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There and back again? A B cell's tale on responses and spatial distribution in teleosts

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ABSTRACT

Teleost B cells are of special interest due to their evolutionary position and involvement in vaccine-induced adaptive immune responses. While recent progress has revealed uneven distribution of B cell subsets across the various immune sites and that B cells are one of the early responders to infection, substantial knowledge gaps persist regarding their immunophenotypic profile, functional mechanisms, and what factors lead them to occupy different immune niches. This review aims to assess the current understanding of B cell diversity, their spatial distribution in various systemic and peripheral immune sites, how B cell responses initiate, the sites where these responses develop, their trafficking, and the locations where long-term B cell responses take place.

1. B cell subsets in teleost fish: a meta-perspective

Teleosts represent the most diverse group of vertebrate species known, displaying a remarkable but still inadequately understood diversity in their immune responses. For instance, what we currently know about teleost B cells largely originates from a limited number of model or economically important teleost species, leaving the vast majority of them either minimally investigated or entirely uncharted. Research conducted within these few species over the past two decades has significantly changed our perspective of teleost B cells. These studies not only unveiled the existence of a teleost-specific B cell immunoglobulin (IgT/IgZ) [1,2] but also challenged a longstanding paradigm in mammalian B cell biology by discovering phagocytic B cells with intracellular bactericidal activity that can bridge innate and adaptive immunity *via* the priming of naïve T cells [3,4]. This pioneering work in teleosts laid the foundation for the discovery of B cells with similar phagocytic function in mammals [5,6].

Thus far, three distinct B cell subsets exclusively expressing IgM, IgD, or IgT [7–10], along with one subset that expresses both IgM and IgD [7–9] have been found in several teleost species. While the double positive B cells are present in most teleost species studied and are thought to represent the major B cell population in fish, the other three subsets of B cells are less studied. Consequently, despite the build-up of knowledge on IgM^+ B cells, distinction between the double positive (IgM^+IgD^+) and IgM-only (IgM^+IgD^-) B cells has not been made in most teleost studies [11–13]. Therefore, the IgM^+ B cell phenotype and function described for most fish species cannot be assigned to either the double positive or the IgM-only subset without uncertainty. Although the *igμ, igδ,* and *igτ* genes, which encode for IgM, IgD and IgT, respectively, have been found in varying numbers and organizations in several teleost species [14], thus far, it is only in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) [9,10,15,16] that these four B cell subsets are reported. Channel catfish (*Ictalurus punctatus*) and medaka (*Oryzias latipes*) are unique in that their genome appears to lack the *igτ* genes and hence, have no IgT B cells [14]. While channel catfish possesses the other three B cell subsets [7], it remains unclear which B cell subsets are present in medaka. Another fascinating exception occurs in members of the Gobiesocidae family, known as clingfishes. In these species, the gene loci encoding immunoglobulin and coreceptor CD79α/CD79β are deleted from the usual chromosomal regions leading to teleost species devoid of B cell receptors and immunoglobulins [17].

2. Diversity of B cells within and between immune sites

The growing availability of anti-IgM antibodies, along with anti-IgT and anti-IgD antibodies for a few species, serves as valuable tools for fish immunologists studying B cells. On certain occasions, antibodies targeting transcription factors such as Pax5, XbpI, and Blimp combined

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with anti-IgM antibody [18–20] or anti-MHC II antibody combined with FACS gating strategies [21] have been used for characterizing teleost B cells. Despite its common application, the suitability of using anti-IgM antibodies to study B cell phenotypes after antigenic stimulation is debated. Crosslinking of the B cell receptor (BCR) with such antibodies could trigger activation signals, which might confound activation signals induced by antigen stimulation, leading to misinterpretation of the resulting B cell phenotype.

Additionally, comprehensive data regarding BCR expression throughout the various stages of B cell differentiation is lacking. For instance, like their mammalian counterparts, fish B cells decrease their surface IgM expression as they differentiate into plasmablasts [8]. However, it remains unclear whether terminal-stage plasma cells in fish, as observed in mammals, completely eliminate surface BCR expression. If so, the use of anti-BCR antibodies may fall short in targeting these effector B cells. An interesting finding in this context, warranting further exploration, is the identification of IgM secretion in a subset of rainbow trout $CD38^{+}IgM^{-}$ cells after *in vitro* antigen stimulation [22], a typical feature of long-lived plasma cells in mammals. The discovery of IgD-only B cells in some teleost species [7,9] further justifies the need for using pan-B cell markers. In this context, a surface proteome study of Atlantic salmon IgM^{+} B cells has identified CD22-like molecule (Ssa02: LOC106582456) as a potential pan-B cell marker that could address issues associated with using anti-IgM antibody [12]. However, it is important to note that the above surface proteome study did not cover IgD-only B cells, prompting a cautious approach when considering its relevance across all B cell subtypes and its applicability to other teleost species.

To date, there is a lack of comprehensive data that compares teleost B cells in the systemic and peripheral immune sites, including those found in the various mucosal-associated lymphoid tissues (MALTs). Thus, it remains uncertain whether B cells from these locations exhibit similar phenotypic and functional characteristics. Despite this, the limited studies conducted have shown differences in B cell subsets and functions within and across lymphoid tissues of fish. A study on channel catfish identified two distinct populations of IgD^{+} B cells in peripheral blood, each with different characteristics in terms of morphology and transcriptional activity [7]. Another study in rainbow trout has reported two populations of B cells in the peritoneal cavity (PerC), each having different requirements for a key B cell survival cytokine, BAFF, indicating different functions of these cells [8]. Similarly, in rainbow trout, Perdiquero et al. identified ten transcriptionally distinct B cell clusters from blood [21]. However, whether these clusters represent functionally different populations, various stages of B cell differentiation, or comprise a combination of these remains an open question. An even more recent study in rainbow trout has reported the presence of IgM-only and IgD-only B cells in the skin and gills, each with varying antibody secretion and antigen-presenting capacities, while the double positive B cells are barely observed in both mucosal sites [9]. These findings collectively show uneven distribution and different functions of B cell subsets across the different immune sites.

