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Role of teleost B cells in viral immunity

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ABSTRACT

Teleost fish possess all the necessary elements to mount an adaptive immune response. Despite this, the important physiological and structural differences between the mammalian and the teleost fish immune system, anticipate significant changes regarding how this response is coordinated and executed. B cells are key players in adaptive immune responses through the production of antibodies. However, recent studies performed in mammals and other species including fish point to many additional functions of B cells within both the adaptive and the innate immune system, in many occasions taking part in the crosstalk between these two arms of the immune response. Furthermore, it should be taken into account that fish B cells share many functional and phenotypical features with innate B cell populations from mammals, which will surely condition their response to antigens. Concerning viral infections, although most studies undertaken to date in fish have been focused on characterizing antibody production, some recent studies have demonstrated that fish B cells are able to interact with viruses at different levels. In this sense, in the current review, we have tried to provide an overview of what is currently known regarding the role of teleost B cells in antiviral immunity.

1. Introduction

The innate immune system encloses non-specific defense mechanisms not dependent upon previous pathogen encounters, which provide a first line of defense and constitute the basis of the immune response in invertebrates and lower vertebrates. In contrast, the adaptive immune system is stimulated by exposure to an antigen, increasing in magnitude and defensive capacities with each successive exposure to this particular antigen. Adaptive immunity involves both humoral and cellular responses. The humoral response is mediated by antibodies (immunoglobulins, Igs) produced by B cells, which can recognize microbial antigens, neutralizing their infectivity and helping in their elimination. In contrast, cellular immunity is mediated by T cells which promote the destruction of pathogens that are localized inside cells such as viruses or intracellular bacteria, thus promoting the elimination of infected cells. Because of this, mammalian immunologists aiming to analyze adaptive responses to viral pathogens have frequently focused on T cell responses, and much less attention has been paid to determining the role of B cells in antiviral immunity. However, it has been known for many years that antibodies can mediate a full protection against many viral diseases and thus the role of B cells in antiviral immunity seems evident [1–3].

The adaptive immune system is based on the presence of recombination-activating gene (RAG)-recombined B cell receptors (BCR) and T cell receptors (TCR) on the surface of B cells and T cells, respectively, and the major histocompatibility complex (MHC), elements present in teleost fish. Despite this, it is obvious that fish and mammalian immune systems have many similarities but do have in fact many differences. For example, neither lymph nodes nor bone marrow are present in fish. Instead, the head kidney assumes hematopoietic functions, being the organ where B cells have been suggested to originate and differentiate in most species [4]. As there are no lymph nodes in teleost, the spleen constitutes the main secondary immune organ in these species. However, the splenic white pulp is poorly developed in comparison to mammalian species and no conventional germinal centers (GCs) have ever been visualized [5]. As GCs are the sites where B cells interact with T follicular helper cells and specialized follicular dendritic cells (FDCs) to mount T-dependent (TD) B cell responses [6], the lack of these structures in teleost fish will significantly affect how B cells respond to antigens, specially TD antigens. Similarly, although fish also possess a mucosa-associated lymphoid tissue (MALT), in all fish species studied to date, the B and T cells present in the mucosa are scattered in a disorganized fashion, in contrast to the mammalian MALT which contains organized structures such as Peyer's patches where mucosal immune responses are initiated [7].

In mammals, many different subsets of B cells are present, playing specific roles in the immune response. Conventional mammalian B cells, catalogued as B2 cells upon the description of the B1 cell

