Fish and Shellfish Immunology 85 (2019) 9–17

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Different IgM$^+$ B cell subpopulations residing within the peritoneal cavity of vaccinated rainbow trout are differently regulated by BAFF

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ARTICLE INFO

Keywords:
Rainbow trout
Peritoneum
B cell
Plasma cell
BAFF
BAFF receptors

ABSTRACT

In teleost fish, IgM$^+$ B cells are one of the main responders against inflammatory stimuli in the peritoneal cavity, as IgM$^+$ B cells dominate the peritoneum after intraperitoneal stimulation, also increasing the levels of secreted IgM. BAFF, a cytokine known to play a major role in B cell biology, has been shown to be up-regulated along with its receptors in the peritoneum of rainbow trout upon antigenic exposure, however, the regulatory mechanisms underneath this response remain unclear. In this study, we have identified two different IgM$^+$ B cell types residing in the peritoneal cavity of previously vaccinated rainbow trout (*Oncorhynchus mykiss*): IgD$^+$IgM^{hi}MHCII^{hi} cells, resembling naïve B cells, and IgD$^-$IgM^{lo}MHCII^{lo} cells, resembling antibody-secreting cells. Based on their membrane IgM levels, these cell types were named IgM^{hi} and IgM^{lo} B cells, respectively. As each of these B cell populations showed a distinct expression pattern for the different BAFF receptors, we studied the effect of BAFF individually on each cell subset. Recombinant BAFF promoted the survival of IgM^{lo} but not IgM^{hi} B cells *in vitro*, resulting in increased levels of IgM-secreting cells. In contrast, BAFF increased the levels of membrane MHC II only on IgM^{hi} B cells, suggesting different functions on these B cell subsets. Moreover, we also showed that peritoneal IgM^{hi} B cells expressed BAFF at levels comparable to those seen on myeloid cells. These results point to BAFF as a main regulator of B cell homeostasis in the peritoneal cavity, suggesting that this cytokine can trigger different signals on different peritoneal B cell subsets in a specific manner.

1. Introduction

Peripheral B cell survival and homeostasis are interactive processes that occur in different microenvironments to provide the settings needed for rapid and robust B cell responses against infections. These responses are mainly orchestrated through signals received by the B cell receptor (BCR) and by cytokines such as those belonging to the BAFF (B cell activating factor) family [1–4]. In mammals, this family includes the ligands BAFF and APRIL (a proliferation inducing ligand) that signal through the receptors BAFF-R (BAFF receptor), BCMA (B cell maturation antigen) and TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor). In addition, the sequence of an ancestral relative of these ligands has been identified in lampreys [5] and teleost fish [6], which has been subsequently named BALM (BAFF and APRIL like molecule). In mammals, BAFF is able to bind the three receptors, while APRIL binds only BCMA and TACI (reviewed in Ref. [7]). BAFF and APRIL play important yet different roles in the development, maturation, homeostasis and differentiation of B cells (summarized in Ref. [8]). In the case of BAFF, several lines of evidence in mammals have demonstrated that, *in vivo*, it promotes the generation and survival of mature B cells [4,9] and increases the number of B cells in secondary lymphoid organs on transgenic mice models [10,11]. *In vitro*, BAFF promotes the survival and increases the life span of immature B cells [12,13]. In concordance with these observations, treatment of mice with recombinant BAFF protein expands the B cell populations in the spleen and augments the concentration of serum IgM [14]. Considering receptor expression throughout B cell maturation, BAFF-R is not expressed on B cell precursors, but is gained on immature B cells upon the appearance of a functional BCR [15], thereby regulating their survival and differentiation [16]. TACI, on the other hand, is a highly inducible receptor, mainly expressed in innate B cell populations such as B1 or marginal zone (MZ) B cells [8]. Hence, BAFF signaling through TACI regulates T-independent responses. Finally, BCMA expression is restricted to plamablasts and plasma cells, being BAFF signaling through BCMA responsible for long-term survival of these cells [17].

The peritoneum is a membrane-bound abdominal cavity of vertebrates, filled with a small amount of serous fluid, that contains the liver,

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http://dx.doi.org/10.1016/j.fsi.2017.10.003
Received 24 May 2017; Received in revised form 3 October 2017; Accepted 4 October 2017
Available online 05 October 2017

the spleen, part of the gastro-intestinal tract and other viscera, depending on the species [18]. In mammals, it is now well established that the peritoneum is populated by different types of immune cells, such as macrophages, dendritic cells, NK cells, T cells, mast cells and B cells [19,20]. In mammals, B cells migrate from the peritoneum to secondary lymphoid organs during the course of infections or inflammatory processes. On the other hand, there is a simultaneous influx of monocytes and macrophages into the peritoneal cavity [21–24]. The B cells populating the peritoneal cavity have been widely studied in mammals because the peritoneal cavity is where most of the B1 cells reside, in coexistence with conventional B2 cells. B1 cells, recognized as elements of the innate immune system, 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 T-independent antigens. Upon activation, B1 cells, as conventional B cells, differentiate to antibody secreting cells (ASCs), although whether they reach a fully differentiated state (as a plasma cell) is still under debate [25,26]. Natural IgM secreted by peritoneal B1 cells exhibits low affinity and poly-reactivity, and is very important on the protection of the organism during the early phase of the immune response against a variety of pathogens [27,28]. Therefore, different subsets of B cells coexist in the peritoneal cavity producing both natural and antigen-induced antibodies, making it a very interesting niche to study because of the diverse functions and many unaddressed questions associated with the development and regulation of these populations [29].

Bony fish constitute the first animal group comprising all the elements of the adaptive immune system. However, some features of their immune system such as the lack of lymph nodes or follicular structures that anticipate important differences in the functionality of fish and mammalian B cells [30–32]. In the peritoneal cavity of teleost and opposing to what happens in mammals, lymphocytes, and especially IgM^+ B cells dominate the peritoneum after 48 h of antigenic stimulation with a pathogenic bacteria [33]. In line with this study, very recent studies from our group have also shown that after intraperitoneal injection of viral hemorrhagic septicemia virus (VHSV) or *Escherichia coli*, IgM^+ B cells dominate the peritoneum in rainbow trout and even differentiate to ASCs [34]. In addition, we have also shown that peritoneal IgM^+ B cells significantly up-regulate the transcription levels of BAFF, APRIL, BALM, BAFF-R and TACI after intraperitoneal injection of VHSV [35]. The effect of the three cytokines was tested on the survival of peritoneal B cells obtained from naïve fish, observing that only APRIL and BALM increase the survival of peritoneal B cells [35], whereas BAFF seemed to produce no effect. Despite these results, whether BAFF regulates other aspects of B cell functionality in the peritoneum such as those described for splenic B cells [31] remains unclear, and this is what we have undertaken in the current study. Thus, we have analyzed the different B cell subtypes present in the peritoneum of vaccinated fish and studied the regulatory effect that BAFF plays on such subpopulations, using rainbow trout as a model. We have identified of two main different B cell types in the peritoneal cavity, with the following phenotypes; $IgD^+IgM^{hi}MHCII^{hi}$ cells, which resemble naïve B cells, and $IgD^-IgM^{lo}MHCII^{lo}$ cells, with enlarged size, and a phenotype similar to that seen in ASCs. These two populations differed in the expression of plasma cell markers as well as in the expression of BAFF receptors. Thus, we analyzed the effect that recombinant BAFF had on these cell types. Strikingly, we observed an increase on the survival of ASC-like B cells accompanied by a detrimental effect on naïve-like B cell survival. On the other hand, BAFF up-regulated the expression of membrane MHC II on naïve-like B cells but not on ASC-like cells, thus demonstrating a differential effect of BAFF on these two different subsets. Finally, we have also shown that naïve-like B cells expressed BAFF at levels comparable to those seen on peritoneal myeloid cells. Our findings shed light on the regulation of B cell responses by BAFF and on the complex mechanism controlling the functionality of diverse B cell subsets in an important yet still widely unknown peripheral immune site such as the peritoneum.

