The Ancient Cytokine BAFF- and APRIL-like Molecule Regulates the Functionality of Teleost IgM\(^+\) B Cells Similarly to BAFF and APRIL

Rocío Simón, Patricia Díaz-Rosales and Carolina Tafalla

*J Immunol* 2021; 206:1765-1775; Prepublished online 24 March 2021; doi: 10.4049/jimmunol.2000762

http://www.jimmunol.org/content/206/8/1765

Supplementary Material

http://www.jimmunol.org/content/suppl/2021/03/24/jimmunol.2000762.DCSupplemental

References

This article cites 33 articles, 9 of which you can access for free at:

http://www.jimmunol.org/content/206/8/1765.full#ref-list-1

Why *The JI*? Submit online.

- Rapid Reviews! 30 days\(^*\) from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

\(^*\)average

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
The Ancient Cytokine BAFF- and APRIL-like Molecule Regulates the Functionality of Teleost IgM$^+$ B Cells Similarly to BAFF and APRIL

Rocio Simón, Patricia Díaz-Rosales, and Carolina Tafalla

TNF superfamily (TNFSF) members, such as BAFF and a proliferation-inducing ligand (APRIL), have been identified in mammals and, recently, in teleost fish as key regulators of B cell homeostasis and activation. Many cartilaginous and teleost fish contain an additional gene, designated as BAFF- and APRIL-like molecule (BALM), of unknown function and lost in tetrapods. In this study, we have performed a wide characterization of the functions of BALM on naive B cells for the first time, to our knowledge, in teleosts using rainbow trout (Oncorhynchus mykiss) as a model. Similar to BAFF and APRIL, BALM increased the survival and promoted the proliferation of peripheral blood IgM$^+$ B cells and cooperated with BCR cross-linking to increase the proliferation rate of IgM$^+$ B cells. BALM also seemed to be a differentiating factor for trout IgM$^+$ B cells, as it increased IgM secretion and increased cell size. Additionally, BALM appeared to increase the Ag-presenting properties of IgM$^+$ B cells, augmenting MHC class II surface expression and upregulating the phagocytic capacity of these cells. Finally, the fact that there was no synergy between BALM and BAFF/APRIL in any of these functions strongly suggests that BALM signals through the same receptors as BAFF and APRIL to carry out its functions. This hypothesis was further supported in competitive BALM binding assays. The results presented provide relevant information for understanding how these TNFSF members cooperate in teleost fish to regulate B cell functionality, helping us to interpret the evolutionary relations between molecules of this family. The Journal of Immunology, 2021, 206: 1765–1775.

The online version of this article contains supplemental material.
duplication and translocation events. This is the case in TNFSF, in which additional orthologs of mammalian TNFSF members have been found in some teleost species (15). Interestingly, new members of this superfamily that appear to be unique to teleosts have also been identified (15). One of these unique teleost TNFSF members is a gene with similarities to both BAFF and APRIL, consequently designated as BAFF- and APRIL-like molecule (BALM) (15). This gene, identified in species such as rainbow trout, three-spined stickleback (*Gasterosteus aculeatus*) and fugu (*Takifugu rubripes*), whereas absent in other species such as zebrafish (*Danio rerio*), seems to code for an ancestral BAFF-like ligand that appeared early in evolution and was then lost in tetrapods, possibly when BAFF and APRIL acquired divergent functions. Remarkably, an ancestral relative of BALM was also identified in jawless fish (16) that possess a primitive adaptive immune system still lacking the cognate T and B cell receptors that appeared in teleost fish. To date, very little is known regarding the effects of this ancestral cytokine on teleost B cells. Transcriptomic studies have revealed that, in homeostasis, rainbow trout BALM is strongly expressed in the spleen, blood, and kidney and weakly in the gills, heart, skin, liver, and intestine (15). In this same species, the transcription of BALM was shown to be upregulated locally in lymphocytes after the i.p. administration of virus particles (17). Additionally, a significant correlation between the transcription of BALM and the progression of the proliferative kidney disease, a parasitic disease known to profoundly dysregulate B cell responses, was found in rainbow trout (18). Regarding functional studies, rainbow trout APRIL and BALM but not BAFF were shown to increase the number of total IgM⁺ B cells in peritoneal leukocyte cultures (17), whereas BALM and not BAFF and APRIL promoted the survival and proliferation of IgM⁺ B cells in the kidney (18).