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population, express both IgM and IgD on the cell surface and predominantly participate in TD antibody responses that require the cooperation of $CD4^+$ helper T lymphocytes for a complete activation. As mentioned before, these TD antibody responses take place in the GCs, where, upon antigen encounter, B cells divide and obtain the capacity to differentiate into antibody-secreting cells (ASCs), either plasmablasts or plasma cells, or memory B cells. During this differentiation, the Igs produced also change through two independent processes. Some activated cells begin to produce antibodies other than IgM or IgD in a process designated as class switch recombination (CSR). Thus, B cells replace the constant region of IgM by that of IgA, IgE or IgG, antibodies with higher affinity and different effector functions depending on what is required based on the particular antigen encountered [8]. Additionally, the variable (V) region of the Ig genes are highly mutated through what has been designated as somatic hypermutation (SHM). In the context of this antibody diversification, B cells that produce antibodies which bind antigens with higher affinity are preferentially expanded. Both CSR and SHM require the action of activation-induced cytosine deaminase (AID) [8]. Within this follicular pathway, B cells eventually exit as high affinity long-lived plasma cells or memory B cells, responsible for the production of isotype-switched antibodies required for immunological memory. This TD pathway provides strong immunological memory, but is relative slow to occur. Thus, it is integrated with additional T-independent (TI) pathways that mainly involve B cell subsets commonly designated as innate B cells, mainly B1 cells and marginal zone (MZ) B cells. These TI responses are activated by direct recognition of the pathogens at mucosal compartments in the absence of T cell cooperation, and are also greatly influenced by cytokines secreted by innate cells such as those belonging to the tumor necrosis factor (TNF) family of ligands [9]. B1 cells arise early during the ontogeny and are considered components of the innate immune system. However, to the light of recent investigations, it seems now evident that these cells play a key role in natural resistance, being essential for the early clearance of pathogens. These innate B cells have a highly poly-specific (poorly mutated) BCR that can bind self-antigens or microbial products such as lipopolysaccharide (LPS), multivalent polysaccharides or large antigens with repetitive structures, being all of them TI antigens. Upon activation, B1 cells also differentiate to ASCs, although whether they reach a fully differentiated state (as a plasma cell) is still a matter of debate [10].

Teleost fish possess a predominant serum Ig (IgM) composed of 2 heavy and 2 light chains each (2H + 2L) bridged by disulphide bonds. These Igs form tetramers, and possess some characteristics of mammalian IgM such as the H chain size [11], low intrinsic affinity for antigen and high functional affinity or avidity [12,13]. IgD has also been identified in most fish species [14,15], although its specific role during the immune response is still largely unknown in fish as it is in mammals [16]. On the other hand, the recent identification of IgT (designated as IgZ in zebrafish) revealed that fish had a unique Ig with new characteristics not observed in other vertebrate Ig class, with its own D and J elements for the generation of diversity [17,18]. Consequently, different B cell subsets can be found, depending on the type(s) of Igs exposed on the membrane. IgD^+IgM^+ B cells are present in all teleost species analyzed thus far and are thought to represent the majority of B lymphocytes in fish. When these cells are activated, as it occurs in mammals, IgD is lost from the cell membrane, giving rise to activated IgM^+IgD^- B cells that have a plasmablast/plasma cell profile. The presence of cells that have lost IgD from the cell membrane has been reported in rainbow trout (*Oncorhynchus mykiss*), demonstrating that these cells had a plasmablast/plasma cell profile [19]. Similarly, the presence of B cells with a plasmablast/plasma cell profile had been reported in rainbow trout through the use of antibodies directed against transcription factors specific of the different B cell developmental stages [20,21]. Furthermore, a distinction between plasmablasts (replicating, low antibody secretors, bearing minimal BCR) and plasma cells (non-replicating, terminally differentiated, high antibody secretors, bearing no BCR) was resolved through the use of hydroxyurea, establishing that upon immunization, both cell types are produced [22,23]. In addition to IgM^+IgD^+ and IgM^+IgD^- B cells, IgD^+IgM^- B cells have also been reported in channel catfish (*Ictalurus punctatus*) [14] and in rainbow trout gills [24], although their immune role has not yet been established. Finally, a lineage of B cells uniquely expressing IgT/Z has been reported in species such as rainbow trout [25] or zebrafish (*Danio rerio*) [26]. Because the ratio of IgT^+ cells/IgM^+ cells is higher in mucosal tissues than in central immune organs, and given the fact that IgT was the main Ig that responded in mucosal compartments to several parasites [25,27,28], IgT has been postulated as an Ig specialized in mucosal immunity [28]. However, it seems plausible that IgT also plays important role outside mucosal compartments given that significant IgT responses have been also reported in the spleen in response to an intramuscular injection with an inactivated virus [29], in the muscle of DNA-vaccinated fish [30] and in the kidney in response to a parasitic infection [31].