2. Materials and methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Piscificatoria Cifuentes (Cifuentes, Guadalajara, Spain) and maintained at the animal facilities of the Centro de Investigación en Sanidad Animal (CISA-INIA, Spain) in a re-circulating water system at 16 °C, with 12:12 h light:dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting, Spain). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for at least 2 weeks. The fish used on this study had been vaccinated in the fish farm against *Lactococcus garvieae* by intraperitoneal injection of 0.1 ml of ICTHIOVAC® LG (Hipra) when they had reached 20 g of weight. The experiments were performed at least 6 weeks after the vaccination date. Some unvaccinated animals from the same source were also used as controls. The experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were previously approved by the Ethics committee from the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). Anesthesia was applied following Zhal et al. recommendations for general anesthesia (narcosis) [36] prior to sacrifice, and all efforts were made to minimize suffering.

2.2. Production of recombinant BAFF

The nucleotide sequence corresponding to the extracellular domain of the rainbow trout BAFF sequence (GenBank Accession number DQ218467.1) together with an N-terminal 6 x histidine tag was synthetized and subcloned into the E3 expression vector (Abyntek). The recombinant plasmids were transformed into BL21 cells and a kanamycin-resistant single positive colony for each clone were then incubated at 37 °C in Luria-Bertani (LB) media. When the OD_{600} reached 0.6, 0.1 mM of isopropyl β-D-thiogalactoside (IPTG, Sigma) was added to induce protein production. After 16 h, cells were harvested, lysed by sonication and dissolved using urea. Thereafter, BAFF was obtained through the use of Nickel columns (Sigma). The protein-containing fractions were pooled, refolded, filtered through 0.22 μm and resuspended in storage buffer (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 0.5 M L-arginine and 2 mM DTT, pH 8.5). Protein concentration was determined in a BCA protein assay (Thermo Fisher Scientific) and the recombinant rainbow trout BAFF (0.3 mg/ml) was aliquoted and stored at −80 °C until used.

2.3. Isolation of peritoneal cavity and spleen leukocytes

Rainbow trout were killed by benzocaine (Sigma Aldrich) overdose. After blood extraction, the abdomen was disinfected with 70% ethanol and the peritoneal cavity injected with 2 ml of cold Leibovitz medium (L-15, Life Technologies) supplemented with 100 I.U./ml penicillin, 100 μg/ml streptomycin, 10 units/ml heparin and 5% fetal calf serum (FCS) (all supplements also obtained from Life Technologies). After gently massaging the abdominal surface, the medium containing peritoneal cells was harvested from the peritoneum with a syringe. The injection and harvest was repeated twice and then a pipette tip was inserted into a peritoneal incision to collect any remaining fluid from the cavity. Single cell suspensions were also obtained from the spleen using 100 μm nylon cell strainers (BD Biosciences) and the same media. All cell suspensions were then placed onto 30/51% discontinuous Percoll (GE Healthcare) density gradients and centrifuged at 500 × *g* for 30 min at 4 °C. The interface cells were collected and washed twice in L-15 containing 5% FCS.

2.4. Flow cytometry

The anti-trout IgD [mAb mouse IgG_1 coupled to R-phycoerythrin (R-PE), 5 μg/ml], the anti-trout IgM [1.14 mAb mouse IgG_1 coupled to fluorescein (FITC), 1 μg/ml] and the anti-trout MHC II β-chain (mAb mouse IgG_1 coupled to allophycocyanin (APC), 2 μg/ml) used in this study have been previously characterized [37,38]. All the mAbs were fluorescently labeled using FITC, R-PE or APC Lightning-Link labelling kits (InnovaBiosciences), following manufacturer's instructions. Splenic or peritoneal cavity leukocytes were incubated with specific antibodies for 30 min in the case of anti-IgM or anti-MHC, or 45 min in the case of anti-IgD, washed three times with staining buffer (PBS containing 1% FCS and 0.5% sodium azide), and analyzed.

A biotinylated version of anti-BAFF (pAb mouse IgG, 1 μg/ml) was also used to determine endogenous BAFF expression by leukocytes as previously reported [29]. To carry this out, cells were incubated for 30 min with biotinylated anti-BAFF pAb, then washed three times with staining buffer and incubated for another 30 min with streptavidin-APC (Thermo Fisher Scientific).

In all cases, isotype controls for mouse mAbs and anti-BAFF pAb, (BD Biosciences) were tested in parallel to discard unspecific binding of the antibodies. All the incubations were performed at 4 °C. During the setting up of the experiments, cell viability was checked by Propidium Iodide (PI) staining. Cell viability was always higher than 95% in our initial experimental conditions.

2.5. Isolation of IgM^{hi}, IgM^{lo} and total B cells

Purified leukocytes were incubated for 30 min on ice with a specific anti-trout IgM antibody (1.14) coupled to FITC [39]. Following two washing steps, cells were suspended in PBS and IgM^{hi}, IgM^{lo} and total B cells were FACS sorted using a BD FACSAria III (BD Biosciences), using first their forward scatter (FSC, relating to size) and side scatter (SSC, relating to complexity) profiles (to exclude the granulocyte gate) and then on the basis of the fluorescence emitted by the anti-IgM mAb. We analyzed the purity of the isolated populations by flow cytometry after sorting and only those samples showing a purity level higher than 95% were used for RNA isolation.

