was named IgT in trout (or IgZ in zebrafish), and it was suggested to represent the last immunoglobulin class to be found in a vertebrate species [12]. During the last five years *ighτ/ighζ* has been cloned and characterized at the gene level in a number of teleosts species [13–18]. However, until very recently its protein structure, production, and potential role in immunity were not reported. In that regard, a 2010 study revealed that rainbow trout IgT is an immunoglobulin specialized in gut mucosal immune responses, while IgM appears to be specialized in systemic immunity [19]. These findings have challenged the paradigm that specialization of immunoglobulin isotypes into mucosal and systemic areas arose during tetrapod evolution. The new capability of measuring not only IgM but also IgT responses will greatly facilitate the evaluation and understanding of teleost immune responses as well as the protective effects of fish vaccines. In this review, we summarize the molecular characterization of newly discovered *ighτ/ighζ* genes in teleosts, as well as recently reported aspects of the protein structure of IgT, its production by a novel lineage of B cells and its role in immunity.

2. Characterization of the genes encoding IgT

2.1. Organization and rearrangement of the igh locus in teleost fish

The immunoglobulin heavy (IgH) and light (IgL) chains are encoded by separate genomic loci, *igh* and *igl*, respectively, and their individual V and C domains are each encoded by independent elements: the variable (*V*), diversity (*D*, only for H chains), and joining (*J*) gene segments for the V domain, and individual constant (*C*) gene segments for the C domains. The V domain of the IgH and IgL chains is functionally divided into three hypervariable sequences termed complementarity-determining regions (CDR) that are located between four relatively stable sequences named framework regions (FR). The diversity of the V domain is provided mostly by the three CDR regions. CDR1 and CDR2 are encoded by the *V* gene alone, while CDR3 is encoded by the *V-J* or *V-D-J* rearrangement junction and thus represents the most diverse CDR [7].

To date, two major organization types of *igh* and *igl* loci have been reported: translocons or clusters [2]. In humans, the *igh* locus is in ‘translocon’ configuration: *V-D-J-Cμ-Cδ-Cγ3-*

Cγ1-Cγ2b-Cγ2a-Cε-Cα. The messenger RNAs for the H chains of IgM (μ) and IgD (δ) are generated by alternatively splicing the recombined *VDJ* to either *Cμ* or *Cδ* in their *igh* loci, whereas the H chains of IgG (γ), IgE (ε), and IgA (α) are expressed through a process known as class-switch recombination. In cartilaginous fish, the *igh* genes are in the 'cluster' configuration with many sets of $V(D)_{2-3}JC$ clusters [1,2].

Until five years ago, the *igh* loci in bony fish were thought to be organized only in 'translocon' configuration, where multiple *V* gene segments are upstream of multiple *D* and *J* segments, followed by a *Cμ* and *Cδ* genes, encoding and generating the μ and δ chains by alternative splicing [20,21]. However, the detailed analyses of the complete *igh* loci from rainbow trout (*Oncorhynchus mykiss*) [10] and zebrafish (*Danio rerio*) [11] revealed a novel genomic architecture that had never been observed in any *igh* loci: upstream of the known (V_H)-*DJCμCδ* elements for μ and δ IgH chains, another set of V_H-*DJC* elements were found, which encoded for the H chain (τ, for teleost fish) of a previously unknown Ig isotype, IgT, named by Hansen and colleagues [10]. In zebrafish this new immunogobulin was termed IgZ [11]. To avoid a mixed terminology, throughout this review we will mostly use the term 'IgT' to refer to IgT/IgZ, and we will use '*ighτ*' for the gene encoding its H chain. The organization of the above mentioned *ighτ-ighμ* locus is strikingly similar to that of the mouse *Tcrd-Tcra* locus encoding T cell receptor δ (TCRδ) and TCRα. In both loci, upstream *V* segments rearrange either to *DJCτ* (or *DJCδ*) to encode τ (or TCRδ) or to *DJCμ* (or *DJCα*) to encode μ (or TCRα) [1,11,12]. Based on the aforementioned arquitecture of the *ighτ-ighμ* locus, and despite of the fact that class switching mechanisms are absent in fish [22,23], B cells of these species were predicted to express either IgT or IgM, which was demonstrated to be the case in rainbow trout at protein level [19] and in zebrafish at gene level [24].

Subsequent reports have showed that IgT orthologs exist in almost all studied species belonging to the main orders of teleost fish (see Table 1), such as fugu (*Takifugu rubripes*) [13], common carp (*Cyprinus carpio*) [14,15], zebrafish [25], stickleback fish (*Gasterosteus aculeatus*) [16,17], grass carp (*Ctenopharyngodon della*) [18], Atlantic salmon (*Salmo salar*) [26,27], Chinese perch (*Siniperca chuatsi*), and orange-spotted grouper (*Epinephelus coioides*). The only exception where IgT has not been found thus far is in the channel catfish (*Ictalurus punctatus*) [28]. As the genome sequence of the channel catfish had not been finished at present time, it is possible that IgT may be found once the genome of this species is completed.