As mentioned above, there are several characteristics of the teleost immune system that seem to anticipate that the main B cell subset found in teleosts, IgM^+IgD^+ B cells, could resemble innate B cell subsets present in mammals. These include the fact that systemically fish rely mostly on IgM responses in the absence of CSR; fish do not form conventional GCs, and there is a poor affinity maturation of antibodies through the course of an immune response [13]. Recent findings by our group further support this statement, as we have established that fish IgM^+ B cells, unlike mammalian B2 cells, constitutively express many pattern recognition receptors (PRRs) [32,33]; strongly respond to pro-inflammatory signals [30,34] and transcribe B1 cell markers not expressed in B2 cells such as CD9 [35]. Furthermore, a high phagocytic capacity has been demonstrated for teleost B cells [36] and later on for mammalian B1 cells [37]. Finally, this year, our group has demonstrated that a high percentage of IgM^+ B cells in rainbow trout express CD5 [38]. CD5 is a specific marker for of B1a cells, a subset of mammalian B1 cells. In mammals, CD5 is known to be physically associated with the BCR [39] and has been postulated as a negative-regulator of BCR signaling because while B1 cells do not proliferate in response to BCR cross-linking in regular mice, B1 cells from CD5-null mice restored this capacity [40]. Thus, the higher expression of CD5 in trout IgM^+ B cells, suggested that this population could also share additional functional and phenotypic traits of mammalian B1 populations. Confirming this hypothesis, our results showed that trout IgM^+ B cells, unlike mammalian B2 cells and similarly to B1 cells, exhibited a large size, high complexity, high surface IgM and low surface IgD expression [38]. Additionally, fish IgM^+ B cells, unlike murine B2 cells, also displayed extended survival in cell culture and did not proliferate after B cell receptor (BCR) engagement [38]. To date, the evolutionary relationship between mammalian B1 and B2 cells still remains unsolved and currently it has not been possible to exclude either of the two different hypothesis that attempt to explain the relation between these two cell subsets as both of them have supporting experimental data. Thus, the lineage hypothesis states that B2 cells have originated from a B1-independent precursor, having evolved independently of B2 cells, whereas in the induced differentiation hypothesis B1 and B2 are thought to have originated from a common phagocytic cell population [41]. In this situation, it seems quite relevant to determine the differences and similarities between these two mammalian B cell subsets and B cells from less evolved vertebrate species. Thus far, the results obtained revealing that fish IgM^+ B cells share many phenotypic and functional traits of mammalian B1 cells, seem to support a common origin for B1 and B2 cells and give weight to the previously formulated hypothesis of a common phagocytic precursor for mammalian B1 cells and fish IgM^+ B cells [41,42].

In this context, in the current review, we have tried to summarize the most interesting aspects of what is known to date regarding how B cells respond to viral infections in fish, focusing on those aspects in which the response of teleost B cells seems to differ from that of

mammals.

2. Antibody production in response to viral infections in fish

IgM is the most studied Ig in teleost fish. IgM has been detected not only in serum, but also in mucus [25,27,28], bile [43] and eggs [44]. Furthermore, the existence of specific IgMs has been demonstrated in all teleost species studied so far [45]. Among these studies, the capacity of teleost fish to produce serum IgMs that can specifically recognize viral antigens has also been demonstrated in a wide range of species in response to viruses [1,46,47]. For example, in the case of fish rhabdoviruses, it is widely recognized that rainbow trout can produce specific and fully functional serum IgMs that are able to neutralize virus pathogenicity *in vitro* [1]. No studies to date have addressed the role of mucus or bile IgMs through the course of viral infections. In the case of IgMs present in eggs, neutralizing IgMs specific for infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, have been identified in common trout (*Salmo gairdneri*) [48].

Although the protective role of virus-specific serum antibodies has been established in different passive immunization experiments using either sera or purified IgMs obtained from fish surviving an infection [49–51] or from vaccinated fish [1,52,53], the precise contribution of these antibodies to the outcome of a viral infection in fish remains unclear in most disease models. Teleost seem to have a more limited repertoire than mammals [54] and a limited secondary response. Moreover, the low temperature of salmonids delays their lymphocyte responses with respect to mammals [55] and while serum IgMs do appear after 10–15 days at 15 °C, it takes more or less 30 days at 5 °C [56]. Although SHM has been reported in fish [31], the antibody response only reaches a 2–3-fold higher affinity after 90 days [13], demonstrating that affinity maturation is much lower than that seen in mammals. Additionally, CSR has never been reported in teleost fish. Thus, despite having established that fish specific IgMs can partially protect from posterior viral encounters, we know that in the context of an experimental infection, specific IgMs peak 6–10 weeks [51,57], far after the mortality had ceased (usually 1 week after infection). These results indicate that the specific neutralizing antibodies produced as response to a viral infection in fish appear too late to play any role in protection of non-immunized fish against an acute infection. Moreover, *in vitro* neutralizing antibodies can only be detected in 54% of the survivor animals and there is no detectable increase in their levels after a secondary infection [57,58]. In this context, the worth of fish producing neutralizing IgMs is not known, but it could be possible that specific antibodies are produced to control viral infections in natural conditions in which the initial viral load is low and the disease takes much longer to progress.