3. Mucosal B cells of teleost fish

Mucosal tissues in both mammals and fish are protected by different mucosa-associated lymphoid tissues (MALTs). Leukocytes aggregating in these sites play a crucial role in targeting mucosal pathogens for elimination, with B cells actively taking part in these mucosal responses. Teleost MALTs encompass a diverse array of tissue types which are located in the gut (GALT) [10], the skin (SALT) [23], the gills (GIALT) [24], the eye [25], the nose (NALT) [26], and the pharynx [27], including the newly discovered pharyngeal tissue named nemausean lymphoid organ (NELO) [28] (Fig. 1). Considering the diverse immune cell populations described in these MALTs, it is probable that they serve as secondary immune sites where antigen-specific B cells become activated and subsequently produce antibodies.

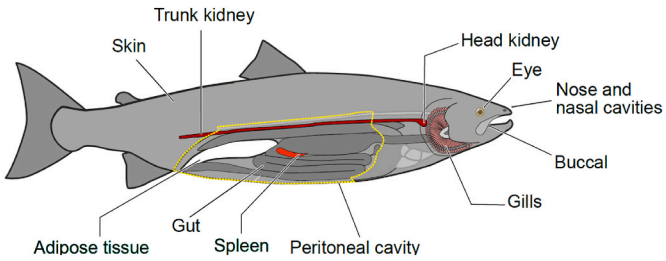

Fig. 1. Presentation of the lymphoid tissues/sites in teleosts and their anatomical location, including the systemic tissues head kidney, trunk kidney, and spleen, the adipose tissue, and the main mucosa-associated lymphoid tissue (MALT) sites described here: eye, nose and nasal cavities, gills, gut, skin, and buccal cavity.

For a long time, IgM was thought to be the only immunoglobulin class that could respond to antigenic challenges at both the systemic and mucosal compartments of teleosts. Since 2010, when the Sunyer group started to unravel the functions of trout IgT [10], the pace of discovery of mucosal immunity has rapidly increased. Many scientific articles have reported its function in salmonids and in other species such as common carp (*Cyprinus carpio)* [29] and zebrafish (*Danio rerio*) [30]. In addition to its role in pathogen clearance, IgT also takes part in the preservation of microbiota homeostasis at mucosal sites [31], which is an interesting function not covered in this review.

3.1. IgT B cells - teleost specific, with a prevailing role in mucosal immunity

Although the genes encoding IgT (or its homologue IgZ in zebrafish and carp) were identified in 2005 [1,2], it took an additional five years to elucidate its specialized role in mucosal immunity. In their breakthrough study, Zhang et al. proved IgT to be an immunoglobulin specialized in gut mucosal immunity [10]. Subsequent research on rainbow trout, employing mainly parasite infection models or vaccine immunization, has consistently shown that pathogen specific IgT titers, upon infections, are prevalent in the mucus from various mucosal sites such as skin [23], nasal [32], buccal [24], and pharyngeal mucosa [28], while IgM continues to dominate in the serum. In this review, we have summarized IgT responses in gut, pharyngeal, gill, and skin mucosa in more detail.

3.2. IgT B cells in gut immunity

In the study by Zhang et al., the parasite *Ceratomyxa shasta*, which specifically infects the trout gut, was used as a model [10]. In the non-infected fish, 54.3% of all gut B cells were IgT positive, while the IgT^{+} cell percentages in blood, spleen, head kidney (HK), and PerC B cells were 16.6–27.8%. Upon infection, the numbers of IgM^{+} cells in the gut of survivor fish remained unchanged when compared to the control, and parasite-specific IgM titers were found only in the serum. In contrast, there was a significant accumulation of IgT^{+} cells in the gut of the infected survivors, with the IgT to IgM antibody ratio being 63-fold higher in gut mucus than in serum. In parallel to the substantial increase in gut IgT^{+} cell numbers, parasite specific IgT titers also increased at this site. The study suggests both a functional and spatial distribution difference between these two B cell subpopulations. Additionally, Zhang et al. found that 48% of the trout intestinal bacteria were coated with IgT, whereas a smaller portion, 24%, was positive for IgM [10]. Similar to IgA in mammals [33,34], IgT is thus a specialized Ig in mucosal immunity and the prevalent Ig isotype coating the microbiota of fish.

3.3. NALT – "nosy" immunity in fish

An interesting model for exploring mucosal immunity in fish has

been the rainbow trout NALT (reviewed in Ref. [35]), and in the first report of the existence of this mucosal site, it was shown to consist of different lymphoid cells [32]. Different from other MALTs, the relative presence of the IgM^+ cells and IgT^+ cells was quite similar, 48.5% and 51.5%, respectively [32]. In the same study, an attenuated viral vaccine administered intranasally showed that trout NALT was capable of mounting a strong immune response, and several adaptive immune genes including MHC, B and T cell receptors, and immunoglobulins were upregulated, indicating an adaptive response. Using a similar model for nasal vaccine delivery, Garcia et al. reported an expansion of IgT, IgM, CD4, and CD8 positive cells in NALT epithelium in vaccinated fish when compared to controls [36]. The authors suggested that this structure, rich in lymphocyte aggregates, might resemble a mammalian structure named organized NALT (O-NALT) [37], which is a site where somatic hypermutation and affinity selection occur.