It is important to highlight that most of the above species possess more than one subclass of IgT, which may be encoded by duplicated *ighτ* genes in the same locus, as for example in stickleback fish [16,17]). Alternatively, these additional IgT subclasses are found in different *igh* loci. For instance, in Atlantic salmon, two *igh* loci were discovered (see Table 1), in each of which, 3 or 5 $V_HD\tau J\tau C\tau$ clusters are upstream of one copy of $V_HD\mu J\mu C\mu C\delta$, encoding for three putatively functional IgT subclasses [26,27]. Similarly, two *igh* loci also exist in rainbow trout [10]. Most of the H chains of IgT contain four C domains (Cτ), whereas stickleback IgTs lack the Cτ2, and in fugu IgT and carp IgT2 they lack Cτ2 and Cτ3. Among the Cτ domains, Cτ1 always maintains its higher similarity to Cμ1, and Cτ4 provides the specificity to the IgT isotype [16]. The reason that only Cτ1 and Cτ4 are always conserved among species may be due to their unique role in binding to IgL chain (through the cysteine residue [Cys] in Cτ1) or to other IgH chains (through the Cys in Cτ4 or secretory tail).

Although the genomic organization and domain numbers of IgT are variable among different species, the phylogenetic studies, based on the sequences of complete C regions or individual C domains, have shown that IgT occupies a separate clade on phylogenetic trees,

distinct from IgM or other Ig isotypes. While a possible relationship between the teleost τ and μ chains has been suggested, they most likely represent distinct IgH lineages with common evolutionary origins [10,11]. Besides the recent findings in rainbow trout [19], the biochemical features and functional roles of IgT and its subclasses in different teleost species await to be investigated.

2.2. Features of the V and C domains of the H chain of IgT

Through analysis of the cDNA clones of trout and zebrafish *igh*τ and *igh*μ, it was shown that the V_H gene segments in their *igh* loci were shared by both *C*τ and *C*μ. However, the upstream *D* and *J* segments were found to be used exclusively by the nearby *C*τ or *C*μ to generate their own CDR3 [10,11], a region with crucial function in antigen recognition. Strikingly, the CDR3 of trout τ chain is longer (5–10 amino acid (aa)) compared to that of μ chain (4–5 aa) [10], which suggested that IgT might recognize a more diverse range of pathogenic epitopes because of the more extended CDR3 in its τ chain [10].

Both membrane and secretory forms of τ chains can be encoded by the same genes. The membrane τ chain can be generated by splicing from a cryptic splice site near the very end of *Cτ4* exon to the first membrane exon (*TM1*), similar to the splice pattern in the mammalian membrane μ chain, but unlike the teleost membrane μ chain, where the *Cμ3* is spliced directly to *TM1*. As a result, teleost membrane μ chain has three C domains, while membrane τ chain retains all four C domains [10,11]. The teleost secretory τ chain is usually predicted to be shorter than its secretory μ chain, despite both of them having four C domains. This is because each of the C domains as well as the secretory tail of τ chain is shorter than the corresponding domain of μ chain (see Fig. 1), which leads to a probable smaller mass of τ chain (theoretical Mw: 58.3 kDa) when compared to that of μ chain (theoretical Mw: 62.8 kDa), without taking into consideration the putative glycosylation or other postranslational modifications in the C domains.

In the IgSF domain, Cys and Trp (tryptophan) residues are crucial for the folding of this domain [29]. The two Cys residues, involved in intra-domain disulfide linkages that form the core Ig domain loop in each of the Cτ1, Cτ2, and Cτ4 domains of all studied τ chains are conserved in their positions (like those of μ chains), and are separated by approximately 50 residues. Interestingly, in the Cτ3 domains of cyprinid fish, the first Cys residue was found 16–17 aa closer to the second one when compared to Cτ3 domains in other fish. However, it was predicted that the atypical Cys residues of this cyprinid Cτ3 domains would not affect the formation of typical IgSF domains [1]. There are several additional Cys residues that exist in the C domains and secretory tails of teleost τ chains, among which the one within Cτ1 is conserved in all teleost, which is predicted to form a disulfide bridge with a Cys from the IgL chain. In mammals, the secretory tails of polymeric IgM and IgA contain a conserved $PX_3\underline{NXS/T}L/VX_3E/DX_4\underline{C}Y$ motif, where the penultimate Cys (C) together with the *N*-linked glycosylation site (NXS/T) were suggested to be typically required for polymer assembly and J chain incorporation [30–33]. Within the shorter secretory tails of teleost τ chains there is also a conserved Cys residue, however, the *N*-glycosylation site and the polymerization motif are missing, which raised an intriguing question: Does the IgT unit τ_2L_2 polymerizes into a dimer like mammalian IgA, or a tetramer like teleost IgM, or just behaves as a monomer like IgG? (Studies described below in section 3.1 address this question). The assembly of IgTs with an H chain only having two conserved additional Cys (mentioned above for zebrafish, Chinese perch, common carp, and grass carp IgT2) are even harder to be predicted based only on the primary sequences of their τ chains.

Conserved Antigen Receptor Transmembrane (CART) motifs have been identified in the transmembrane domains of all antigen receptors (including membrane Ig and TCR) of jawed vertebrates. These motifs are known to interact with other proteins that play a role in the

assembly and/or the signaling properties of lymphocyte antigen receptors [34]. As shown in Fig. 1, the transmembrane regions and especially the CART motifs of teleost τ chains, are very similar to those of teleost and mammalian μ chains. This suggests that membrane IgT has signaling capacities similar to those found in other BCRs. In order to shed light on the specific roles of IgT and IgM in fish immunity, future studies will have to investigate differences in the signaling properties between membrane IgM and IgT.