To date, not many studies have addressed the production of IgD or IgT during the course of viral infections and most of them have exclusively focused on analyzing the transcriptomic responses of these Igs. For example, IgT mRNA levels were significantly increased in the head kidney of gilthead sea bream (*Sparus aurata*) challenged with nodavirus [59] and in the gills of Atlantic salmon (*Salmo salar*) infected with infectious salmon anemia virus (ISAV) [60]. When grouper (*Epinephelus coioides*) larvae were immunized with a nodavirus inactivated vaccine, IgT transcription levels were also induced in specific organs, depending on the route of immunization used [61]. IgT transcription was also significantly increased in the head kidney and the intestine of Atlantic salmon orally immunized with an alginate-encapsulated vaccine against infectious pancreatic necrosis virus (IPNV) [62]. However, the production of virus-specific IgT has still not been demonstrated. Similarly, although a secreted form of IgD was identified in serum and different mucosal organs in species such as rainbow trout [15], whether pathogen-specific IgDs play a role during the course of an infection remains to be demonstrated. Nevertheless, the regulation of IgD at the transcriptional level has been demonstrated during the course of viral infections in teleost fish. In this sense, IgD mRNA levels were significantly up-regulated in freshwater carp (*Catla catla*) [63] and rohu (*Labeo rohita*) [64] upon intramuscular injection with an inactivated rabies virus formulated with adjuvant.

3. Natural antibodies

Natural antibodies refer to those Igs that are present prior to the body encountering a cognate antigen, providing a first line of defense against infection and thereby, allowing time for a specific antibody response to be established [65]. The presence of natural antibodies has been demonstrated in a wide range of fish species including teleost and cartilaginous fish [66]. In teleost, these antibodies preferentially recognize trinitrophenyl (TNP) [66]. Remarkably, in rainbow trout, these anti-TNP antibodies were able to protect rainbow trout cells from both viral hemorrhagic septicemia virus (VHSV) or IPNV infection *in vitro* [67], demonstrating that these antibodies, produced by B cells in homeostasis, are able to protect fish in the early stages of infection, until a specific response is mounted. In this sense, in rainbow trout, it was shown that as the number of IgM antibodies recognizing IPNV increased in serum during the course of a viral infection, the number of natural IgMs binding targets such as galactosidase, poly I:C or phosphorylcholine significantly decreased while the number of total IgMs remained constant [68], thus suggesting a shift on the specificity of these Igs. The capacity of natural antibodies to control an infection has also been demonstrated in carp (*Cyprinus carpio*). In this case, purified IgMs from non-infected fish were shown to neutralize *Trypanosoma carassii,* an extracellular protozoan parasite *in vitro* [69]. Furthermore, also in carp, natural antibodies were shown to be dependent on the genetic background and to increase with age and exposure to non-sterile environments [70]. In the case of goldfish (*Carassius auratus*), natural antibodies to the extracellular A-layer protein of *Aeromonas salmonicida* were found in variable numbers among unchallenged individuals [71]. Remarkably, only those individuals that contained high numbers of natural antibodies against this protein in serum were naturally resistant to a subsequent infection. Furthermore, it seems that there are fish species in which natural antibodies could play a fundamental role during the course of a viral infection due to impairments in the production of specific antibodies. This is the case of cod (*Gadus morhua*), as its levels of serum IgM are much higher than those of other species in homeostasis while immunization and challenge experiments carried out in this species generally result in poor acquired antibody responses [72].

4. Ig repertoires in response to viral antigens

As mentioned before, the generation of antibody diversity constitutes the basis of the specific humoral immune response and is critical for the generation of an adequate protection [73]. This antibody diversity is achieved through different processes. Prior to exposure to an antigen, the initial generation of a broad antibody repertoire is achieved early in B cell development by rearrangement of the V, D and J gene segments to generate Igs with unique Ig heavy- and light-chain variable regions (IGHV and IGLV) [74]. After antigen encounter, a second strategy to increase the Ig repertoire is through junctional diversity, a process through which different sizes are generated in the heavy chain sequences by imprecise V(D)J recombination. Terminal deoxynucleotidyl transferase (TdT) is one of the enzymes responsible for the generation of this junctional diversity, through the addition of non-templated (N) nucleotides to the single-strand DNA ends [75]. Finally, during B cell differentiation, the genes encoding the variable domains of the heavy and light chains undergo a high degree of point mutations through SHM, mostly through the action of AID [76].