2.6. Gene expression analysis in FACS isolated populations

To analyze the gene expression pattern of B cell subsets isolated from the spleen and peritoneum, RNA isolation and expression of individual genes was performed as previously described [38]. Briefly, total RNA from FACS isolated cells was isolated using the Power SYBR Green Cells-to-Ct Kit (Thermo Fisher Scientific) following manufacturer's instructions. RNAs were treated with DNase during the process to remove genomic DNA that might interfere with the PCR reactions. Reverse transcription was also performed using the Power SYBR Green Cells-to-Ct Kit according to manufacturer's instructions. Minus reverse transcriptase controls were included in all the assays to rule out amplification of genomic DNA. To evaluate the levels of transcription of BAFF receptors (BAFF-R, BCMA and TACI), Pax 5 and Blimp1 in these samples, real-time PCR was performed with a LightCycler® 96 System instrument using SYBR Green PCR core Reagents (Applied Biosystems) and specific primers (Table 1). Each sample was measured in duplicate under the following conditions: 10 min at 95 °C, followed by 45 amplification cycles (15 s at 95 °C and 1 min at 60 °C). A melting curve for each PCR was also included to ensure only a single product had been amplified. The expression of individual genes was normalized to the relative expression of trout housekeeping gene EF-1α elongation factor as described before [31].

2.7. ELISPOT

ELISPOT was used to quantify the number of IgM-secreting B cells following a method previously described [31]. Peritoneal cavity leukocytes were incubated with BAFF (3 μg/ml) or left unstimulated (control) at 20 °C for 48 h in culture 96-well plates. ELISPOT plates containing Inmobilon-P membranes (Millipore) were activated with 70% ethanol for 30 s, coated with anti-trout IgM mAb (clone 4C10) at 2 μg/ml in PBS and incubated overnight at 4 °C. To block non-specific binding to the membrane, plates were then incubated with 2% BSA in PBS for 2 h at RT. Thereafter, leukocytes from individual fish incubated with BAFF or control media for 48 h were added to the wells in triplicate at a concentration of 1×10^5 cells per well. After 24 h of incubation at 20 °C, cells were washed away 5 times with PBS and plates were blocked again with 2% BSA in PBS for 1 h at RT. After blocking, biotinylated anti-trout IgM mAb (clone 4C10) was added to the plates and incubated at 1 μg/ml for 1 h at RT. Following additional washing steps (5 times in PBS) the plates were developed using streptavidin-HRP (Thermo Scientific) at RT for 1 h, washed again with PBS and incubated with 3-amino 9-ethylcarbazole (Sigma Aldrich) for 30 min at RT in the dark. Substrate reaction was stopped by washing the plates with tap water. Once the membranes had dried, spots were examined and counted under a light magnifying glass.

Table 1
Primers used for real-time PCR analysis in this study.

Gene	Primer name	Primer sequence (5′-3′)
Pax5	RT-Pax5-F	ACGGAGATCGGATGTTCCTCTG
	RT-Pax5-R	GATGCCGCGCTGTAGTAGTAC
Blimp1	RT-Blimp1-F	GGCAGTGGACCTGTGGAAGG
	RT-Blimp1-R	CGCAGGTGGACCTTGAGGTT
BAFF-R	RT-BAFFR-F	TGTCTGGATATCAATGGTCGTCATA
	RT-BAFFR-R	CTTTAGCTGGAGGGTTAAGTCTTGC
BCMA	RT-BCMA-F	ATGTCAGAAGGACAGTGTGGACTGG
	RT-BCMA-R	CGGCTCTGGGGCTTTGCTCT
TACI	RT-TACI-F	GCATCGAGTACTGTGCTTCTCTAGG
	RT-TACI-F	AAGTCAGGCTGTTGGGTCTTACATT
EF-1α	RT-EF1a-F	GATCCAGAAGGAGGTCACCA
	RT-EF1a-R	TTACGTTCGACCTTCCATCC

2.8. Statistics

Data handling, analyses and graphic representation was performed using Microsoft Office Excel 2010. Statistical analyses were performed using ANOVA followed by a post hoc two-tailed Student's *t*-test when the ANOVA indicated that the variances of both groups differed significantly. The differences between the mean values were considered significant on different degrees, where * means $P \leq 0.05$, ** means $P \leq 0.01$ and *** means $P \leq 0.005$ (GraphPad Prism 4 software).

3. Results

3.1. Identification of two different B cell populations in the peritoneum of vaccinated fish

Prior to establishing the effect of BAFF on peritoneal leukocytes, we performed a phenotypic characterization of the IgM^+ B cells residing in the peritoneal cavity of previously vaccinated fish. To carry this out, we isolated leukocytes from the peritoneum and labeled them with anti-IgM, anti-IgD and anti-MHC II mAbs. In order to analyze B cells, in our samples we gated an $FSC^{lo}SSC^{lo}$ population, which we named lymphoid gate, since it excludes granulocytes and myeloid cells (Fig. S1). We first analyzed the staining of membrane IgM against the size of the lymphocytes (FSC) and, interestingly, we could identify two different populations; one exhibiting high levels of IgM and small size and another one showing lower levels of IgM and a significant larger size (Fig. 1A). Based on their IgM levels, these populations were subsequently named IgM^{hi} and IgM^{lo} B cells. We later analyzed the levels of membrane IgD on these populations, observing that IgM^{hi} cells showed a positive