3.4. GIALT and SALT– homing B cells with diverse phenotypes

Although IgT^+ B cells dominate teleost mucosal tissues, a recent study of rainbow trout revealed that B cells exclusively expressing either IgD or IgM were also present in gills and skin and found to be evenly distributed within the two tissues [9]. In contrast, double-positive IgD^+IgM^+ cells, were almost non-existent at these sites. Both the IgD-only and the IgM-only cells showed plasmablast profiles, expressing higher levels of *irf4* and *IL1b* as well as different *Blimp1* transcript isoforms than the blood IgM-only cells did. In mammals, the transcription factor Blimp-1 is a key factor driving the maturation of B cells into Ig-secreting cells [38], and the same role is reported in fish [39]. After 48 h of cultivation, both the gill and skin IgD- and IgM-only B cells maintained their antibody production, thus confirming a plasmablast/plasma cell phenotype.

Another novel lymphoid structure, situated within the primary lamella of the salmon gills, is the interbranchial lymphoid tissue (ILT) (reviewed in Ref. [40]). While T lymphocytes are identified as the dominant leukocytes in the ILT and are shown to proliferate there, B cells also inhabit this immune site. Using infectious salmon anemia virus bath immersion, Austbø et al. detected both IgM and IgT transcripts in the ILT, where IgM was most highly expressed [41]. No clear alteration in transcript levels for IgM was detected during the sampling period, while IgT expression increased slightly at the last sampling on day 24. The latter may indicate a potential clonal expansion of IgT^+ B cells in the ILT.

It is thus clear that under homeostatic conditions, IgT and IgM B cell numbers at different MALTs, such as the skin and gut, are not that different. After an insult, like an infection, however, there is a pronounced increase in IgT positive cells, without changes in IgM positive B cell numbers. Whether this is caused by the infiltration of the IgT positive cells to mucosal sites, local B-cell proliferation, or both is a pending question. If there is a migration to these sites, there must be cells producing chemokines that attract only IgT positive cells, and not the IgM positive B cells. This is an interesting research topic that warrants further studies.

3.5. The polymeric Ig receptor (pIgR) taking part in immunoglobulin transport is present in teleosts

A common feature of all teleost MALTs is the presence of diffusely organized lymphoid cells spread along mucosal territories [42]. Also, the polymeric Ig receptor (pIgR), which in mammals is known to transport immunoglobulins to mucosal surfaces has been detected at mucosal sites of various fish species, including grass carp (*Ctenopharyngodon Idella*) and flounder (*Paralichtys olivaceus*) [43,44]. More direct evidence for the role of pIgR in teleost has emerged through studies of trout gut [10], skin [23], and NALT [32]. In the work of Zhang et al., a polyclonal anti-trout pIgR antibody was used to verify the presence of the secreted form of the receptor in the gut mucus [10]. Coimmunoprecipitation experiments with IgT- or IgM-specific antibodies demonstrated a direct interaction between the pIgR and IgT/IgM. Using the same antibody, the trout pIgR was detected in the skin epithelial layer and again shown to associate with both skin mucus IgT and IgM [23]. Immunofluorescence microscopy analysis of the skin revealed that the pIgR receptor was localized between the epidermis and the mucus layer, and in the same images both IgT and IgM stained positive.

4. Peritoneal cavity B cell responses

Not only mucosal immune sites, but also the PerC has received more attention from research on teleost B cells. The PerC immune response is important to delineate due to the intraperitoneal (IP) delivery of most vaccines for fish in aquaculture. Recent studies have highlighted a prominent role played by the PerC micromilieu in activation and differentiation of B cells after IP stimulation, indicating that the HK and spleen may not consistently be the primary locations for antibody production.

Korytar et al. investigated changes in cell populations in the PerC of rainbow trout within three days after IP delivery of live or inactivated *Aeromonas salmonicida* [45]. Myeloid cells dominated first, while there was an increase in IgM^+ cells in the PerC of infected trout already from 6 to 12 h, peaking at 72 h, before adaptive immune responses kick in. Fish receiving inactivated bacteria had a similar, but somewhat delayed response. The authors suggested different roles for B cells at this early stage, including production of proinflammatory cytokines for the onset of inflammation, further, regulation and phagocytosis during the resolution of infection, and the production of natural antibodies. An additional investigation in rainbow trout by Castro et al. observed an increase in IgM^+ B cell numbers, the amount of total IgM antibody secreting cells (ASC), and differentiation (as determined by a reduction of MHC-II expression and an increase in IgM^+ cell size) at 3 and 6 days after IP *E. coli* or viral hemorrhagic septicemia virus infection [46]. When a stimulation with *E. coli* lipopolysaccharide (LPS) was used in the same study, the IgM^+ cell numbers also increased, but there was no indication of differentiation of B cells. Interestingly, IgM^+ cells were found to be the main phagocytic cells in the PerC after two days.

Investigations into Atlantic salmon peritoneal B cells have mainly focused on ASCs after infection. In a study with salmon alphavirus 3 (SAV3), the number of leukocytes, the percentage of IgM^+ cells, and the number of ASCs significantly increased in the PerC 14 days after IP infection [11]. The fraction of ASCs was reduced, apparently due to a stronger increase in other leukocyte numbers. At later time points, up to 9 weeks after infection, all these parameters remained high in the PerC [11]. In a follow-up investigation, the fraction of total IgM ASCs in isolated leukocytes from the PerC increased 6 weeks after IP delivery of infectious SAV3 or inactivated SAV1, while only the infection significantly increased the frequency of SAV3-specific ASCs, which was evident at 13 weeks post infection [47]. Van der Wal et al. presented similar findings after IP infection of Atlantic salmon with the bacterial pathogen *Piscirickettsia salmonis,* under comparable conditions. A major increase in leukocyte numbers, total IgM ASCs, *P. salmonis*-specific ASC, and non-specific (*Y. ruckeri*-specific) ASC frequency was found in the PerC up to 6 weeks after infection [48]. Thus, Atlantic salmon showed a strong local peritoneal response of leukocytes and both specific and non-specific ASCs after IP infection, where the bacterial *P. salmonis* infection resulted in higher numbers of IgM ASC at 3 and 6 weeks compared to the viral SAV3 infection.