As part of the analysis of the immune response, studies aimed at characterizing the immune repertoire of the IGHV have provided highly valuable information to understand the complex processes of BCR gene dynamics during the immune response. In the past, the diversity of the

Ig response was estimated through a technique designated as CDR3 spectratyping. CDR are the regions of the recombined BCR that bind to their specific antigen. Among them, the CDR3 region of the VH gene is the most hyper-variable region of the BCR genes [77], thus the determination of CDR3 size by spectratyping (also known as Immunoscope) was a powerful tool to analyze the BCR repertoire, commonly used both in mammals and fish [29]. This technique was based on the fact that B cell clones differ in CDR3 length, and thus, the CDR3 length distribution analysis is an estimate of the overall diversity and any deviation from a bell-shaped Gaussian distribution is indicative of clonal expansions. These clonal expansions can be monoclonal or oligoclonal depending on whether there is one single or several expanded peak(s) [78]. However, in the last decade, the huge improvement of sequencing methods that gave rise to the new generation sequencing strategies, has facilitated that CDR3 clones were subsequently sequenced to gain additional information on how the clonal diversity of B cells was affected by a certain disease or through a specific physiological process [79]. Alternatively, sequencing through Illumina MiSeq (2×300) of a pooled sample of PCR products obtained using a set of isotype-specific primers for IgM, IgD and IgT, respectively, and a set of IGHV subgroup-specific primers that can amplify all members of the 13 known IGHV groups was recently used to study the clonal diversity and SHM processes in rainbow trout Igs from fish infected with a myxozoan parasite [31].

Concerning viral infections, in 2013, Castro et al. studied the repertoire of IgM, IgD and IgT in the spleen of homozygous isogenic rainbow trout that had been challenged with VHSV using CDR3-length spectratyping and subsequent 454 GS FLX pyrosequencing [29]. In this work, the authors established that in healthy fish a bell-shaped Gaussian distribution of CDR3 lengths is observed for IgM, IgT and IgD, as also observed in non-disturbed mammalian systems. However, when CDR3 spectratyping was performed in infected fish, the authors demonstrated a selection of some specific peaks for IgM and IgT but not for IgD which included some private (only observed in one fish) and public (observed in all fish) responses. This selection of specific peaks is indicative of the expansion of specific B cell clones in response to the virus. Furthermore, although there were only a few amplified μ and τ junctions (VDJ combinations), some of these clones were significantly amplified during the response to the virus, again suggesting the selection of specific B cell clones and the generation of IgM- and IgT-secreting cells in the spleen of the infected animals. Therefore, this study provided evidence on the fact that IgT does not only play a role in mucosal responses, but can also be involved in the specific response generated in the spleen in response to a virus infection. In Atlantic salmon, a deep sequencing protocol was established to characterize the repertoire of IgM heavy chain variable region during the early developmental stages and after infection with piscine myocarditis virus (PMCV), the causative agent of cardiac myopathy syndrome [80]. In this case, a protocol based on the construction of IgM-VR libraries through a 5′-RACE strategy and posterior sequencing through Illumina MiSeq was developed. This protocol covered the entire repertoire and provided with a new pipeline for sequence analyses of IgM repertoires in Atlantic salmon. Concerning the changes in the IgM repertoire observed after 10 weeks of infection with PMCV, the authors found that shared clonotypes were markedly increased after viral infection. Interestingly, shared clonotypes comprised more than 1/3 of all transcripts and all highly represented clonotypes were shared. Finally, they observed that these shared clonotypes were significantly increased in head kidney and blood, but not in the spleen in response to viral infection [80]. These results suggest that upon exposure to PMCV, salmon organize a public response in which the shared clonotypes are expanded.