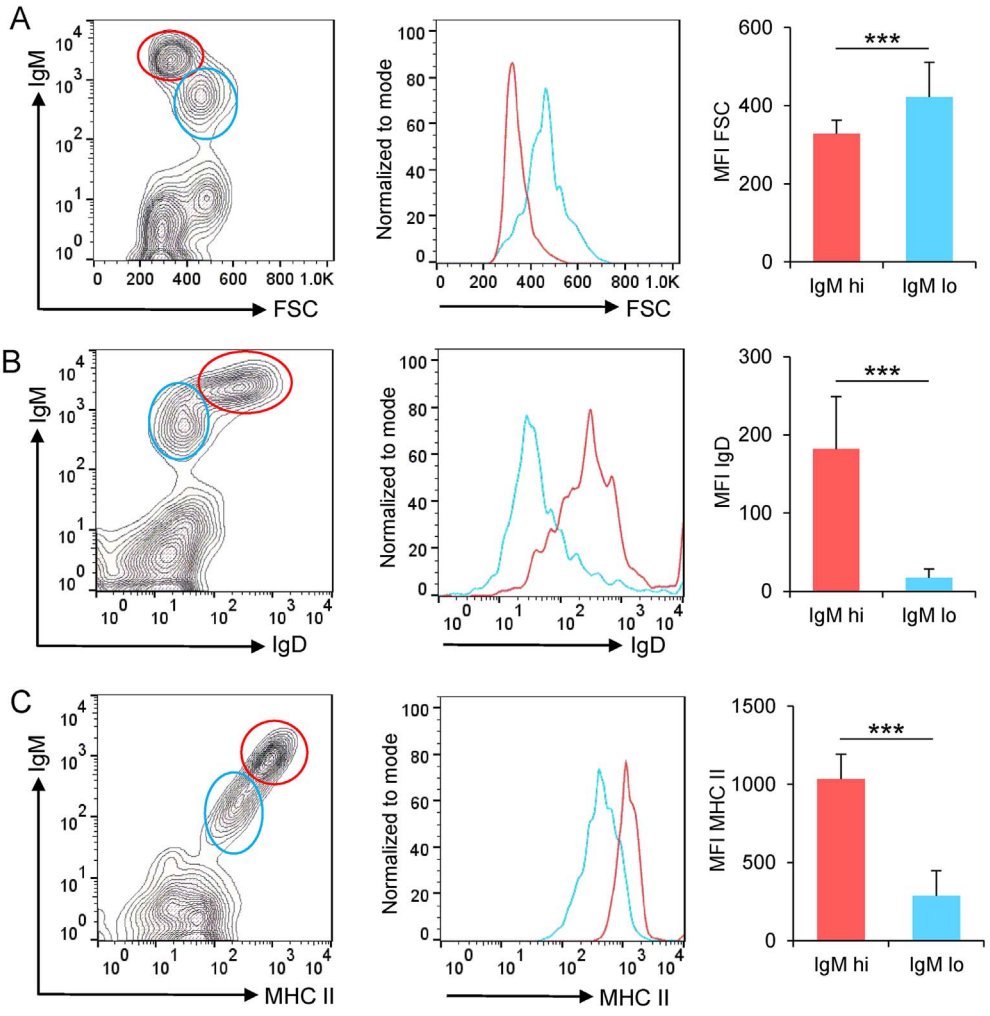

Fig. 1. Identification of IgM^{hi} and IgM^{lo} B cell subsets in the peritoneum. Leukocytes from the peritoneal cavity of rainbow trout were isolated and a flow cytometry analysis was carried out. Cells were stained with anti-IgM (**A**), anti-IgM plus anti-IgD (**B**) or anti-IgM plus anti-MHC II (**C**) mAbs. For each labelling, density plots profiles are shown (left panels), in which IgM^{hi} (red) and IgM^{lo} (blue) population gates are shown. Representative histogram (middle panels) showing the intensity of the FSC (**A**), IgD (**B**) and MHC II (**C**) on IgM^{hi} and IgM^{lo} populations are also included. The mean fluorescence intensity (MFI) for each parameter was quantified and is shown as bar plots (right panels) as mean + SD (n = 12 fish, from four independent experiments containing three animals each). Statistical differences were evaluated by a one-way ANOVA followed by two-tailed Student's *t*-test, where *** means $p \leq 0.005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

staining for IgD, while IgM^{lo} cells had no surface IgD (Fig. 1B). Finally, the level of membrane MHC II on both populations was evaluated, and we could see that although IgM^{hi} cells presented high levels of membrane MHC II, IgM^{lo} cells showed a significantly reduced amount of surface MHC II. Thus, the phenotype of these two populations found in the peritoneum were IgD^{+}IgM^{hi}MHCII^{hi}, which resembles a naïve B cell phenotype, and IgD^{-}IgM^{lo}MHCII^{lo}, with enlarged size, which is a phenotype similar to that seen in plasmablasts/plasma cells [40].

When non-vaccinated fish from the same source were analyzed, we found that the IgM^{lo} compartment was severely diminished (Fig. S2), representing less than 10% of the total peritoneal cavity resident IgM^{+} B cells, while in vaccinated animals this population accounted for almost 40% of total IgM B cells, thus suggesting that intraperitoneal antigen encounter triggered by the vaccine was crucial for the expansion and maintenance of this population.

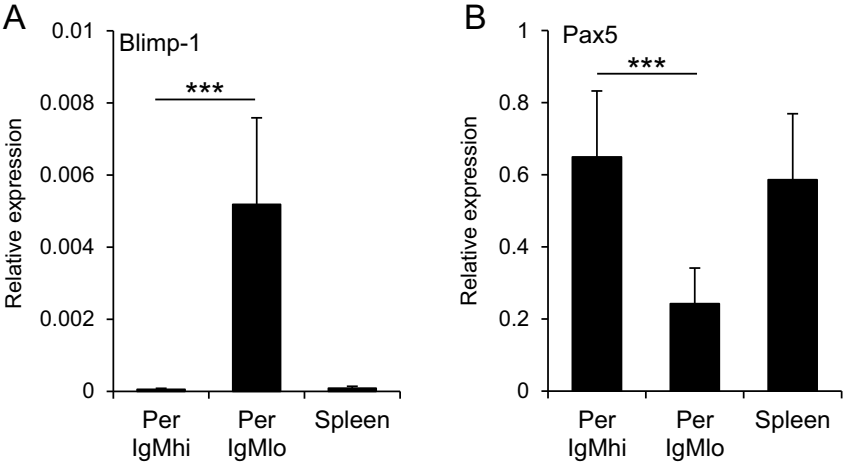

Fig. 2. Expression of plasma cell markers on IgM^{hi} and IgM^{lo} peritoneal B cells. Leukocytes from the spleen and the peritoneal cavity of rainbow trout were isolated and labeled with an anti-IgM mAb. IgM^{+} B cells from the spleen and IgM^{hi} and IgM^{lo} subpopulations from the peritoneum were FACS isolated and RNA was then extracted from all collected samples as described in Materials and Methods. The transcription levels of Pax5 (**A**) and Blimp-1 (**B**) genes relative to the endogenous control gene EF-1α were calculated for each subpopulation. Results are shown as mean + SD (n = 9, from three independent experiments containing three animals each). Statistical differences were evaluated by a one-way ANOVA followed by two-tailed Student's *t*-test, where * means $p \leq 0.05$ and ** means $p \leq 0.01$.

3.2. Expression of Blimp-1 and Pax5 on IgM^{hi} and IgM^{lo} peritoneal B cells

To further characterize the phenotype of these two IgM^{+} B cell populations found in the peritoneum of vaccinated fish, we FACS isolated IgM^{hi} and IgM^{lo} cells from the peritoneal cavity, as well as IgM^{+} B cells from the spleen, and analyzed the expression of two key regulators of plasma cell differentiation, namely B lymphocyte-induced maturation protein-1 (Blimp-1) and Paired Box 5 (Pax5) transcription factors through real time PCR. Blimp-1 has been shown to be sufficient to repress Pax5 expression during plasmacytoid differentiation and this process is required to drive differentiation of naïve B cells to IgM-secreting cells [41]. Transcription of Blimp-1 was only detected on IgM^{lo} peritoneal B cells, but not on IgM^{hi} or splenic B cells (Fig. 2A). On the contrary, IgM^{hi} B cells from the peritoneum showed high transcription levels of Pax5, comparable to those seen on splenic B cells, while the transcription of Pax5 was significantly down-regulated in IgM^{lo} peritoneal B cells (Fig. 2B). Therefore, the pattern of Blimp1 and Pax5 transcription is also consistent with IgM^{lo} cells representing a subset of B cells that is differentiated to plasmablast/plasma cell.