Although the previous mentioned investigations observed that the effect of stimulation was less pronounced or at best comparable to infection, this is not always the case. Shi et al. observed an increase in the proportion of IgM^+ cells in the PerC of olive flounder after IP injection with both live and inactivated *Vibrio anguillarum*, where the inactivated bacterium resulted in higher proportions [49]. Additionally, Simon et al. showed that IP stimulation of rainbow trout with TNP-LPS

significantly increased both total IgM and specific ASC frequencies in the PerC up to 4 weeks post injection [50]. Notably, this corresponded with an increase in percentages of both $IgM^{+}IgD^{+}$ and $IgM^{+}IgD^{-}$ cells.

It is interesting that wherever the B cell responses in the teleost PerC after IP challenge are compared with responses in the HK or spleen, the peritoneal B cell responses show higher frequencies of ASC. Jenberie et al. showed that whereas the leukocyte and total IgM ASC number increased in the salmon PerC up to 9 weeks post SAV3 infection, in HK and spleen, leukocyte numbers remained at the same level and the number of total IgM ASCs decreased [11]. In their following paper, the frequency of specific ASCs is increased in the PerC, HK, and spleen at 13 weeks post SAV3 infection, still the frequency in the PerC was higher [47]. After *P. salmonis* infection of Atlantic salmon, increases in leukocyte, total IgM ASC, and specific ASC numbers were most pronounced in the PerC, although total ASC numbers were also clearly increased in the HK at all investigated time points. Increases of specific ASC numbers were observed in all investigated organs [48]. Finally, Simon et al. showed an increase in total IgM ASC frequency only in the PerC of TNP-LPS stimulated trout. Specific ASC frequencies were found to be increased in PerC, HK, and spleen, and again the highest increase was observed in the PerC [50].

It is thus clear that an IP insult results in a major increase of specific and non-specific ASCs in the teleost PerC. This response clearly differs from mammalian peritoneal B1 cells that, although they can be locally activated, migrate away from the PerC to secondary lymphoid organs [51]. These observations also indicate that the HK and spleen might not be, at least at early time points, the main production sites of antibodies. It would be very interesting for future research to investigate not just the number of local ASC, but also the actual number of antibodies they secrete locally and to evaluate to what extent these antibodies participate in local and systemic immune responses.

5. Activation of B cells

Whether locally or systemic, the humoral immune response is initiated when B cells, expressing specific BCRs, recognize antigen. The membrane Ig recognizes antigens in their native conformation but has in itself no signaling properties. Instead, signaling relies on the associated molecules Igα (CD79A) and Igβ (CD79B), which recruit the signaling machinery through their cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) [52]. Binding of antigen to the BCR is necessary, but for most antigens additional signals are needed to achieve full B cell activation, leading to proliferation and ASC formation. This second signal can be obtained through the interaction of the B cell presenting the endocytosed antigen to antigen specific T helper cells through MHC II or by intrinsic TLR engagement of B cells [53]. In addition, B cell activation can occur when multivalent antigens with repetitive structures crosslink the BCR. In fish, however, the specific co-receptors and costimulatory mechanisms crucial for B cell responses remain inadequately understood. Furthermore, the molecular events that follow BCR engagement are poorly characterized in teleost B cells.

The presence of co-stimulatory receptors and mechanisms was recently presented when we found that, amongst others, CD40, CD22, CD79A, CXC4R-like, CXC5R-like, and TLR2 are parts of the B cell surface proteome of naive Atlantic salmon B cells from blood [12]. Zebrafish CD79 is reported to have a conserved role in B cell development, and its expression, together with that of surface IgM, decreased after bacterial infection, which is consistent with the maturation of a plasma cell response [54]. However, the functionality of these surface proteins on teleost B cells has not been extensively studied.

Engagement of CD40 on B cells by the CD40 ligand (CD40L) on cognate T helper cells is important for activation of the T-dependent humoral response [55]. In zebrafish and flounder (*Paralichthys olivaceus*), CD40/IgM + lymphocytes have been identified [56–58]. *In vitro* studies with Nile tilapia (*Oreochromis niloticus*) leukocytes using an antibody against CD40L to block its activity, suggest that CD40L has a role in T-dependent B cell responses [57]. Granja et al. added soluble CD40L to trout splenocytes which activated IgM^{+} B cells. In a series of *in vitro* experiments, synergistic effects of CD40L and a T-independent antigen (TNP-LPS), but not a T-dependent antigen, was found on B cell responses [59]. This questions the involvement of T helper cells and suggests that the $CD40^{-}$CD40L signal might be provided by other cell types.

5.1. Activation of teleost B cells by TLR ligands

In mammals, it is known that intrinsic TLR signaling has important roles in both B cell activation and antibody secretion. Such signaling was shown to be particularly important for immunity against viral infection in animals [60]. In a few studies, the levels of TLR transcripts in fish B cells have been analyzed. In B cells of Atlantic salmon and rainbow trout, nuclei acid sensing TLRs, such as TLR3, TLR9, TLR21, TLR22, TLR7, and TLR8 are expressed [16,39]. The TLR repertoire of B cells in rainbow trout also includes TLR1, 2, and 5 [39], while in Atlantic salmon TLR2 is part of the B cell surface proteome [12]. No major differences in TLR profile were apparent for Atlantic salmon B cells from HK, spleen, and blood [16]. Altogether, it appears that teleost B cells have the ability to respond directly to a range of different pathogen associated molecular patterns (PAMP), possibly impacting B cell functionality. Indeed, *in vitro* stimulation experiments using purified B cells have shown that the TLR ligand CpG increases IgM secretion and differentiation into plasmablasts of salmonid B cells [16,61]. In addition, the antigen presenting capacity appears to be affected by increased gene expression of co-stimulatory molecules such as CD83, CD86, and CD40 [16,61]. The data also suggest that CpG-induced cytokines secreted by myeloid cells can potentiate the direct effects of CpG on Atlantic salmon B cells [16,62].