5. Teleost B cells as antigen presenting cells (APCs)

B cells not only produce antibodies against invading pathogens, but also contribute to the clearance of pathogens through different actions. For example, B cells are professional antigen presenting cells (APCs) and as such, they play a key role in the activation of T cells. Thus, mammalian B cells acquire antigens through the BCR or through pinocytosis mechanisms, to then present them to T cells in the context of MHC II expressed on their surface. However, in the past, studies designed to define the role of B cells as APCs in mammals have generated discrepant results, and consequently there is still a debate on whether B cells on their own are sufficient to activate T cells *in vivo* and whether this interaction renders T cells immunogenic or tolerogenic [81]. It seems now clear that when B cells encounter non-specific antigens, the outcome is T cell tolerance, whereas when they encounter a specific antigen, they can efficiently activate cognate T cells [82]. In fish, it has been demonstrated in species such as rainbow trout or zebrafish that IgM^+ B cells express MHC II on the cell surface [33,83]. Furthermore, Zhu et al. elegantly demonstrated in zebrafish that B cells have the capacity to present both soluble and particulate antigens to specific T cells [83]. Thus, the fact that teleost B cells have a high phagocytic capacity [36], qualifies them to more effectively present particulate antigens than mammalian B2 cells. Concerning whether teleost B cells are able to present viral antigens to T cells, only indirect evidence is available. Thus, it has been demonstrated that incubation of rainbow trout splenocytes with VHSV provokes the specific up-regulation of surface MHC II expression on IgM^+ B cells, not observed in IgM^- B cells or in response to Poly I:C [33]. In these experiments, a significant up-regulation of CD80/86 and CD83 mRNA levels in IgM^+ B cells that was not visible in the IgM^- cell fraction was also demonstrated [33]. Thus, altogether, these results suggest that teleost IgM^+ cells act as APCs during the course of a VHSV infection.

6. Evidence of direct interaction of B cells with fish viruses

In mammals, it has been widely demonstrated that B cells have evolved to directly sense microbes through innate receptors such as Toll-like receptors (TLRs) and that this TLR-mediated activation of B cells contributes to the establishment of an adequate humoral response [84]. However, there are important differences in the pattern of TLRs transcribed in different B cell subsets from diverse mammalian species.

TLRs known to recognize highly conserved structures of viral origin in both mammals and fish include TLR3, 7, 8 and 9. These TLRs localize within endosomal compartments and detect foreign nucleic acids [85]. Among them, TLR3 is the receptor for dsRNA and together with TLR4 (responsible for LPS sensing in mammals), they are the only TLRs that signal through the TRIF pathway and thus induce interferon β (IFN-β) [86]. Interestingly, salmonids and other species such as pufferfish (*Fugu rubripes*) lack TLR4 [87]. Furthermore, in those species in which TLR4 is present such as zebrafish, TLR4 does not seem to be involved in LPS detection [87] Concerning the response to viral stimuli, TLR3 is the main TLR responsible for the sensing of viruses, along with the fish-specific TLR22 also known to sense dsRNA [88]. Human naïve tonsil or blood B cells lack TLR3 [89,90], although it is expressed in human plasma cells [90]. Additionally, a subset of B cells located in the upper respiratory tract of humans was shown to express TLR3 and respond to viral stimuli [91]. In rainbow trout, IgM^+ B cells from different tissues were shown to express all the TLRs known to date, including TLR3 and TLR22 [32]. This suggested a high capacity of teleost B cells for sensing viral ligands, and to verify this hypothesis, the effects of VHSV on the functionality of splenic IgM^+ cells was performed, comparing the effects to those provoked by a TLR3 agonist such as Poly I:C [33]. These experiments demonstrated that VHSV enters IgM^+ B cells and starts viral transcription even though viral translation is interrupted. Consequently, this early virus-B cell interaction induces the transcription of Mx, type I IFN (IFN1) and the chemokine CK5B in these cells. Furthermore, this induction of IFN1 was dependent on an endosomal receptor, probably TLR3 [33]. Additionally, this direct interaction of VHSV with trout IgM^+ B cells induced NF-κB activation, and as a

consequence pro-inflammatory cytokines such as interleukin 1β (IL-1β) were secreted. Thus, as also shown in mammals, as part of their immune tasks, teleost B cells also secrete cytokines upon antigen sensing to modulate the early immune response of other cell types.