2.3. Expression of ight gene

As a first step to evaluate the function of IgT, its expression during development and in specific organs of adult fish was studied in most of the species shown in Table 1. Compared to IgM, detectable expression of IgT1 was observed earlier and increased more rapidly during the early developmental stage of zebrafish [11], while the earlier IgT expression was not seen in other teleost species [10,13,15], which might be due to differences in the time points studied in all the above mentioned species [1].

The expression of IgT1 in adult zebrafish was found almost exclusively localized in primary lymphoid organs (i.e., head kidney and thymus) while IgM expression could be detected in both primary and secondary lymphoid organs [11,25]. However, the expression patterns of trout IgT, fugu IgT, carp IgT1 and IgT2, grass carp IgT1, and Zebrafish IgT2, are quite similar to that of IgM, i.e., they are detectable in both primary and secondary lymphoid organs, or in some cases even non-lymphoid organs (i.e. muscle and brain in carp [15]), although IgM was always the predominant isotype [10,13,15,18,25]. Interestingly, in common carp the relative expression level of IgT2 was higher than that of IgT1 in the mucosal organs (intestine and gills). Combined with the strong expression of IgT2 in goblet cells of intestine and gill epithelium detected by *in situ* hybridization, this suggests that IgT2 may play important roles in mucosal surfaces of fish [13,15].

In summary, both IgT and IgM can be expressed from the very early developmental stages (as early as 4 days post fertilization) of teleost fish, with the expression of IgT increasing more rapidly when compared to that of IgM, which may suggest that IgT plays a significant role in protecting fish larvae. In most adult fish, IgT and IgM can be expressed constitutively in both primary and secondary lymphoid organs, with IgM always being the dominant isotype and in some cases (i.e. after parasite infection) IgT being more highly expressed in mucosal organs [15,19]. This suggests that IgM may protect adult fish from infection through systemic immune responses and IgT may be specialized in mucosal immunity. As shown below this appears to be the case at least in rainbow trout [19].

3. Biochemical and functional characterization of IgT

The first account for a mucosal immune system in fish dates from 1942, when Duff observed protection in rainbow trout orally immunised with *Aeromonas salmonicida* antigens [35]. In 1969, Fletcher and Grant reported for the first time the presence of immunoglobulin in gut and skin mucus in plaice (*Pleuronectes platessa* L.) [36] and, since then, the evaluation of mucosal antibody responses in fish has always been limited to IgM [37].

Since IgT was discovered in 2005 [10,11], up until recently nothing was known about the protein structure of this immunoglobulin, the cells producing it, and most importantly, its role in teleost immunity. Below we summarize the latest findings concerning the biochemical and functional characterization of this new teleost immunoglobulin class.

3.1. Biochemical features of IgT

Antibodies raised against rainbow trout IgT have recently enabled its protein identification and biochemical characterization from several body fluids [19]. It was shown that trout IgT

has a molecular mass of ~180 kDa under nonreducing conditions, being the heavy and light chain masses of ~70–75 kDa and ~25 kDa respectively. These masses are in close agreement with the theoretical masses obtained from the IgT primary sequences [19]. As predicted from the analysis of its amino acid sequence [12], gel filtration analysis confirmed that serum IgT eluted at the position expected for a monomer (~180 kDa) compared to IgM, which is found in tetramers. When the same study was conducted in trout gut mucus, most of the IgT was eluted as a multimer, in very close proximity to IgM. Surprisingly, immunoblot analysis under nonreducing conditions revealed that the mucus IgT multimers were associated by non-covalent bonds. In contrast, serum and mucus IgM were found in various redox forms that differ in the number of monomers associated through disulfide bonds [38]. The mechanisms that hold these subunits together in mucosal IgT are at this point unknown although they may relate to the presence of a piece of the trout polymeric Ig receptor (pIgR) bound to it (see below section 4).

IgT and IgM concentrations substantially differed in serum and gut mucus. The IgM concentration in serum (~2.5 mg/ml) was ~34 fold higher than that of gut mucus, whilethe IgT levels in gut mucus (~7 μg/ml) were double to those found in serum. In essence, the IgT/IgM ratio was much higher in the gut mucus (9.5%) when compared to that in serum (0.15%), thus suggesting that IgT could play a role in gut mucosal immunity [19]. Overall, the above mentioned biochemical characteristics of IgT greatly resemble those of human IgA, which is found in monomeric form in serum, while in the gut mucosa it forms polymers [39]. In addition, like IgT, human IgA is the prevalent immunoglobulin in the gut [39]. The lack of similar studies on other species limits our understanding of IgT across teleosts and it remains to be investigated whether the trout IgT features are universally present in other fish.