The role of TLR versus BCR signaling in mediating effects of PAMP on teleost B cells is challenging to study. Some studies were performed with B cells purified using an anti-IgM antibody, which in itself may cross-link the BCR and activate the B cells prior to the subsequent TLR stimulation, thus not giving a clear answer to this [16]. Using only FAB fragments of anti-IgM for cell purification, which should prevent BCR crosslinking, has been approached [61]. This model suggested that CpG-mediated TLR signaling alone is sufficient to increase surface MHC II expression and transcription of co-stimulatory molecules. When BCR-stimulation using anti-IgM was combined with CpG, a synergistic effect on proliferation of B cells from blood was evident [61]. Developing antibodies to other cell surface markers than IgM and a methodology for negative selection of teleost B cells would make such studies more feasible. Altogether, CpGs have profound direct effects on the immune functions of salmonid B cells, suggesting an application of these TLR-ligands as vaccine adjuvants [63]. In Atlantic salmon, this has been explored showing that CpG in combination with dsRNA (poly I:C) boosted levels of neutralizing antibodies in a salmonid alpha virus vaccine compared to controls [64,65]. Based on the TLR profile of salmonid B cells, more ligands should be explored for effects on B cell functions both *in vitro* and *in vivo*.

5.2. Spatial biology of teleost B cell activation

Since both spleen and HK are secondary lymphoid tissues in teleosts, they seem logical locations for activation of B cells. Although the teleost spleen is regarded as the main secondary lymphoid organ, the tissue is less organized and without definite regionalization such as in spleens of other jawed vertebrates (reviewed in Ref. [66]). It is thus questioned, how and where antigen presentation and initiation of the adaptive immune response are organized. Aggregates of melanomacrophages (melanomacrophage centers -MMCs) are found mainly in spleen and HK of fish and can retain antigens after vaccination or infection (reviewed in Ref. [67,68]). In rainbow trout, a recent study found that infection with the parasite *Ichthyophthirius multifiliis* induced aggregates of proliferating antigen specific B cells closely associated with CD4 T cells nearby

spleen MMCs. Together with IgM CDR3 repertoire analysis of B cells within these areas, their data suggest that systemic IgM responses are induced within such microstructures [69]. It is not known in detail if similar processes take place in association with HK MMCs.

In trout HK, both developing B cells and ASC are present [70]. In Atlantic salmon, a population of MHCII + cells is present which has been shown to endocytose antigen in the periphery, such as the PerC, and over time these cells accumulated mainly in the HK and not the spleen [71]. These data support that the HK also functions as a secondary lymphoid tissue in teleost. However, the teleost kidney is a complex organ, and the HK and the posterior kidney harbor distinct B cell populations in rainbow trout. Mostly B cell precursors and ASC are present in HK, while plasmablasts and partially activated B cells are more prevalent in the posterior kidney [72]. The characteristics of these B cell populations were delineated by analyzing the expression of B cell transcription factors Pax5 and Blimp-1 together with properties of proliferation and Ig-secretion. In a recent study on Atlantic salmon infected with SAV3, a significant increase in IgM secreting cells occurred only in the posterior kidney at 2 weeks post infection, while a response in HK and spleen emerged later [47]. Although the posterior kidney is not included in most studies of teleost B cell responses, these findings emphasize the importance of exploring the role of this tissue in relation to HK as secondary immune site for B cell activation.

6. In search of affinity maturation in teleosts

An important feature of adaptive immune responses is that after activation of immune cells, the responses become more specific to the insult. B cells mainly accomplish this through affinity maturation that leads to antibodies with higher specificity and affinity. This is achieved through antigen-driven clonal selection of B cells that have the highest affinity for the pathogen epitopes and somatic hypermutation of the variable regions of the BCR. Both these processes occur mainly in germinal centers (GC) in mammals, whose presence in fish has been debated [73]. This, coupled to the relatively low antibody affinity observed in fish, has led to discussions on whether fish have affinity maturation and where the processes for affinity maturation in fish might occur.

Affinity maturation of the teleost antibody response was initially doubted [73–75], although it has later been clearly demonstrated, first in rainbow trout [76] and more recently in channel catfish [77]. When we are looking for the location of B cell affinity maturation, we need to find B cells that undergo somatic hypermutation and selection. Activation-induced cytidine deaminase (aicda, also referred to as AID), the enzyme that drives the processes of somatic hypermutation and class switching in mammals, is also present in fish. This enzyme was identified and found to be functionally active in catfish and zebrafish [78,79]. Selection of B cells involves helper cells that present the antigen (like follicular dendritic cells (FDC) in mammals) and provide signals for differentiation, survival, or cell death (like follicular T helper cells (Tfh) in mammals). Taken together, a possible location of affinity maturation for teleost B cells should have indicators of aicda expression in B cells, cells capable of antigen storage and presentation, and supporting cells.

A structure that has long been debated to be a prototype GC and thus a location of affinity maturation in teleosts, is the beforementioned MMC. MMC are aggregates of melanomacrophages (MM) that are supported or encapsulated by reticular cells [80]. They have auto fluorescent properties, have been implied to be involved in immune responses, and can trap antigen [81,82]. Recent work by Magor and his group has shown that aicda expressing B cells aggregate within catfish MMC [79]. Through high throughput sequencing of zebrafish spleen and kidney MMC, the same group found hundreds of VDJ mutations mainly affecting the antigen binding loop, which were dominated by few clonotype lineages, indicating SHM and selection [83]. In addition, they observed that MM in zebrafish and goldfish (*Carassius auratus*) MMC (can) trap antigen, and MMC in goldfish kidney express genes corresponding with an FDC profile (BAFF, FcR-like, CR1-like, MFGE8, and a putative CXCL13) [83]. In rainbow trout, aggregates around MMC have also been shown to exhibit evidence of aicda expression, higher levels of apoptosis, and somatic hypermutation as determined through IgHμ V sequencing [69]. Together, this presents a strong case for MMC as locations of affinity maturation in teleost fish, but whether they are true predecessors to the mammalian GC remains a pending question.