The fact that teleost IgM$^+$ B cells are equipped with a wider range of innate receptors and owing that they have retained several innate functions not maintained in mammalian B2 cells, strongly suggests that, in teleost, IgM$^+$ B cells are implicated in the initial recognition of pathogens to play a role both in innate functions and in the early onset of adaptive mechanisms. In correlation with this hypothesis, we have seen that IgM$^+$ B cells are one of the main leukocyte types attracted to the sites of inflammation in response to stimulation with viral antigens. This was initially visualized when examining the local immune response to a DNA vaccine against VHSV [30]. Within this study, we observed that both IgM$^+$ and IgT$^+$ B cells were the main cell subsets that were infiltrated in the muscle, surrounding the injection area. These cells were visualized by immunohistochemical techniques and flow cytometry after a protocol for their isolation from the muscle was optimized [30]. Similarly, IgM$^+$ B cells constituted the main leukocyte subset within the peritoneal cavity after the injection of inactivated VHSV in rainbow trout [34]. In this case, these B cells were shown to differentiate to ASCs locally in response to the virus [34]. Altogether, these results demonstrate a key role in the early stages of antigen recognition and in initiation of the inflammatory responses.

7. Effect of viral antigens on B cells in mucosal surfaces

As mentioned above, scattered lymphocytes (both T and B cells) have been detected both in the lamina propria or residing between epithelial cells in mucosal tissues [92]. However, not many studies have focused on studying how B cells in this mucosal surfaces interact with viral antigens. In 2013, Ballesteros et al. identified IgM$^+$ and IgT$^+$ B cells all along the digestive tract of rainbow trout with the exception of the stomach, and studied the B cell response in the different segments in fish orally vaccinated with an alginate-encapsulated DNA vaccine against IPNV [93]. In naïve fish, IgM$^+$ B cells were mostly located in the lamina propria although scattered IgM$^+$ intraepithelial lymphocytes (IELs) could also be detected in the pyloric caeca region. On the other hand, IgT$^+$ B cells were primarily localized as IELs all along the digestive tract. These results challenged the established line of thought that teleost IELs are exclusively T cells [94]. Given that no organized lymphoid structures such as mammalian Peyer's patches have ever been described in the teleost fish intestinal tract, if these T IELs are in contact with B IELs remains unknown. Interestingly, ten days after a single oral IPNV vaccine administration, a significant increase of both IgM$^+$ and IgT$^+$ B cells was observed, especially in the pyloric caeca region [93]. Whether this increase in the number of B cells is a consequence of local proliferation or a result of recruitment of cells from central immune organs should be further investigated. However, these results demonstrated that both IgM$^+$ and IgT$^+$ B cells respond to an oral viral antigen in the digestive tract and pointed to the pyloric caeca as a key segment in regards to antigen uptake given that VP2 transcription levels were highest in this segment [95] and concerning the organization of an early local immune response mediated by B cells. Also in rainbow trout, a transcriptomic study performed in gills following VHSV infection demonstrated the up-regulation of both IgM and IgT [96], again suggesting the involvement of both B cell subsets in the early antiviral response within mucosal surfaces. In sea bass, IgT expression has also been shown to increase in both gills and spleen after infection with nodavirus [97] confirming the involvement of IgT in mucosal surfaces also in response to viral infections. Interestingly, results obtained from Aquilino et al. [96] revealed that MHC II transcription levels in sorted IgM$^+$ B cells from gills were significantly higher in cells from virus-infected fish that those from in mock-infected animals, again suggesting a role for local IgM$^+$ B cells in antigen presentation processes.

8. Production of B cell-stimulating factors during viral infections in fish

In mammals, B cell functionality is modulated by cytokines released by innate cells, such as macrophages, dendritic cells or granulocytes, upon the early recognition of common pathogen patterns through invariant receptors. Among them, cytokines belonging to the TNF family of ligands such as B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL) play a major role in B cell homeostasis, activation and differentiation processes in mammals [98]. Concerning the receptors through which they signal, mammalian BAFF binds to transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI, also known as TNFRSF13B) and to BAFF receptor (BAFF-R, also known as BR3 or TNFRSF13C) with a strong affinity and to B cell maturation antigen (BCMA, also known as TNFRSF17) with a lower affinity. Then again, mammalian APRIL binds TACI and BCMA with a high affinity [98]. Interestingly, APRIL also signals in some B cell subsets through the polysaccharide side chains of heparan sulfate proteoglycans (HSPGs), structurally unrelated to TNF receptors [99]. All these different receptors are preferentially expressed in B cells and their expression profile depends on the B cell subset, the anatomical location or the stage of differentiation [100,101], thus conditioning the response of these cells to BAFF and/or APRIL.