3.2 A new lineage of B cells in teleost fish and its role in innate immunity

The antibodies raised against trout IgT enabled the identification and characterization of a new lineage of B cells uniquely expressing surface IgT. These IgT^+ B cells did not express any IgM or IgD transcripts and were not stained by monoclonal antibodies against IgM [19]. While the IgM^+ B cell population has been identified in most analyzed teleosts species, thus far, the $IgM^-/IgD^-/IgT^+$ and the IgM^-/IgD^+ B cell subsets have been definitively characterized with the corresponding monospecific antibodies only in rainbow trout [19] and catfish [40,41], respectively. Hence, each teleost fish species appears to contain two or more major subsets of B cells, in rainbow trout the $IgM^+/IgD^+/IgT^-$ (IgM^+ B cells) and $IgM^-/IgD^-/IgT^+$ (IgT^+ B cells), or in catfish the IgM^+/IgD^-, IgM^+/IgD^+ and IgM^-/IgD^+ subsets. Thus, the presence of the IgT^+ and IgD^+ B cell subsets needs to be confirmed in other teleost fish species. The rainbow trout IgT^+ B cell subset represents ~16–27% of all B cells (including both IgT^+ and IgM^+ B cells) in the main systemic lymphoid organs, whereas IgM^+ B cells comprise about ~72–83% of all B cells. In contrast, in trout gut, the percentage of IgT^+ and IgM^+ lymphocytes was around 54% and 46% of all B cells, respectively. Combining the higher IgT/IgM ratio in gut mucus than serum, these results were strong indications that constitutive IgT was produced more efficiently in trout gut which may play specific functions in the mucosal immunity. In trout, both flow cytometry and immunofluorescence analyses showed no double stained IgT^+/IgM^+ cells. These results confirmed the aforementioned prediction that teleost fish exist two mutually exclusive B cell lineages that express either IgT or IgM. Hence, it appears that teleosts use a different mechanism for generating B cell isotype diversity when compared to tetrapod species. As teleost *igh*τ-*igh*μ and mammalian *Tcrd-Tcra* loci have comparable genomic structures, it is possible that the mechanism involved in the commitment of fish B cells to expressing either IgT or IgM resembles that involved in generation of αβ or γδ T cells. From a morphological

perspective, sorted IgT^+ B cells displayed typical lymphocyte features that make them indistinguishable from IgM^+ B cells under both light and electron microscopy.

Hu and colleagues have raised polyclonal antibodies against a novel subtype of zebrafish IgT termed as IgT2 [25]. They showed that double positive $IgM^+/IgT2^+$ accounted for about 2% of the blood lymphocytes. Whether this double positive population indeed co-expresses both IgM and IgT2, remains to be demonstrated. At the transcript level, *in vivo* LPS treatment up-regulated IgT2 after 12h in most lymphoid organs examined, including kidney, spleen, gill, gut, and skin, while IgT1 was only up-regulated in kidney and was detectable in spleen [25]. This is in agreement with gene expression studies in adult zebrafish where IgT1 expression was limited to the primary lymphoid organs [11]. In the future, specific monoclonal antibodies against zebrafish IgM, IgT1 and IgT2 will help to unravel their differential roles in immunity.

Teleost IgM^+ B cells displayed innate immune functions such as the ability to phagocytose particles and kill internalized bacteria [42]. Similarly, IgT^+ B cells were equally able to engulf latex beads or bacteria and had also an intracellular killing capacity [19]. In addition, both trout IgM^+ and IgT^+ B cells proliferated *in vitro* following stimulation with LPS and *Vibrio* bacterin. Furthermore, after 7 days, stimulated IgT^+ B cells differentiated into larger lymphocytes with greater IgT-secreting capacity than the small IgT^+ B lymphocytes. The latter suggests that the IgT^+ B cell subset was able to respond to pathogen-associated molecular patterns (PAMP) and differentiated into antibody-secreting cells in the same way as IgM^+ B cells did.

3.3. Prevalent IgT coating on the surface of intestinal bacteria

Animal gastrointestinal tracts are populated by complex microbial communities whichlive in symbiosis with the host's mucosal environment. Antibodies are known to play a pivotal role in the maintenance of gut homeostasis in mammals [43,44]. In addition, gut microbial communities modulate the nature of mucosal and systemic immune responses (reviewed in [45]). In mammals, IgA and to a lesser extent, IgM and IgG, coat commensal intestinal bacteria [46], precluding their translocation into the intestinal epithelium by a process known as immune exclusion [43]. Similar to mammals, the gut of fish is filled with high densities of bacteria [47]. Analogously to mammalian IgA, rainbow trout IgT was shown to coat a significantly higher percentage of gut bacteria (48%) when compared to IgM (24%) [19]. The prevalence of IgT over IgM on the coating of intestinal bacteria reinforced the concept that IgT in trout behaves as a mucosal immunoglobulin. In addition, these results revealed for the first time a potential immune exclusion role of immunoglobulins in a non-mammalian species.

The use of a model that combines germ-free mice and non-replicable labelled bacteria has considerably highlighted the importance of antibody-mediated responses against gut bacteria. Using this model, a highly specific anti-commensal IgA response in germ-free mice was observed, although sequential (boosting) doses of commensals lacked the classical prime-boost effect seen in systemic vaccination [48]. Thus, in mammals gut IgA responses to commensals are different when compared to that to pathogens. It will be interesting in the future to analyze whether teleost fish respond to commensals in a similar fashion, as this will have important implications for the application of probiotics in aquaculture.

3.4 IgT responses against pathogens

Up until now, IgM was thought to be the only antibody isotype responsible for specific immunity in teleost fish both in systemic and mucosal compartments. This paradigm was changed recently when rainbow trout IgT was shown to be specifically involved in gut

antibody responses against *Ceratomyxa shasta,* an intestinal parasite [19]. This parasite is endemic to rivers of the Northwest Coast of the United States, and causes severe infections in the gut of rainbow trout [49]. We reported that the gut of fish that survived to *C. shasta* infections (3 months post-parasite exposure), contained large accumulations of IgT^+ B cells whereas the number of IgM^+ B cells did not change with respect to control fish [19]. The lamina propria of surviving fish was enlarged and IgT^+ B lymphocytes even penetrated the intestinal epithelium. At that point, the majority of *C. shasta* trophozoites were located in the gut lumen, and therefore, they appeared to have been expelled from the host's intestinal tissue. These observations were coupled to very large increases in the same area of IgT but not IgM, both at the protein and gene levels. More critically, gut mucus from surviving fish contained parasite-specific IgT titres but no specific IgM titres. Conversely, the serum of surviving fish had parasite-specific IgM, but not IgT titres. Whether IgT is responsible for eliciting protection against the parasite will require further work. Overall, this model provided the first evidence in teleost fish or any other non-tetrapod species, for a compartmentalization of immunoglobulin isotypes into mucosal (IgT) and systemic (IgM) areas in response to pathogenic challenge.