3.3. Expression of BAFF and APRIL receptors on IgM^{hi} and IgM^{lo} peritoneal B cells

Since homologue sequences to BAFF-R, BCMA and TACI have been very recently reported in teleost [42], it was important to analyze the expression pattern of these receptors in the different peritoneal B cell subsets prior to establishing the effect that BAFF had on these populations. Through real time PCR analysis, we observed that IgM^{hi} B cells showed a high transcription level of BAFF-R, comparable to that seen on splenic B cells, while IgM^{lo} cells presented significantly lower transcription level of this receptor (Fig. 3). On the contrary, BCMA expression levels were significantly higher on peritoneal IgM^{lo} cells in comparison to those found on IgM^{hi} B cells and splenic B cells (Fig. 3). Finally, TACI expression levels were undetectable in the three populations analyzed (Fig. 3).

3.4. BAFF induces the secretion of IgM by peritoneal B cells through supporting the survival of IgM^{lo} plasma cells

Having demonstrated the existence of a resident IgM plasma cell subset in the peritoneum, our next step was to analyze the effect of BAFF on IgM secretion, since BAFF has been shown as a potent stimulator of IgM secretion in the absence of a cognate antigen [14]. To carry this out, we performed ELISPOT analysis using peritoneal leukocytes that had been cultured *in vitro* in the presence of BAFF or left unstimulated for 48 h. We observed a highly significant increase in the number of IgM-secreting cells after treatment with BAFF (Fig. 4A). The effect of BAFF on Blimp-1 expression was also analyzed in FACS isolated total IgM^{+} B cells and in this case, no up-regulation was observed in response to the cytokine treatment (Fig. 4B), thus suggesting that BAFF is not a differentiation factor for plasma cells, since up-regulation of Blimp-1 is an essential step for the terminal differentiation of plasma cells in mammals [43]. Because BAFF has been shown to promote IgM secretion through increased survival of splenic plasma cells in humans [44] and teleost [31], it was plausible that BAFF could also be promoting IgM secretion through the survival of intraperitoneal resident IgM plasma cells. To test this hypothesis, we analyzed the survival of peritoneal B cells after 3 days of stimulation *in vitro* with BAFF or control medium. We first observed that BAFF did not alter the number of total IgM^{+} B cells present in peritoneal leukocyte cultures from these fish (Fig. 4C) as had been previously reported using peritoneal cultures from non-vaccinated fish [35]. However, when the effect of the individual subpopulations was studied, we observed a significantly decrease in the number of IgM^{hi} B cells in response to BAFF that went along with a highly significantly increase in the number of IgM^{lo} B cells (Fig. 4D and E). The decrease of IgM^{hi} and the increase of IgM^{lo} B cell numbers was clear in cell cultures from all fish examined individually (Fig. 4F). This differential effect of BAFF on both populations gave as a result a shift in the ratio of IgM^{hi}: IgM^{lo} B cells, moving from approximately 60: 40 to a 50: 50 after exposure to BAFF (Fig. 4G).

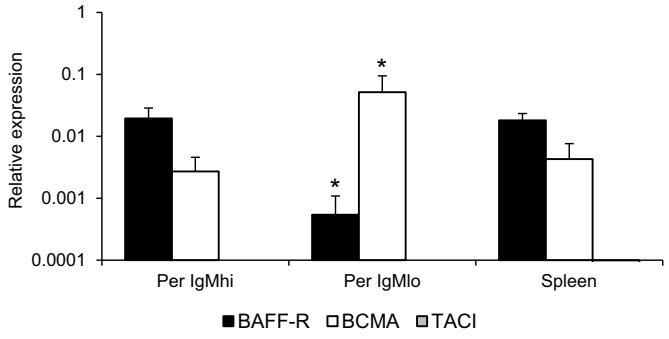

Fig. 3. Expression of BAFF receptors on IgM^{hi} and IgM^{lo} peritoneal B cells. Leukocytes from the spleen and the peritoneal cavity of rainbow trout were isolated and labeled with an anti-IgM mAb. IgM^{+} B cells from the spleen and IgM^{hi} and IgM^{lo} subpopulations from the peritoneum were FACS isolated and RNA was then extracted from all collected samples as described in Materials and Methods. The transcription levels of BAFF-R, BCMA and TACI genes relative to the endogenous control gene EF-1α were calculated for each subpopulation. Results are shown as mean + SD (n = 9, from three independent experiments containing three animals each). Statistical differences with values observed in splenic B cells were evaluated by a one-way ANOVA followed by two-tailed Student's *t*-test, where * means $p \leq 0.05$ and ** means $p \leq 0.01$.

3.5. Effect of BAFF on surface MHC II levels

Since BAFF has been shown to enhance the antigen presenting capacity and to increase the levels of surface MHC-II on spleen B cells from mouse and fish [31,45,46], we aimed to explore whether BAFF was also able to modulate MHC-II on peripheral B cell subsets from the peritoneum. After 72 h incubation with BAFF, IgM^{hi} B cells displayed significantly increased surface MHC II levels, while IgM^{lo} B cells did not exhibit changes on their MHC-II expression levels (Fig. 5A and B). These data indicate that only IgM^{hi} B cells which express higher membrane MHC-II levels up-regulate its expression after incubation with BAFF.

3.6. Peritoneal teleost IgM B cells produce BAFF

Mammalian unstimulated B cells do not express BAFF, but it has been shown that a subpopulation of teleost B cells from the spleen of unstimulated fish are able to express membrane BAFF [31]. This prompted us to analyze whether the different B cell subsets found in the peritoneum of vaccinated fish were able to produce BAFF and to what extent. Thus, we studied the presence of BAFF in peritoneal IgM^{-}, IgM^{lo} and IgM^{hi} cells through flow cytometry using an anti-rainbow trout BAFF polyclonal antibody. An average 7.9% of the cells within the myeloid gate, cells showing large size and high complexity were producing BAFF (Fig. 6A). Since no IgM^{+} cells were detected within the myeloid gate (Fig. S3), our results suggest the production of BAFF by peritoneal resident dendritic cells and macrophages (Fig. 6B and C). Within the lymphoid gate, we studied BAFF production in IgM^{-}, IgM^{lo} and IgM^{hi} cell populations (Fig. 6A). We verified that IgM^{hi} B cells were the lymphocyte cell type mainly responsible for BAFF production (Fig. 6B and C), with an average, 7.7% of these cells being positive for surface BAFF expression, whereas the percentage of IgM^{-} lymphocytes and IgM^{lo} B cells producing this cytokine was negligible (Fig. 6B and C). Furthermore, the levels of membrane BAFF seen in IgM^{hi} B cells, estimated by the mean fluorescence intensity (MFI) values, were equivalent to those found in myeloid cells (Fig. 6C).