To gain further insight into the role that BALM plays in the teleost immune system, in the current study, we have investigated the effects of BALM on a wide range of B cell functions, using rainbow trout as a model and comparing its effects to those exerted by the related cytokines BAFF and APRIL. We focused our studies on IgM⁺ IgD⁻ naive B cells (designated from this point forward as IgM⁺ B cells) and excluded from the analysis other less abundant B cell subsets, such as IgT⁺ or IgD⁺ B cells, or cells with a plasmablast phenotype (IgD⁻ IgM⁺ IgHcl⁻) (15). Our results demonstrated that blood IgM⁺ B cells responded to BALM in a similar manner as BAFF and APRIL, provoking significant effects on survival, proliferation, IgM secretion, cell size, MHCII surface expression, and phagocytic capacity. Interestingly, our results also revealed that BAFF exerted some effects on blood IgM⁺ B cells not seen in splenic IgM⁺ B cells, demonstrating some specific effects of this cytokine, depending on the cell source. Finally, we combined the three cytokines in all of these studies to establish possible cooperative or synergistic effects. We found that the combination of BAFF and APRIL promoted the survival of blood IgM⁺ B cells at levels significantly higher than those reached by either of the cytokines alone, suggesting that BAFF and APRIL signal through different receptors to carry out this function. However, no other cooperative effects were identified between cytokines in cell survival or other functions studied, suggesting that BALM does not signal through a receptor different from those already used by BAFF and APRIL. This was also supported by binding experiments in which we established that the preincubation of blood leukocytes with BAFF or APRIL significantly decreased the binding of BALM to IgM⁺ B cells. Altogether, these results provide relevant information for understanding the role of these cytokines on B cell functionality in fish, helping us to interpret the evolutionary relations between molecules of the TNFSF family.

**Materials and Methods**

**Experimental fish**

Rainbow trout (*O. mykiss*) of 250 g were obtained from Piscicultura Cifuentes (Guadajalara, Spain) and maintained at the animal facilities of the Animal Health Research Center of the National Institute for Agricultural and Food Research and Technology in an aerated recirculating water system at 16°C, with 12:12 h light-dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting, Cojobar, Spain). Prior to any experimental procedure, fish were acclimated to laboratory conditions for at least 2 wk. All of the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for use of laboratory animals and have been approved by the National Institute for Agricultural Food Research and Technology Ethics Committee (PROEX002/17).

**Leukocyte isolation**

Rainbow trout were killed by benzocaine (Sigma-Aldrich) overdose, and blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with Leibovitz L-15 Medium (L-15; Thermo Fisher Scientific) supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific), 5% FCS (Thermo Fisher Scientific), and 10 IU/ml heparin (Sigma-Aldrich). PBLS were obtained by centrifugation (500 x g for 30 min at 4°C) of diluted blood on 51% continuous Percoll (GE Healthcare) density gradients. The interface cells were collected, washed twice in L-15 containing antibiotics and 5% FCS, and adjusted to 2 x 10⁶ cells/ml.

**Production of recombinant cytokines**

The nucleotide sequence corresponding to the extracellular domain of the rainbow trout BAFF sequence (GenBank accession number DQ218467.1), APRIL (GenBank accession number NP_001118434.1), or BALM (GenBank accession number ABC45841.1), together with an N-terminal 6xHis tag were synthesized and cloned into the E3 expression vector (Abyntek Biopharma) (Supplemental Fig. 1A). The recombinant plasmids were transformed into BL21 cells, and transformants screened by kanamycin resistance. A single positive colony per each cytokine was then incubated at 37°C in Luria-Bertani media containing kanamycin (Sigma-Aldrich).

When the OD₆₀₀ reached 0.6, 0.1 mM isopropyl β-D-thiogalactoside (Sigma-Aldrich) was added for 16 h to induce protein production. Cells were harvested by centrifugation, and cell pellets were then lysed by sonication and dissolved using urea. Thereafter, cytokines were obtained through one-step purification process using a nickel column (Sigma-Aldrich). BAFF and APRIL were refolded, filtered through 0.22 µm and then resuspended as previously described (11, 13). For the refolding of BALM, 4.5 ml of the protein was dialyzed into 450 ml of 50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, and 0.5 M l-arginine (pH 8.0). The dialysis was performed for 4 h using a 14-KDa cut-off dialysis membrane. At this point, the buffer was changed, and the protein was dialyzed for an additional 16 h. After dialysis, the sample was centrifuged at 13,000 rpm for 30 min and filtered through 0.22 µm. In all cases, the protein concentration was determined by a BCA Protein assay (Thermo Fisher Scientific) using BSA as a standard. The recombinant rainbow trout cytokines were then aliquoted and stored at −80°C until use.