The sequences of BAFF and APRIL have been identified in many cartilaginous and teleost fish species (reviewed in Ref. [102]), however, not many functional studies have been performed to date. In addition to BAFF and APRIL, a third molecule with high similarity values with BAFF but containing a D–E loop characteristic of APRIL was identified in 2007 by Glenney and Wiens in some teleost fish species [103]. Thus far, this molecule has been reported in salmonids, pufferfish, three spined stickleback and lamprey [103,104], but then again, many aspects of its functionality remain unclear. Finally, BAFF-R, TACI and BCMA sequences have been identified in rainbow trout [105]. Thus, the levels of transcription of the three receptors have been studied in different B cell subsets in this species [105,106].

Most of the studies aimed at clarifying the role of these cytokines on B cell functionality have also been undertaken in rainbow trout. Thus, we know that BAFF promotes the survival of splenic IgM$^+$ B cells, up-regulates the expression of membrane MHC II on these cells and increases IgM secretion [107]. Interestingly, in addition to being produced by myeloid cells, in rainbow trout, it was shown that some IgM$^+$ B cell subsets are able to secrete BAFF even in homeostasis, pointing to an autocrine regulatory system [107]. APRIL, on the other hand, has been shown to be capable of inducing the proliferation of splenic IgM$^+$ B cells by itself, in contrast to the situation in mammals where APRIL is only a co-stimulator factor [108]. Additionally, APRIL was shown to increase IgM secretion without promoting a terminal differentiation to plasma cells, to increase the levels of surface MHC II on splenic IgM$^+$ B cells and to augment the capacity of these cells to process antigen [109]. Finally, thus far, the only functional effect reported for BALM is the fact that it increased the survival of peritoneal IgM$^+$ B cells in rainbow trout [106].

Concerning whether these cytokines are regulated in fish during the course of viral infections, some studies were performed in the peritoneal cavity of rainbow trout. As mentioned before, our group demonstrated that the peritoneal injection of VHSV induced a significant increase in the number of IgM$^+$ B cells in the peritoneal cavity and their differentiation to ASCs [34]. In this situation, the levels of transcription of BAFF and BAFF-R are significantly increased in the peritoneal cavity [106]. Furthermore, when the transcription of these cytokines and their receptors was studied in sorted IgM$^+$ B cells from VHSV-injected fish, we found that the levels of transcription of BAFF, APRIL, BALM, BAFF-R and TACI were up-regulated in comparison to the levels observed in sorted IgM$^+$ B cells from fish injected with saline alone [106]. Interestingly, these effects seemed specific of peritoneal IgM$^+$ B cells, since the same response was not observed in splenic B cells. Therefore, it

seems that the BAFF-APRIL axis plays a key role in the expansion and activation of IgM^+ B cell populations in the peritoneal cavity during the course of a viral infection.

9. Concluding remarks

B cells are not only implicated in the immune response to viral pathogens through the production of antibodies, but can also directly interact with viruses, thus playing an important role in the presentation of their antigens to T cells and on the early regulation of the surrounding immune cells through the secretion of a wide range of immune factors. Although this is true both in mammals and in fish, it seems plausible that these additional functions of B cells that are part of the crosstalk between the innate and the adaptive immune systems, prevail in teleost fish. The reason for this is that teleost B cells seem better equipped than mammalian B2 cells to directly sense pathogens [32]; they phenotypically and functionally resemble mammalian B1 cells [38] and therefore retain many innate functions such as a high phagocytic capacity [36] or the capacity to secrete of antimicrobial peptides [110] and pro-inflammatory cytokines [111], no longer present in mammalian conventional B cells. In addition to this, although specific IgMs have been shown to have protective capacity against some viral infections, in acute infections, specific IgMs are produced far after mortalities due to viral infections have occurred. On the other hand, whether specific IgTs or IgDs are produced during the course of a viral infection has not been yet demonstrated. Thus, although in the past years our knowledge on how teleost B cells interact with pathogens and react against them has greatly advanced, there are still many aspects of this complex interaction that remain unclear. Thus, in our opinion, future research in this field should be focused on clarifying if teleost fish are capable of establishing a long term B cell memory and determining the precise role of IgD and IgT during the course of viral infections. Additionally, concerning the optimization of antiviral vaccines, it will be important to determine whether correlates can be established between the different B cell functions and protection.

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