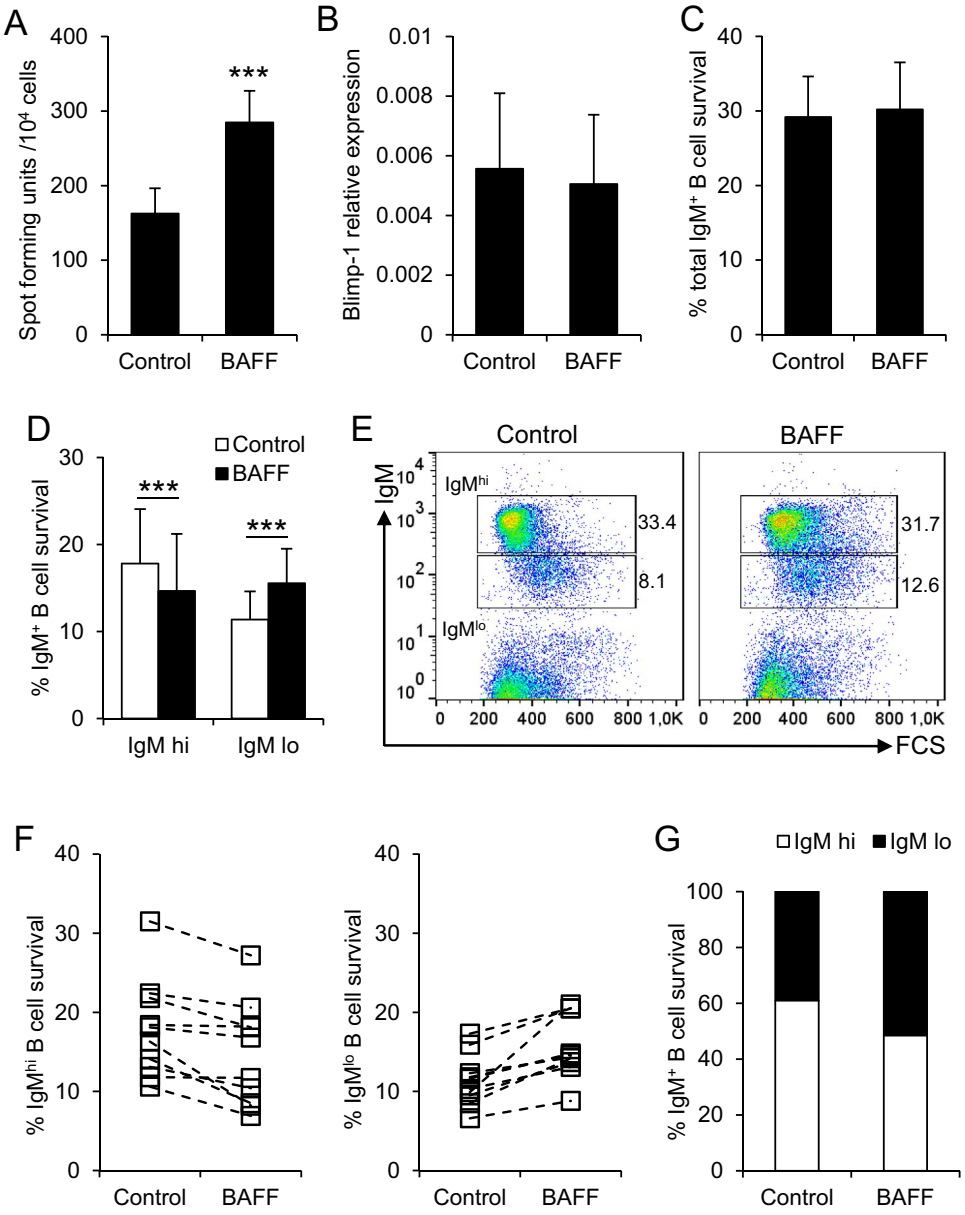

Fig. 4. Rainbow trout BAFF promotes IgM secretion in peritoneal leukocytes through increasing the survival of IgM^{lo} plasma cells. (**A**) Splenocyte cultures were treated with BAFF (3 μg/ml) or left unstimulated (control) for 48 h and then plated in ELISPOT plates previously coated with anti-trout IgM mAb, for a further 24 h. After incubation, cells were washed away and a biotinylated anti-trout IgM mAb was used to detect numbers of spot forming cells. Quantification of spot forming cells is shown as mean + SD. (**B**) Peritoneal leukocyte cultures were treated with BAFF (3 μg/ml) or left unstimulated (control) for 24 h, and then RNA from IgM$^+$ FACS isolated B cells was extracted as described in Materials and Methods. The transcription of Blimp-1 relative to the endogenous control EF-1α was calculated for each sample, and shown as mean + SD. (**C**) Peritoneal cavity leukocytes were incubated with recombinant BAFF (3 μg/ml) or left unstimulated (control) for 3 days at 20 °C. After this time, cells were labeled with an anti-IgM mAb and analyzed by flow cytometry. The percentage of live IgM^{hi} and IgM^{lo} B cells among the lymphocyte gate was then determined. Quantification of average B cell survival is shown as mean + SD. A representative dot plot for each experimental condition is also included (**D**). (**E**) Total IgM B cell survival was quantified and shown as mean + SD. (**F**) The percentage of surviving IgM^{hi} (left panel) and IgM^{lo} (right panel) B cells for each individual fish under control or BAFF stimulation conditions was determined and plotted, as well as the average survival for IgM^{hi} and IgM^{lo} populations (G). In each assay, n = 9, from three independent experiments containing three animals each. Statistical differences were evaluated by one-way ANOVA followed by a two-tailed Student's *t*-test, where *** means $p \leq 0.005$.

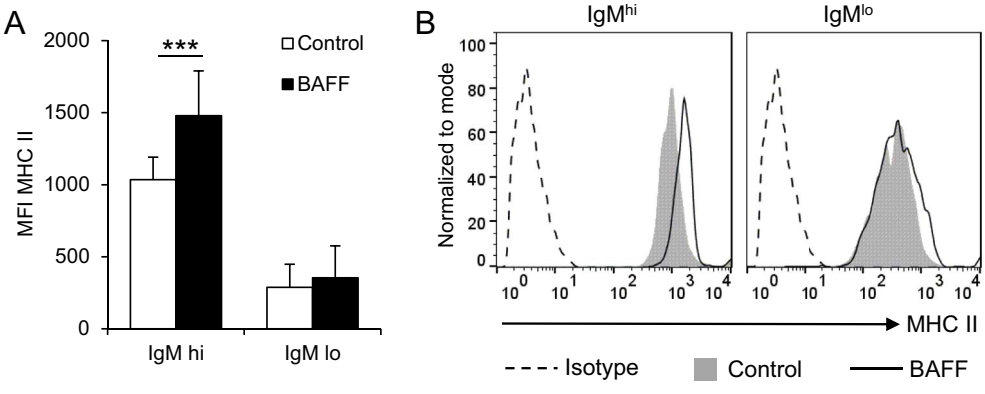

Fig. 5. BAFF up-regulates membrane MHC II on peritoneal resting B cells. (**A**) Peritoneal cavity leukocytes were incubated with BAFF (3 μg/ml) or left unstimulated (control) for 3 days at 20 °C. After this time, cells were labeled with anti-IgM and anti-MHC II mAbs and analyzed by flow cytometry. Mean fluorescence intensity (MFI) for membrane MHC II was measured on IgM^{hi} and IgM^{lo} B cells, and average values were plotted as mean + SD (left) (n = 9, from three independent experiments containing three animals each). (**B**) Histograms from one representative experiment, showing MHC II MFI on IgM$^+$ gated B cells are shown. Statistical differences were evaluated by one-way ANOVA followed by a two-tailed Student's *t*-test, where *** means $p \leq 0.005$.