To verify the correct folding of the recombinant cytokines, 5 µg of each protein were analyzed by PAGE under reducing (SDS-PAGE) or native non-reducing conditions, followed by silver staining (Supplemental Fig. 1B, 1C). Briefly, gels were fixed in 50% methanol plus acetic acid, sensitized with 0.02% sodium thiosulfate, stained with 0.2% silver nitrate, and developed with 2% sodium carbonate plus 0.94% formaldehyde. Gel images were acquired in a ChemiDoc Imaging System with Image Lab Touch software (Bio-Rad Laboratories).

LPS contamination in the recombinant proteins was negligible (<4 ng/ml), as established using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific).

**Cell stimulation**

PBLS were cultured at 20°C in L-15 medium supplemented with antibiotics and 5% FCS in 24- or 96-well plates (Nunc) in the presence of recombinant rainbow trout BAFF, APRIL, or BALM at final concentrations of 3, 1, and 10 µg/ml, respectively. A wide range of BALM doses were initially tested to select the optimal dose on the basis of its effect on B cell survival (Supplemental Fig. 2A, 2B). BAFF and APRIL doses were selected according to previously published results from our group (11, 13). Cells were incubated with the different stimuli for different time periods depending on the experiment. In some experiments, leukocytes were stimulated with an unabeled mAb against trout IgM (clone 1.14, mouse IgG1) (19) at a final concentration of 10 µg/ml, as previously described (20). Nonstimulated controls were always included. Finally, in some experiments, an irrelevant...
protein with a similar molecular mass to that of recombinant APRIL (25.2 kDa), also bearing an N-terminal His tag was produced in the same conditions and used to verify that the effects exerted by BAFF, APRIL, and BALM were specific. In other experiments, denatured BALM (incubated for 2 h at 95°C) was used as a negative control (Supplemental Fig. 2C, 2D).

Flow cytometry
PBLS seeded in 96-well plates at a density of 2 × 10^6 cells/ml were incubated for 72 h at 20°C with BAFF, APRIL, or BALM or with media alone, as described above. After 3 d, cells were washed in staining buffer (phenol red-free L-15 medium supplemented with 2% FCS) and coincubated with FITC-conjugated anti-IgM (1.14) (1 μg/ml) and specific mAbs against trout MHCII β-chain (mAb mouse IgG1 coupled to allophycocyanin, 2 μg/ml) or trout IgD (mAb mouse IgG1 coupled to allophycocyanin, 10 μg/ml), as previously characterized (21, 22). Thereafter, cells were washed twice with staining buffer and analyzed on a FACS-casta flow cytometer (BD Biosciences), incorporating an anti-IgM mAb and FITC-conjugated anti-IgM (1 μg/ml) and labeled with 10 μg/ml of antibiotinylated BALM or left unstimulated for 30 min at 20°C. Thereafter, cells were washed with serum-free L-15 medium, seeded on poly-L-lysine coated slides, and incubated for 1 h at RT. After gently washing with PBS, slides were fixed in 4% paraformaldehyde for 30 min at RT and then incubated for 1 h at RT with blocking solution (PBS containing 5% BSA and 0.5% saponin). Fixed cell slides were then incubated with anti-IgM (1.14) coupled to allophycocyanin (10 μg/ml) and FITC-streptavidin (5 μg/ml) for 1 h at RT. Samples were counterstained with 1 μg/ml DAPI (Sigma-Aldrich) for microscopy. For the competitive binding assays, PBLS were first preincubated with “cold” (nonbiotinylated) APRIL (1 μg/ml), BAFF (3 μg/ml), or BALM (10 μg/ml) for 30 min at 20°C followed by biotinylated BALM. After 30 min at 20°C, cells were stained as described above. Laser scanning confocal microscopy images were acquired with an inverted Zeiss Axiovert LSM 880 microscope with Zeiss ZEN software. Cell images were then analyzed in 15 digital fields (n = 500 cells) for each condition from at least four different individuals and processed with Zeiss ZEN and Adobe Photoshop CS6 software packages.