When addressing teleost affinity maturation and possible GC precursors, we should also consider extrafollicular (EF) antibody responses in mammals. Usually, antibody responses in mammals start with an EF response where B cells are activated, differentiate into plasmablasts, and produce the first peak of antibodies, followed by the formation of GC after B and T cell migration, resulting in the development of long-lived plasma cells (LLPC) [84]. Some infections, however, barely activate GC responses and result in EF responses [85,86]. Interestingly, EF responses show similarities with teleost antibody responses: low to modest affinity maturation and development of memory B cells, but no to low development of LLPC nor long term antibody production [84]. Infections in mucosal tissue or parenchymal organs of mammals might lead to local aggregates of lymphoid cells that can range from disorganized foci of T, B, and myeloid cells to tertiary lymphoid tissues that resemble lymph node T and B cell zones and can include FDCs [84]. These responses are probably mainly EF-like, except if a tertiary lymphoid tissue includes functional FDC and Tfh, and it is interesting to consider teleost (local) B cell, MM, and MMC responses within this spectrum.

If we consider MMC as locations for affinity maturation of B cells, their spatial distribution will be of interest. Historically, they have been described as being localized in the spleen, (head) kidney, and liver of teleost fish [67,68,87]. More recently, additional locations came into view, as Dang et al. described MMC in spleen, kidney, liver, pancreas, and gills of shorthorn sculpin [88]. It should be noted that MM aggregates in salmonids show less structure, lacking a capsule, and display a more random distribution than in other teleost species. However, they still associate with leukocytes and reticular cells [67,89,90], and show evidence of somatic hypermutation [69], so should be considered MMC.

Possible locations of affinity maturation without mention of MMC are the zebrafish intestine and rainbow trout NALT. Based on VDJ sequencing and clonal diversity analysis, Waly et al. observed mutations and dominating clonotypes in intestine samples comparable to the MMC samples we discussed earlier, suggesting affinity maturation somewhere in the zebrafish intestine [83]. In addition, Garcia et al. describe lymphocyte aggregates in the rainbow trout NALT with $CD4^+$, IgM^+, $CD8\alpha^+$, or IgT^+ cells and high aicda mRNA expression [36]. Intranasal vaccination with live attenuated infectious haematopoietic necrosis virus (IHNV) expanded B cells, increased the aicda expression, and induced both proliferation and apoptosis of B cells, suggesting ongoing affinity maturation.

Since local responses seem to be prominent in teleosts, it might be worth investigating whether any affinity maturation is occurring there. The range of possible local aggregates of lymphoid cells with EF responses that could include some affinity maturation in mammals suggests that this is possible. Especially the major B cell responses in the PerC and MM responses as observed in oil-adjuvanted vaccine induced lesions in Atlantic salmon [91] could be interesting to evaluate since the presence and mechanics of possible local affinity maturation would be of great interest for (IP) vaccine design and evaluation.

7. Locations of long-lived plasma cells: what unique factors in the head kidney attract and sustain ASC?

An important consideration in vaccine design is the development of immunological memory. While the presence of humoral memory responses in fish is well accepted, the suitability and accuracy of applying the term ‘long-lived plasma cell,’ as is commonly used in mammals, to describe a subset of antibody-producing B cells in teleost species without a clear understanding of their phenotype, survival niche, metabolic

program, and the necessary soluble survival factors, requires careful consideration. Consequently, there have been inconsistencies in terminologies used to describe fish B cells engaged in antibody production, such as plasmablasts, ASC, plasma cells, short-lived plasma cells, and long-lived plasma cells [8,9,11,48,92–95]. Although there appears to be no standardized criteria for choosing one terminology over the other, characteristics like an increased cytoplasmic-to-nuclear ratio, enhanced antibody production and affinity, larger size and increased internal granularity (FSC Vs. SSC), increased/decreased MHC II expression, reduced surface expression of BCR, and/or insensitivity to hydroxyurea (HU) have been used to describe antibody-producing B cells in fish [7,46,70,92,95]. A significant increase in antibody secreting cell numbers has been observed in fish upon various insults [11,46,48,93], however, what exactly 'instructs' the formation of a long-lived response remains uncertain. In a recent *in vitro* stimulation study, rainbow trout splenic B cells exhibited a preference for activation by TNP-LPS (a model T-independent antigen used in mammalian studies) than TNP-KLH (a model T-dependent antigen used in mammalian studies) [59]. While the fate of these activated B cells transitioning into plasma cells remains undetermined, contrasting findings have been reported in the same fish species where TNP-KLH induced a higher number of plasma cells than TNP-LPS [93,96]. These discrepancies suggest a lack of consensus regarding the development of ASC and lacking comprehension of plasma cell biology in fish.