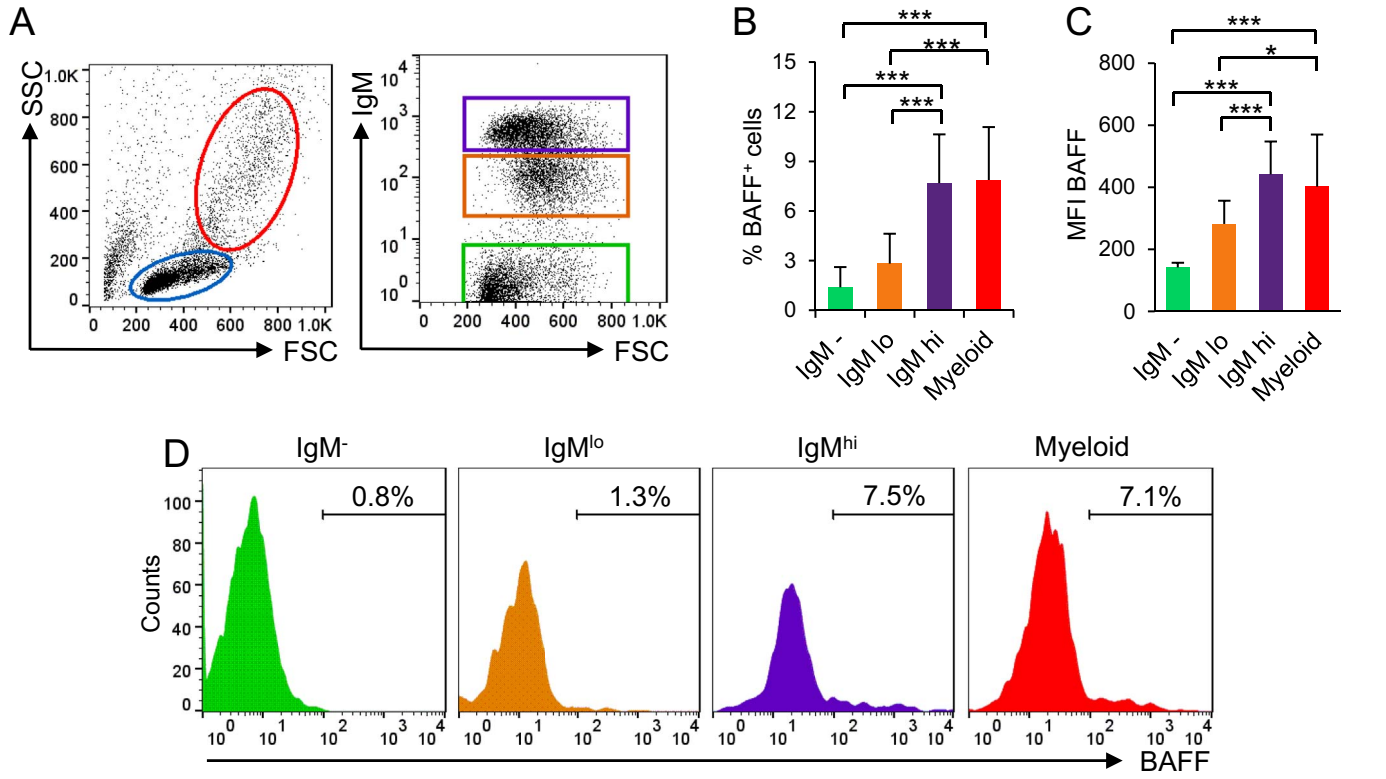

Fig. 6. BAFF expression by peritoneal B cells in physiological conditions. (**A**) Rainbow trout leukocytes isolated from the peritoneal cavity were stained with an anti-IgM mAb together with an anti-BAFF pAb, and analyzed by flow cytometry. Lymphoid (blue gate) and myeloid (red gate) populations were gated, and then IgM^- (green gate), IgM^{lo} (orange gate) and IgM^{hi} (purple gate) cells were further selected from the lymphoid population. The average percentage of $BAFF^+$ cells within each compartment was calculated (**B**) as well as the mean fluorescence intensity (MFI) of BAFF on those populations (**C**) (shown as mean + SD, n = 9, three independent experiments containing 3 animals each). (**D**) A representative histogram showing the level of BAFF expression in each population is shown. Statistical differences were evaluated by one-way ANOVA followed by a two-tailed Student's *t*-test, where * means $p \leq 0.05$ and *** means $p \leq 0.005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Since its discovery in 1999 [14], BAFF has been extensively studied and shown as an important regulator of B cell homeostasis and survival (reviewed in Ref. [7]). In BAFF-deficient mice or mice in which BAFF has been neutralized *in vivo*, B cell development and functionality is severely perturbed [4,47,48], showing that BAFF plays an indispensable role in B cell lineage survival. Interestingly, the role played by BAFF in some B cell subsets is not exactly the same. For instance, peritoneal cavity B1 cells do not need BAFF for development [47], and memory B cell survival and function are completely independent of BAFF, being the only mature B2 subset whose survival is independent on this ligand [49]. In agreement with this, a mouse model in which BAFF is inhibited in adult animals showed a complete depletion of immature and mature B2 cells, while memory B cells and peritoneal cavity B1 cells remained unaffected [50], thus suggesting different regulatory roles for BAFF in the peritoneal B cell compartment.