Statistics
Data handling, statistical analyses, and graphic representation were performed using Microsoft Office Excel 2010 and GraphPad Prism version 7.02 (GraphPad Software). Statistical analyses were performed to compare values obtained in each experimental group using a two-tailed paired Student t test with Welch correction when the F test indicated that the variances of both groups differed significantly. The differences between the mean values were considered significant on different degrees, where *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.005.

Results
BALM increases the survival of peripheral blood IgM+ B cells
We have previously reported that BAFF and APRIL increase the survival of rainbow trout splenic IgM+ B cells (11, 13), but their effects on rainbow trout peripheral blood IgM+ B cells had never been tested. In this work, we chose to examine the effects of rainbow trout BALM on blood IgM+ B cells because the blood is the tissue that contains the higher percentage of IgM+ B cells in rainbow trout (24). As all experiments were performed in parallel with BAFF and APRIL, the results obtained would also allow us to establish whether BAFF and APRIL exerted differential effects on IgM+ B cells, depending on the source. Our experiments revealed that all three cytokines were capable of significantly promoting the survival of blood IgM+ B cells, as both the percentage of IgM+ B cells (Fig. 1A, 1B) and the total number of IgM+ B cells (Fig. 1A, 1C) in cultures significantly increased after 3 d of incubation.

BALM induces the proliferation of blood IgM+ B cells by itself or in cooperation with BCR cross-linking
We have previously described that APRIL exerts lymphoproliferative effects of splenic IgM+ B cells (13), whereas BAFF only seemed to act as a survival factor for these cells (11), as reported in mammals (25). To establish whether BALM exerted lymphoproliferative effects on IgM+ B cells that might partially explain the increased B cell survival, we performed a proliferation test by means of EdU incorporation. Our results show that all three cytokines are capable of inducing the proliferation of a small subset of peripheral blood IgM+ B cells (Fig. 2A). Surprisingly, in contrast to what was observed for splenic IgM+ B cells (11), BAFF significantly induced the proliferation of a subset of peripheral blood IgM+ B cells (Fig.
To confirm that BAFF is in fact exerting different effects on splenic and blood IgM+ B cells, we performed a proliferation assay in parallel with blood and splenic IgM+ B cells from the same fish. This experiment confirmed that BAFF has no lymphoproliferative effects on splenic IgM+ B cells, but is capable of inducing the proliferation of peripheral blood IgM+ B cells (Supplemental Fig. 4).

To analyze whether cytokines could be increased with BCR cross-linking to induce a higher proliferation rate, anti-IgM was added to some of the wells alone or in combination with the different cytokines, as previously described (20, 23). As reported in those studies, anti-IgM alone is not sufficient to induce the proliferation of trout IgM+ B cells (Fig. 2B, 2C). However, when BCR cross-linking was combined with BALM, APRIL, or BAFF stimulation, a significant increase in the induced proliferation rate was observed in comparison with cultures treated with the cytokines alone (Fig. 2B, 2C).

### BALM increases the number of IgM-secreting cells

Both BAFF and APRIL were shown to significantly increase the number of IgM-secreting cells in splenocyte cultures (11, 13). Therefore, we investigated whether the three cytokines had a similar effect on PBL cultures. All three cytokines significantly increased the number of IgM-secreting cells in cultures after 3 d when compared with the number of IgM-secreting cells found in nonstimulated cultures, as verified in an ELISPOT assay (Fig. 3A, 3B). Taking into account the effects of the three cytokines on B cell survival, this increase in the number of IgM-secreting cells could be a consequence of more B cells (which would also include IgM-secreting cells). Alternatively, the cytokines could be inducing the differentiation of blood IgM+ B cells to plasmablasts/plasma cells. In both mammals (26) and rainbow trout (14), activated B cells that start their differentiation toward plasmablasts/plasma cells increase their size. Our results show that all three cytokines significantly increased the size of blood IgM+ B cells (Fig. 3C), suggesting that APRIL, BAFF, and BALM induce the differentiation of rainbow trout blood IgM+ B cells to plasmablasts/plasma cells.

### BALM increases the Ag-presenting properties of peripheral blood IgM+ B cells

Given their professional APC nature, B cells constitutively express MHCIIm on the cell surface (27). Thus, we next studied whether APRIL, BAFF, and BALM could have an effect on the levels of surface MHCIIm expression in blood cells using a specific anti-trout MHCIIm Ab (22). Our results clearly show that all three cytokines significantly increased the levels of surface MHCIIm on peripheral blood IgM+ B cells (Fig. 4A–C). Notably, this effect exerted by the cytokines was not visible in the case of IgM− cells (Fig. 4D).