Trout vaccination trials against *Yersinia ruckeri* through two different routes, bath vaccination and intraperitoneal (i.p.) injection, provided interesting results regarding the differential stimulation of IgT and IgM expression [50,51]. Bath vaccination resulted in increased IgT expression levels in the spleen 72h post-vaccination in vaccinated fish compared to controls, but no differences in IgM expression were found [51]. Conversely, IgT expression was not up-regulated neither in the spleen nor in the head-kidney of trout that received the *Yersinia* vaccine i.p; however IgM transcript levels were significantly higher in the head-kidneybut not the spleen of vaccinated compared to control fish [50]. In a recent study, trout surviving primary infections with *Y. ruckeri* were re-exposed to the same pathogen to study secondary immune responses [52]. Gene transcription in the spleen revealed no changes in IgM or IgT levels during secondary infection. However, this study was conducted by i.p. injection of *Y. ruckeri* and therefore, it is possible that no mucosal sites were directly stimulated. As described above, *C. shasta* has a strong tropism for the gut of trout. Thus, direct stimulation of the mucosal site may be important to induce strong IgT responses. This is indeed the case for mammalian mucosal IgA, which require direct stimulation of the mucosal area [53]. In carp, different infection models were tested in order to investigate the role of the two IgT subtypes, IgT1 and IgT2 present in this species. Expression analyses showed that IgT1 was more abundant in systemic organs while IgT2 was prevalently expressed at mucosalsites. Moreover, infection with the mucosal parasite *Lernea* induced IgM and IgT2 gene expression but no IgT1 [15]. Future work on different gut and systemic pathogens is needed to understand further the nature of compartmentalized IgT and IgM responses in teleosts.

4. Transport of fish immunoglobulins to mucosal surfaces

In mammalian mucosal tissues, secretion of antibodies to the external mucus lining is accomplished through a unique cooperation between the mucosal B cell system and the polymeric Ig receptor (pIgR), which is expressed basolaterally on epithelial cells [54]. Both mammalian IgA and IgM are transported across mucosal epithelia to the outer mucus layer via pIgR. At the apical pole, pIgR-IgA/IgM is cleaved releasing the secretory form of IgA (sIgA) or IgM (sIgM) which remain bound to a portion of the pIgR, called secretory component (SC) [55].

Teleost pIgR has been cloned in different species such as fugu [56], carp [57], orange-spotted grouper [58] and trout [19]. They all share common molecular characteristics: they are considered type I transmembrane proteins consisting of a ligand-binding extracellular

region that includes two immunoglobulin-like domains (ILDs). In mammals, pIgR contains 5 ILDs (for more details about fish pIgR see review by Rombout and colleagues in this issue). As previously stated, the SC of mammalian pIgRis known to be associated to sIgA or sIgM. In the case of rainbow trout, an anti-trout pIgR antibody was used to verify the presence of a trout secretory component-like molecule (tSC) in the gut mucus but not in the serum, with the molecular mass of tSC being of ~38 kDa. More critically, tSC was found associated to IgT and IgM of the gut mucus by co-immunoprecipitation studies using anti-IgT or anti-IgM antibody. Thus, similar to mammalian sIgA and sIgM, trout mucosal IgT and IgM need to associate to pIgR for their transport into the gut lumen. Similarly, IgM binding to pIgR has also been demonstrated in fugu [56] and grouper [58].

In higher vertebrates, the joining chain (J chain) is thought to be critical for the interaction of pIgR with mucosal immunoglobulins. Braathen and colleagues showed that the Jchain from divergent tetrapods including mammals, birds, and amphibians efficiently induced polymerization of human IgA whereas the J chain from nurse shark (a lower vertebrate) did not [59]. Interestingly, in teleost species, a J chain-like molecule has not been identified thus far, despite of the availability of several teleost genomes [19,56]. This probably indicates that teleosts are devoid of a J chain. It should be pointed out that while amphibians have a J chain, this molecule does not associate with IgX (an intestinal amphibian immunoglobulin) [60]. Accordingly, it is possible that in teleost fish and amphibians, the J chain is not required for the interaction of pIgR with their respective mucosal immunoglobulins. In addition, whether pIgR is the sole transport mechanism for fish mucosal antibodies awaits further investigation.