A study investigating ASC insensitivity to HU revealed significant distinctions among HK, spleen, and peripheral blood, where a majority of HK ASC displayed resistance to HU, suggesting they were non-replicating plasma cells [70]. In an unidentified trafficking mechanism, this study established the scientific basis for the identification of a potential long-term survival niche for plasma cells in the HK. Surprisingly, this niche has remained undefined for over two decades, resulting in a lack of understanding about its specific cellular composition, distribution, and cytokine milieu. While little attention is given to defining plasma cell survival niches, significant focus is placed on identifying their origins [68,73,80,83]. A recent study revealed organized secondary lymphoid microstructures adjacent to splenic MMC as contributors to the genesis of these cells [69]. Moreover, the possible existence of similar survival niches in other important immune sites, like MALTs, has been overlooked. Plasma cells have been reported at mucosal sites in various fish species [28,32,97], but their sensitivity to HU has not been examined, leaving uncertainty about whether these ASC are truly plasma cells. If they are, this raises questions about whether a comparable survival niche exists at mucosal sites or if MALTs use a different mechanism to support ASC survival. A study conducted by our team identified antigen-specific ASC in the PerC of Atlantic salmon upon IP SAV infection. The prolonged presence of these cells (13 wpi) in the PerC seemed to be antigen independent as virus RNA was undetectable in the PerC adipose tissue [47]. This finding suggests a B cell phenotype resembling plasma cells. Additionally, the same study found a higher frequency of Ag-specific ASC in the PerC than in systemic tissues of infected fish. This suggests the presence of a niche in the PerC that selectively favors their survival or a mechanism actively sequestering Ag-specific ASC in the PerC. Consistent with this, in mammals, Ag-specific memory B-1a cells persist indefinitely in the PerC waiting for subsequent infection [98].

8. Trafficking of B cells to and from sites of ongoing infection

Varying B cell subsets and differentiation stages are present in different tissues, and the orchestration of adaptive immune responses requires trafficking of B cells within and between these tissues. Exploring the patterns and timing of B cell trafficking offers insights into the factors that impact the efficiency of the immune response. Such analyses can also aid in optimizing vaccine formulations and regimens, ensuring the targeted delivery of necessary cell populations to designated locations [99,100]. The trafficking trajectory of B cells in teleosts commences in the HK, where their genesis and development start [72]. Subsequently, B cells are proposed to migrate to peripheral immune tissues where they commit a series of developmental events, including maturation, activation, and differentiation into effector antibody-secreting cells before relocating back to their origin in search of a long-term survival niche [70,95]. The molecular cues and structures supporting B cell trafficking in teleosts remain poorly understood. Nonetheless, increased number of B cells at sites of infection or antigen challenge as well as in target tissues undergoing active pathological processes have been documented in several studies, some of which have already been discussed [11,45,48–50,101–103]. While the timing and extent of the increase in B cell numbers exhibit variability, the observations point at the role B cells have in immune surveillance and targeted immune responses. The expanded B cell population within these specific microenvironments seems to function by containing infections at the point of entry or mitigating damage caused by infections in target organs. It remains uncertain whether B cells achieve this directly through their ability to ingest and kill pathogens, indirectly by producing antibodies that enhance the phagocytic activity of other cells, or through a combination of both mechanisms. Findings such as that nearly 80% of cells internalizing bacteria in the PerC after IP infection with *E. coli* were B cells [46], along with a substantial increase in PerC antibody-secreting cell numbers following various infection models [8,11,48], indicate their pivotal role in both mechanisms.

In this context, studies involving IP challenge have contributed significantly to advancing our understanding of B cell trafficking in fish. It is now firmly established that B cells represent one of the main cell types that undergo a notable increase in numbers in PerC in response to various IP antigenic stimulations or infections as previously discussed [8,11,45,46,49]. Despite consistently observing elevated B cell numbers in the PerC across these studies, the question of whether these increased numbers result from migration from other immune sites or stem from local activation and proliferation requires further exploration. In a study investigating the homing of leukocytes in Atlantic salmon after IP stimulation with OVA and CpG, the injected materials were exclusively found in $MHCII^{+}/IgM^{-}$ cells in HK and spleen [71]. This study did not analyze the immune response in the PerC, leaving out information about local phagocytic cells. Despite this, given the robust phagocytic ability of IgM^{+} B cells in the PerC, the above finding implies that B cells encountering antigens IP might remain within the PerC, where they become activated and undergo differentiation-a hypothesis that requires further investigation. An important question that arises from this is how immune cells, such as B cells, move in and out of the PerC.

After IP stimulation of turbot (*Scophthalmus maximus)*, the trafficking of bead-containing PerC neutrophils and macrophages to systemic lymphoid organs mainly occurs through the visceral peritoneum as evidenced by rapid attachment of these cells to the peritoneal folds that connect visceral organs [104]. While not explored in this study, it is tempting to speculate that B cells might employ a similar route of trafficking as the myeloid cells. Although PerC leukocytes in fish and mammals are freely migrating between the systemic and PerC compartments, they differ in their transit points. In mammals, omental milky spots, besides functioning as peripheral secondary immune sites, act as gateways for leukocytes moving between the PerC and systemic compartments [105]. Milky spot-like structures with secondary immune function have been described in fish [106]. Nevertheless, further investigation is necessary to understand their potential involvement in the trafficking of leukocytes. Apart from these structural bases, the trafficking of leukocytes in mammals is mediated by various chemoattractants [107,108] and presumably in fish, as IP injection of TNF-α promotes expression of different CC and CXC chemokines in endothelial cells that recruit and activate different phagocytic cells in gilthead seabream (*Sparus aurata*) and zebrafish [109].

While several teleost studies have documented an increase in PerC B cells following IP stimulation, little attention has been placed on the trafficking of these cells in and out of the PerC. Studies on PerC adipose

tissue in Atlantic salmon and rainbow trout have revealed differential transcription of a wide range of immune genes, including those involved in antigen presentation [48,106,110], hinting at potential antigen presentation capabilities. Additionally, others have shown B cells exhibiting a plasmablast phenotype and an increased presence of ASC within the PerC after IP challenge [8,11,46,110], indicating local activation and differentiation. Notably, despite accumulating evidence showing an active immunological role of the PerC against IP administered antigen, specific structures that provide mechanistic support for activation and differentiation of B cells have not been conclusively illustrated. Existing models describing the distribution of B cells in teleost species have overlooked the potential contribution of peripheral immune sites, such as PerC, in B cell activation and differentiation [70,72,95]. This has inspired us to propose a model outlining the dynamic interplay between the PerC and systemic sites concerning B cell activation and maturation after IP challenge (Fig. 2).