In the case of peritoneal immune responses in fish, it has been very recently demonstrated that after intraperitoneal injection of VHSV or *E. coli*, IgM^+ B cells dominate the peritoneum [34]. Furthermore, in the case of VHSV, these peritoneal IgM^+ B cells significantly up-regulate the transcription levels of BAFF, APRIL and BALM after viral encounter [35]. These observations suggested that BAFF family ligands play an important role in the development of B cell responses within the peritoneal cavity, what led us to continue our analysis of the effects of BAFF on rainbow trout peritoneal B cells. First, we analyzed the composition of the peritoneal cavity B cell compartment in fish that had been previously vaccinated as part of the fish farm vaccination schedule. Hence, as a first step, we confirmed the presence of two different B cell types by multicolor flow cytometry analysis: an $IgD^+IgM^{hi}MHCII^{hi}$ population and an $IgD^-IgM^{lo}MHCII^{lo}$ population (with enlarged size), the former resembling a naïve B cell phenotype and the latter resembling an ASC phenotype. Corresponding to their levels of membrane IgM, we named these populations IgM^{hi} (naïve B cell-like phenotype) and IgM^{lo} (ASC phenotype), and to corroborate that they in fact corresponded to naïve and ASC B cells, respectively, we FACS isolated them from the peritoneum and analyzed the expression of Pax5 and Blimp-1 genes. This analysis corroborated both phenotypes, since IgM^{lo} B cells showed high expression of Blimp-1 and low expression levels of Pax-5, whereas IgM^{hi} B cells expressed undetectable levels of Blimp-1 and high levels of Pax-5, in a very similar trend to that seen on B cells from the spleen, which is mainly populated by naïve IgM^+ B cells. Blimp-1 has been shown to be sufficient to repress Pax5 expression during plasmacytoiyd differentiation, being this process required to drive differentiation of naïve B cells to IgM-secreting cells [41]. Moreover, the expression of Blimp-1 has been associated with the commitment of B lymphocytes to plasma cells *in vitro* and *in vivo* [43,51], whereas it has been shown to be repressed during the differentiation of B cells to memory phenotypes [52]. In this context, our results unveil that the peritoneal IgM^{lo} B cell population represents a lymphocyte subset terminally differentiated to IgM ASC. This shows that two different B cell populations co-inhabit the peritoneum in fish that had been intraperitoneally vaccinated at an early life stage for long periods of time given the fact that our experiments were performed with fish from 6 weeks to 12 weeks post-vaccination, observing no significant differences on the percentages of each B cell subset. On the contrary, fish from the same source and of similar size that had not been vaccinated in the fish farm contained IgM^{hi} B cells in the peritoneum but only very few IgM^{lo} B cells, thus suggesting that the exposure to the vaccine antigen plays a pivotal role in the differentiation of this peritoneal B cell subset. In mammals, the peritoneum has been shown as the main site of production of natural IgM protective against infection [53], and it has been demonstrated that

peritoneal cavity B cells are the precursors of splenic IgM natural antibody-producing cells [54]. Similarly, the coexistence of B1a, B1b and B2 cells, showing different phenotypes and functions, in the peritoneum of mammals have been extensively reported [55], hence suggesting that different B cell types co-exist in the peritoneum playing different roles during the immune response.

Having established the existence of these two B cell subsets in the peritoneum of vaccinated fish, we aimed to assess whether BAFF could play a differential role in their survival and regulation. To carry this out, we first analyzed the expression of BAFF receptors on such subsets. IgM^{hi} showed high transcription levels of BAFF-R, low transcription levels of BCMA and undetectable levels of TACI, similar to the profile observed for splenic B cells. Conversely, IgM^{lo} presented low transcription levels of BAFF-R and high transcription levels of BCMA, together with undetectable levels of TACI. In mammals, BCMA is highly expressed on plasma cells and is required for survival of long-lived plasma cells [17,56]. These results are also in line with previous studies in mammals that demonstrated that terminally differentiated plasma cells show increased expression of BCMA and lowered expression of BAFF-R and TACI [17,44].

When peritoneal cavity leukocytes were incubated with BAFF *in vitro*, secretion of IgM was significantly augmented. As the differentiation of B cells towards plasma cells or ASCs requires expression of the Blimp-1 transcriptional receptor [57], but no up-regulation of Blimp-1 expression was detected on FACS isolated IgM^+ B cells from BAFF-treated peritoneal cavity leukocyte cultures, IgM secretion seems to be up-regulated as a consequence of ASC survival rather than differentiation. Further analysis confirmed that after 3 days of culture with BAFF, the number of IgM^{lo} B cells was increased, but, surprisingly, the number of IgM^{hi} B cells was diminished. As a consequence, the size of the IgM^+ B cell pool was almost identical in control or BAFF-treated cultures as established before in non-vaccinated fish [35]. The different effects of BAFF on the survival of IgM^{hi} and IgM^{lo} B cells could indicate that BAFF might be signaling through different receptors expressed on IgM^{hi} cells and IgM^{lo} cells, thus exerting different regulatory roles on these populations. In mammals, it is well known that signaling through different BAFF receptors renders different effects on specific B cells. Regarding the IgM^{hi} population, we have very recently demonstrated that BAFF promotes the survival of splenic B cells [31], which present a very similar phenotype to that seen of this peritoneal cavity population, so further investigation as to why peritoneal IgM^{hi} B cells behave differently than splenic B cells requires further investigation. Finally, given the positive effects on IgM^{lo} B cell survival, we analyzed whether BAFF had lymphoproliferative effects on these cells. BAFF did not promote the proliferation of this B cell subset (data not shown), as expected, since terminally differentiated plasma cells do not proliferate [40]. Thus, the effects played by BAFF on peritoneal B cells are based only on differential survival on IgM subpopulations.

As we had previously observed that BAFF increased the levels of surface MHC II on splenic resting mature (IgD^+IgM^+) B cells [31], we studied this effect on the two subpopulations identified in the peritoneum of vaccinated fish. BAFF up-regulated the levels of membrane MHC II on IgM^{hi}, but not on IgM^{lo} B cells, thus suggesting that BAFF promotes the increase of MHC II on IgM^+ resting B cells in both lymphoid tissues and peripheral sites. IgM^{lo} B cells do not increase their MHC II levels in the presence of BAFF, most probably due to the fact that plasma cells lose their ability to present antigen [58].

In mammals, BAFF is predominantly produced by myeloid cells although recent studies have demonstrated its expression by a wide variety of hematopoietic and non-hematopoietic cell types (reviewed in Ref. [8]). Concerning cells of the B lineage, only activated B cells can produce BAFF [59]. However, we have recently demonstrated that in teleost BAFF is produced by a subset of splenic B cells found in unstimulated fish [31]. In this context, we also studied the capacity of IgM^{hi} and IgM^{lo} peritoneal cavity B cell subsets to produce BAFF. Interestingly, we observed that IgM^{hi} B cells produced BAFF in a proportion of cells and in levels comparable to those achieved by peritoneal myeloid cells, while IgM^{lo} B cells produced negligible BAFF amounts similarly to IgM^- populations. This suggests that together with myeloid cells, IgM^{hi} B cells are the main source of BAFF on peritoneal cavity leukocytes, which could represent a strategy to maintain the B cell pool at peripheral sites in homeostasis. We have previously shown that the expression of BAFF is up-regulated after intraperitoneal antigen stimulation [33], pointing to an important role played by BAFF on the immune response at the peritoneal cavity. Here we show that, in response to BAFF, IgM^{hi} B cells are able to up-regulate their MHC II surface levels to augment their antigen presentation capacities, while peritoneal cavity plasma cells (IgM^{lo}) showed an increased survival rate in response to the cytokine, which results in increased IgM secretion levels.

In conclusion, we have demonstrated that different teleost B cell populations co-exist in the peritoneum of vaccinated fish, which respond differently to BAFF. Our results reveal a complex network through which BAFF or BAFF-related cytokines regulates B cell function in the peritoneum of fish.

Acknowledgements

This work was supported by project AGL2014-54456-JIN from the Spanish Ministry of Economy and Competitiveness (MINECO), the European Research Council (ERC Starting Grant 2011 280469), and the European Commission under the 7th Framework Program for Research and Technological Development (FP7) of the European Union (Grant Agreement 311993 TARGETFISH).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.10.003.

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