To understand further the presence and transport of IgT in mucosal sites, it is important to understand where IgT antibody-secreting cells (ASCs) are localized within systemic and mucosal lymphoid tissue, as well as potential routes of IgT transport from systemic compartments into mucosal surfaces. IgT-ASCs could be present locally at the mucosal sites (*in situ* production) or originate *ex situ* in other organs, or both. With regards to the trafficking routes, while it seems likely that IgT can reach the gut lumen through its transport from locally produced IgT (gut lamina propria or epithelium), it is possible that IgT, or mucosal IgM for that matter, are derived from systemic organs. In that regard, there is evidence that in Antarctic fish, plasma IgM could be transported across the hepatocytes to be secreted into the bile, and through the bile, reach the intestinal epithelium [61]. Analogously, in rats, the liver shows a remarkable capacity fortransport of dimeric IgA from blood into the bile [62,63]. Thus, a similar mechanism may be responsible for IgT (and IgM) transport in fish. Hence, generating a geographical map of both IgT and IgM ASCs during primary and secondary immune responses in teleost, as well as analyzing the transport routes of IgT and IgM, would substantially complete the present picture of teleost antibody responses both in systemic and mucosal sites.

5. Future perspectives

While significant advances have been made on the identification and characterization of *ighτ* gene orthologs, a considerable amount of intriguing questions remainto be answered. While it is impossible to summarize them all here, we propose some priority areas that need to be addressed: 1) To understand further the role of IgT in immunity, it is necessary to unravel how many subtypes of IgT (including chimeric IgM-IgT molecules) are present in teleosts, to develop monospecific antibodies against each subtype, and to investigate if different B cell subsets express unique IgT subtypes. 2) It will be critical to identify which heavy and light chain genes (since three IgL isotypes have been reported in several teleost species, see the summarized Table 1 in [64]) are involved in the assembly of IgT and IgM, and which are the dominant Ig products expressed in systemic and mucosal sites before and

after pathogenic challenge or vaccination. 3) To understand further the functions of teleost immunoglobulin molecules, it will be crucial to compare the immune repertoires of IgT, IgM and IgD in systemic and mucosal sites of naïve and infected animals. 4) Since there is no class switch in teleost fish, it will be critical to elucidate how, where and when B cells commit to expressing either IgT or IgM, and the reason why IgT^+ B cells do not express IgD. For example, which transcription factors control such mechanism? Is this commitment decided in early stages of development or can it be modified in adults? To which degree can PAMPs influence this commitment?

While some important biochemical and functional properties of IgT have finally been unearthed, we are far from understanding the specific contributions of IgT in teleost immunity. Below we propose some priority areas that need to be addressed: 1) Trout mucosal IgT is polymeric although no disulfide bonds are involved in holding the monomers together, thus, which are the molecular mechanisms involved in generating polymeric IgT? Is the SC of pIgR required for IgT polymerization? 2) What are the phenotypic differences between IgT^+ and IgM^+ B cell subsets? It will be fascinating to perform microarray and deep sequencing studies on normal and activated IgT^+ and IgM^+ B cells to elucidate which genes are differentially expressed in these two B cell lineages. 3) Both IgT^+ and IgM^+ B cells can phagocytose large particles. Can they present phagocytosed antigen to T cells? 4) IgT has been shown to be the dominant immunoglobulin in the gut mucosa of rainbow trout. Is IgT also the prevalent immunoglobulin system in other mucosal sites, including the skin and gills? 5) If IgT also coats commensal bacteria found in other mucosal sites, including the skin surface, then does IgT play a role in immune exclusion of skin bacteria? Can fish probiotics modulate the expression of IgT in mucosal and systemic sites? Will probiotics affect the capacity of gut IgT to coat commensal bacteria? 6) How do the different B cell subsets home and traffic during the course of an immune response? Which chemokines and chemokine receptors are involved in the homing of IgT^+ and IgM^+ B cells in systemic and mucosal tissues? 7) What are the specific contributions of IgT and IgM antigen-specific responses in primary and secondary responses? Are there differences in the kinetics and extent of the affinity maturation of IgT and IgM during the course of an immune response? 8) Can IgT activate the classical pathway of the complement system? This point will be really interesting as mammalian IgA appears to be poor activator of the complement system. 9) How are IgT mucosal responses modulated by different routes (especially oral and bath) of vaccination? Can we find specific adjuvants that improve the efficacy of mucosal responses? This research will be critical for the development and design of a new generation of vaccines that not only induce effective systemic response, but also protective mucosal immunity.

The list of new captivating research opportunities with the IgT system is obviously much larger than that proposed above. It is clear that the field of mucosal immunoglobulins in teleost fish is facing a new era. We cannot wait to see how the next review on IgT will look like in a few years from now. We now have the tools for a more integrated understanding of immunoglobulin responses in teleost fish. While discovering new and fascinating roles of immunoglobulinsin fish immunity, this research will be fundamental for the future design of novel immunotherapies for aquaculture.