9. Conclusion

Even though understanding B cell biology of teleosts is fundamental to elucidating antibody responses and improving vaccine design for aquaculture, we have been relying heavily on assumptions and extrapolations from the mammalian paradigm. Recent research has yielded valuable information, but also highlighted the need to keep an open mind and be willing to challenge assumptions, especially when investigating spatial distributions of B cells. When investigations only focus on 'the usual suspects', we risk missing out on information regarding other important locations for B cell responses. The PerC and its associated AT, new MALTs, and MMC are some examples of locations whose importance in B cell responses has only been appreciated in more recent years. Additional holistic omics approaches combined with tailored reagents targeting different B cell phenotypes will be crucial to further evaluate teleost B cell biology and spatial distributions in the coming years. These tools, together with evolving models and a willingness to keep challenging them, will allow us to obtain a more complete story on the special distribution of teleost B cells and whether they are really going there and back (to the HK) again, or whether they occasionally find a different location to end their quest.

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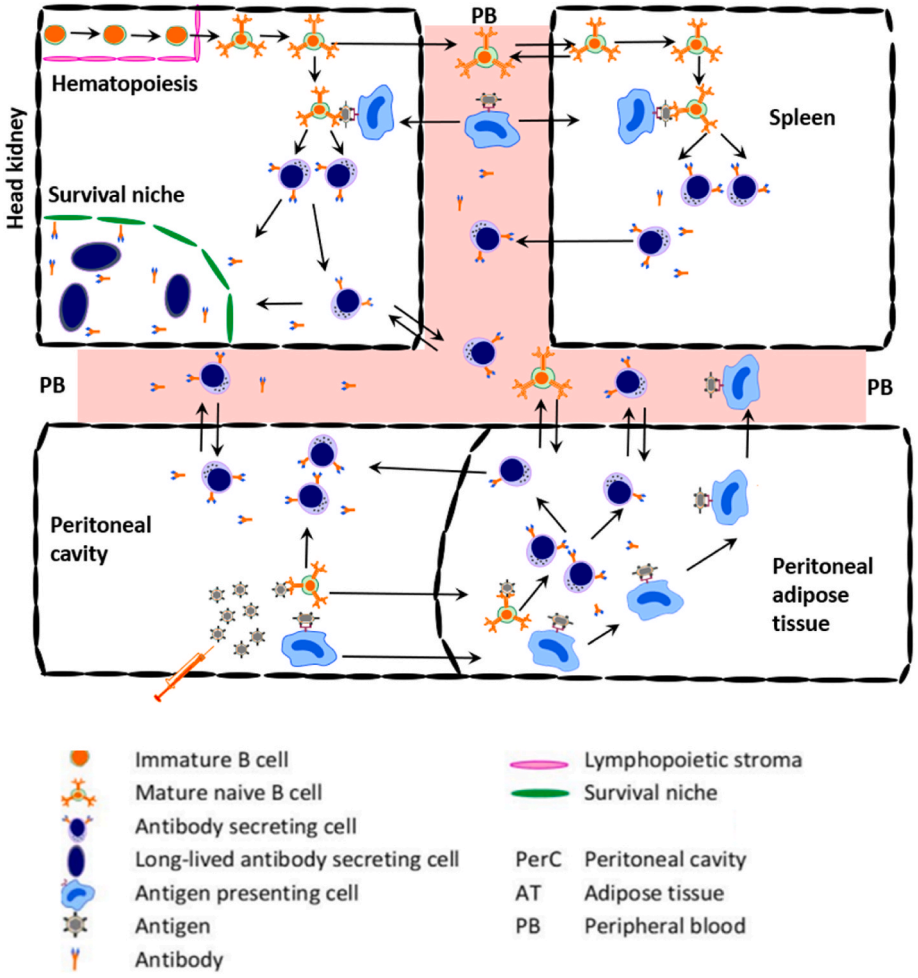

Fig. 2. A model illustrating antibody secreting cells (ASC) trafficking within the peritoneal cavity (PerC), head kidney (HK), and spleen after intraperitoneal (IP) challenge. B cells develop and mature in the HK. Mature naïve B cells exit the HK and travel through the bloodstream in pursuit of foreign antigens throughout the body. Resident PerC B cells and other antigen presenting cells (APC) sample antigens that are administered IP. Upon antigen encounter, resident PerC B cells mature into ASC in PerC adipose tissue (AT). The AT ASC migrate back to the PerC or to the systemic sites for antibody secretion and to search for a survival niche in the HK to terminally differentiate into long-lived ASC. Similarly, antigen encountered PerC APCs migrate to the PerC AT to present antigen to AT resident naïve B cells or to transit to the systemic immune tissues. The Ag encountered APCs migrate from the PerC AT to the HK and spleen via the peritoneal fold connecting the viscera and to the blood for antigen presentation and activation of naïve B cells. These B cells mature into ASC in the systemic sites, secrete IgM, and migrate to a survival niche in the HK or are redistributed via the blood to the periphery, such as PerC, for antibody production.

CRediT authorship contribution statement

Shiferaw Jenberie: Conceptualization, Writing – original draft, Writing – review & editing. **Yorick A. van der Wal:** Conceptualization, Writing – original draft, Writing – review & editing. **Ingvill Jensen:** Writing – original draft, Writing – review & editing. **Jorunn B. Jørgensen:** Writing – original draft, Writing – review & editing, All authors read and approved the final manuscript.

Declaration of competing interest

The authors confirm that no financial or other conflicts exist.

Data availability

No data was used for the research described in the article.

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