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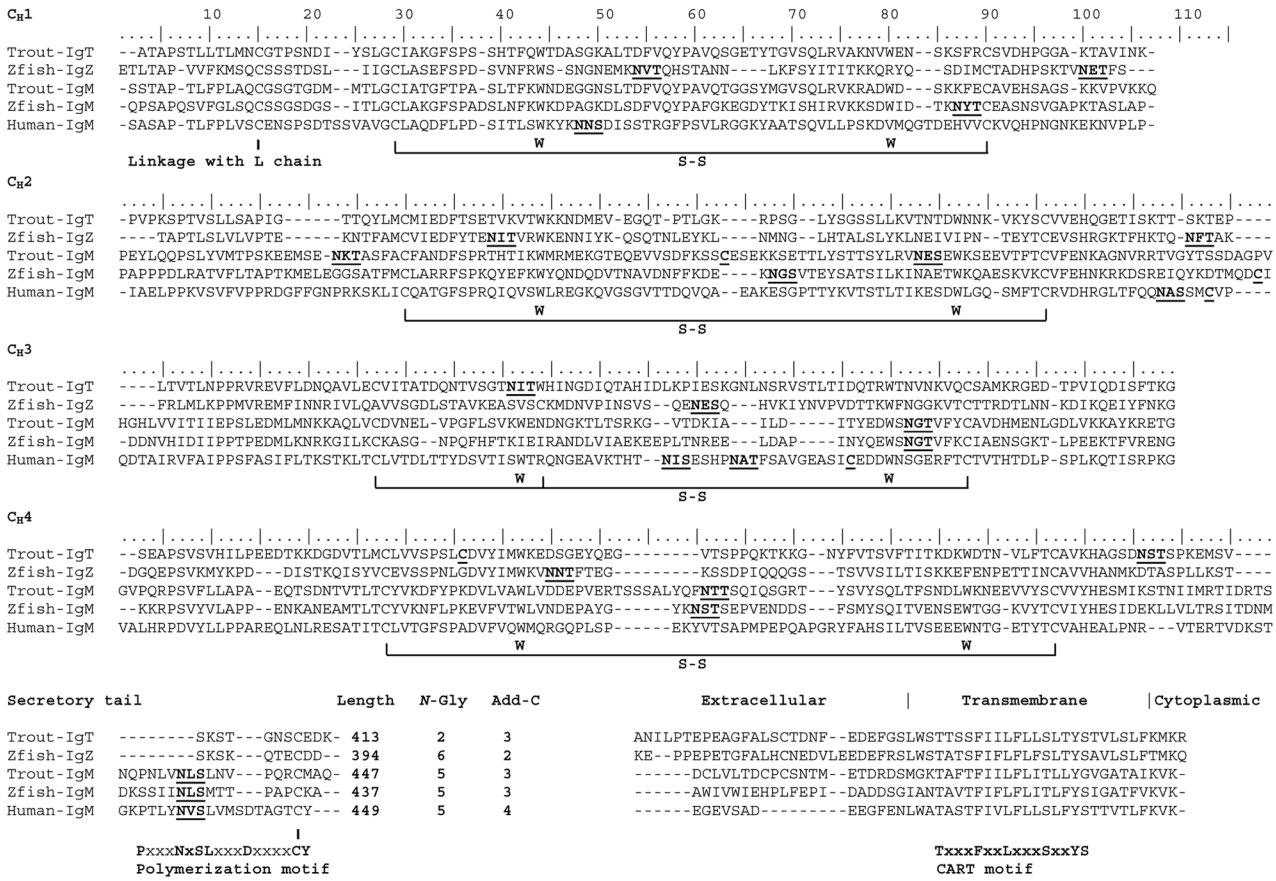

Fig. 1.

Amino acid sequence alignment of the heavy chains of IgT/IgZ and IgM. Gaps are indicated by dashes to optimize the alignment. The conserved (identical and similar) residues are shaded. The conserved cystein (S-S) and relatively conserved tryptophan (W) residues required to form the fold of each IgSF domain are shown below the alignment. The conserved cystein residues involved in the disulfide bond linking with IgL chain (in C_H1 domains) and the ones important for Ig polymerization (in secretory tails) are indicated, respectively. The polymerization and CART motifs located in the secretory tail and transmembrane region of human IgM are indicated below the alignment. The *N*-linked glycosylation sites are in bold and underlined in each sequence. The numbers of *N*-linked glycosylation sites (N-Gly) and additional cysteins (Add-C) are shown at the end of each sequence. The GenBank accession numbers for the aligned IgH sequences are: rainbow trout τ (Trout-IgT, AY870263 and AY870265), zebrafish ζ (Zfish-IgZ, AY643752 and AY643750); rainbow trout μ (Trout-IgM, AY870258 and U04615), zebrafish μ (Zfish-IgM, AY643753and AY643751), and human μ (Human-IgM, X14940 and X58529).

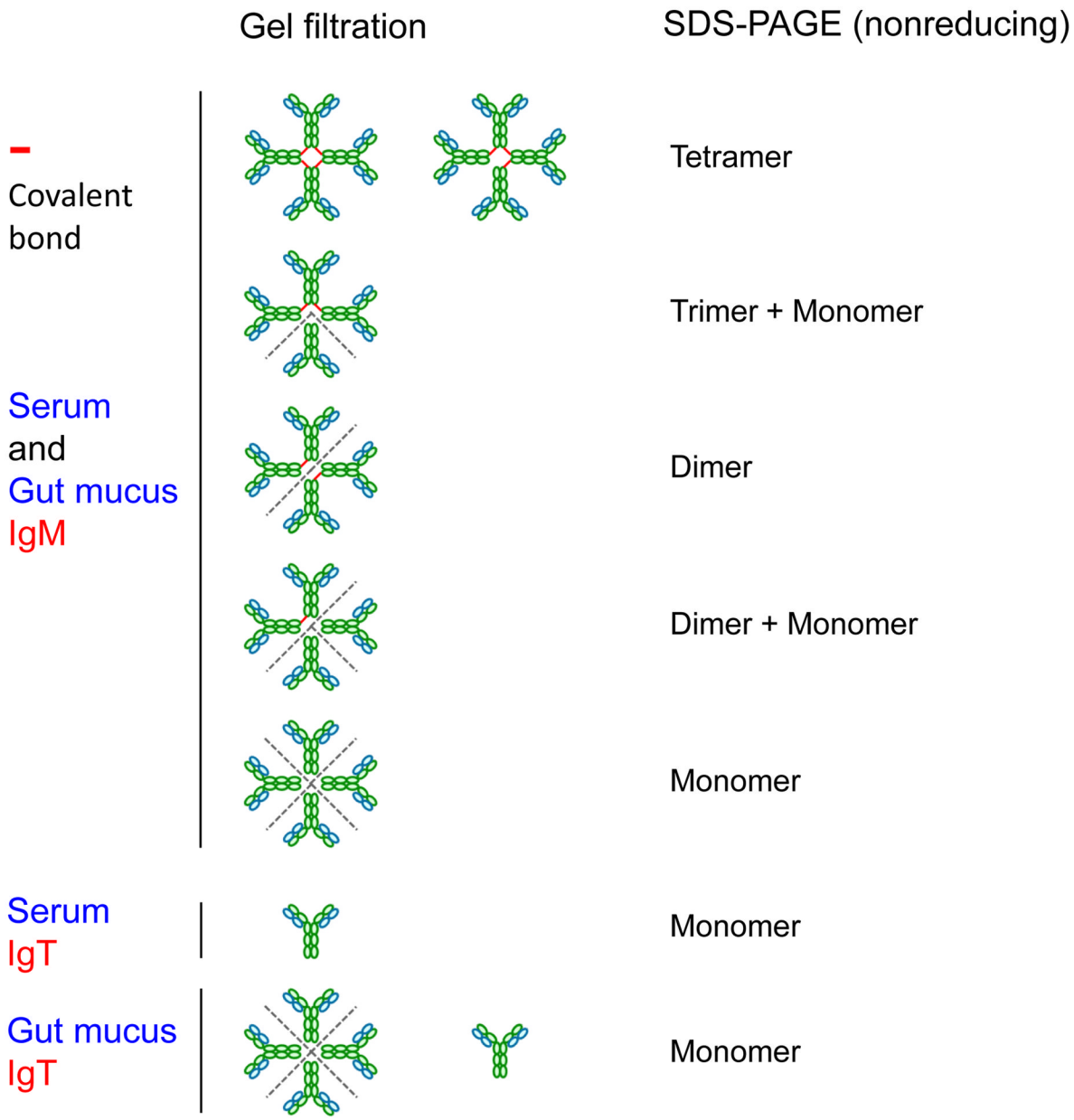

Fig. 2.

Redox forms of trout IgM and IgT in serum and gut mucus. Distinct chromatographic (gel filtration) and electrophoretic (nonreducing SDS-PAGE) behaviors of trout IgM and IgT are shown diagrammatically. Note the number and location of covalent bonds between the subunits of IgM are different in various redox forms [38]. The dotted lines indicate the areas where the non-covalent bonds are broken by SDS-PAGE under nonreducing conditions. The putative secretory component (SC) of a pIgR is not shown.

Table 1

Organization of teleost *igh* loci and the constant regions encoded by *igh*τ

Order	Species	Potentially functional IgT Subtypes	Organization of *igh* locus	Constant region	Reference[a]
Salmoniforms	*Oncorhynchus mykiss*	IgT1 IgT2	$V_HD\tau J\tau C\tau$-$V_HD\mu J\mu C\mu C\delta$ Data not available	Cτ1-Cτ2-Cτ3-Cτ4	[10] [11]
	Salmo salar	IgT4, IgT5 IgT2	locus A: $(V_HD\tau J\tau C\tau)_5$-$V_HD\mu J\mu C\mu C\delta$ locus B: $(V_HD\tau J\tau C\tau)_3$-$V_HD\mu J\mu C\mu C\delta$	Cτ1-Cτ2-Cτ3-Cτ4	[26,27]
Cypriniformes	*Danio rerio*	IgT1 IgT2	$V_HD\tau J\tau C\tau$-$D\mu J\mu C\mu C\delta$ Data not available	Cτ1-Cτ2-Cτ3-Cτ4	[11] [25]
	Ctenopharyngodon idella	IgT1 IgT2	$V_HD\tau J\tau C\tau$-$D\mu J\mu C\mu C\delta$ Data not available	Cτ1-Cτ2-Cτ3-Cτ4	[18] DQ478943
	Cyprinus carpio	IgT1 IgT2 (chimeric IgM-IgT)	Data not available	Cτ1-Cτ2-Cτ3-Cτ4 Cμ1----------Cτ4	[15] [14,15]
Tetraodontiformes	*Takifugu rubripes*	IgT	$V_HD\tau J\tau C\tau$-$D\mu J\mu C\mu C\delta$	Cτ1------H[b]----Cτ4	[13]
Gasterosteiformes	*Gasterosteus aculeatus*	IgT1, IgT2, IgT3, IgT4	$(V_HD\tau J\tau C\tau$-$D\mu J\mu C\mu C\delta)_3$-$V_HD\tau J\tau C\tau$	Cτ1-------Cτ3-Cτ4	[16] [17]
Perciformes	*Siniperca chuatsi*	IgT	Data not available	Cτ1-Cτ2-Cτ3-Cτ4	Q016660
	Epinephelus coioides	IgT	Data not available	Cτ1-Cτ2-Cτ3-Cτ4	GU182366

[a] When no literature is available the GenBank accession number is showed.

[b] H, the putative hinge region: GPV(KPTV)5.