Rainbow trout IgM+ B cells have been shown to exert a potent phagocytic capacity (28), a trait that strongly conditions their Ag-presenting abilities (29). Therefore, we also established whether BALM, APRIL, or BAFF had an effect on the phagocytic capacity of peripheral blood IgM+ B cells. To this aim, blood cells were incubated with recombinant BALM, APRIL, or BAFF or with media alone for 72 h. After this time, cells were incubated with 1 μm of crimson red-labeled polystyrene beads for 3 h, and the phagocytic activity of IgM+ B cells was determined thereafter through flow cytometry. We observed that the prestimulation of IgM+ B cells with the cytokines significantly increased the percentage of phagocytic...
FIGURE 2. Lymphoproliferative effects of APRIL, BAFF, and BALM on blood IgM⁺ B cells. (A) PBLs were incubated with APRIL (1 μg/ml), BAFF (3 μg/ml), BALM (10 μg/ml), or media alone (Control) during 3 d at 20°C. After this time, cells were labeled with EdU (1 μM) and incubated for a further 24 h. At that point, cells were labeled with anti-IgM mAb, and the number of IgM⁺ B cells with incorporated EdU (proliferating cells) was evaluated by flow cytometry. Representative dot plots for each condition are included. (B) In other experiments, PBLs were incubated with the different cytokines, as described above, in the presence or absence of anti-IgM mAb (10 μg/ml). In this case, cells were only labeled with EdU. Representative dot plots for each condition are included. (C) Graph showing the percentage of EdU⁺ cells (mean ± SD) among blood leukocytes (n = 9 fish). Individual values are shown as black dots. Asterisks above bars denote significant differences between treated and control cells, whereas asterisks above brackets indicate significant differences between groups, as indicated. *p ≤ 0.05, **p ≤ 0.005.
IgM⁺ B cells in leukocyte cultures when compared with unstimulated cultures (Fig. 5A, 5C). Along with this increase in the percentage of phagocytic cells, the mean fluorescence intensity (MFI) of internalized beads in blood IgM⁺ B cells was also significantly higher in cytokine-treated cells compared with untreated cells (Fig. 5B), indicating that the average number of particles internalized per IgM⁺ B cell was higher when IgM⁺ B cells were prestimulated with the cytokines. Interestingly, when we analyzed the effects of cytokines on the IgM⁻ population, we also found a significant upregulation in the percentage of phagocytic cells in response to the three cytokines (Fig. 5A, 5E); however, in this case, only BALM was capable of significantly increasing the average number of particles internalized per IgM⁻ B cell (Fig. 5D). Altogether, our results point to a role for BALM, APRIL, and BAFF in the regulation of the Ag-presenting capacities of peripheral blood 

**FIGURE 3.** APRIL, BAFF, and BALM activate the number of IgM-secreting cells and increase the size of IgM⁺ B cells. (A and B) ELISPOT analysis of IgM-secreting cells in blood-cultured cells treated with APRIL (1 μg/ml), BAFF (3 μg/ml), BALM (10 μg/ml), or media alone (Control). Cells were cultured with the appropriate stimuli for 48 h and then plated in ELISPOT plates previously coated with anti-IgM mAb (2 μg/ml) for a further 24 h. After incubation, cells were washed away and a biotinylated anti-IgM mAb (1 μg/ml) was used to detect number of spot-forming cells. Duplicates from a representative individual (A) and quantification of spot-forming cells (B) from six independent fish are shown (mean ± SD). (C) Blood leukocytes were incubated with APRIL (1 μg/ml), BAFF (3 μg/ml), BALM (10 μg/ml), or media alone (Control) during 3 d at 20°C. At this point, the size of the B cells was calculated by flow cytometry. Graph showing MFI values for forward scatter (FSC) is shown (mean ± SD; n = 7 fish). Individual values are shown as black dots. Asterisks denote significantly different values between cells treated with the cytokines and controls. *p ≤ 0.05, ***p ≤ 0.005.

**FIGURE 4.** APRIL, BAFF, and BALM upregulate MHCII surface expression on IgM⁺ B cells. Blood leukocytes were incubated with APRIL (1 μg/ml), BAFF (3 μg/ml), BALM (10 μg/ml), or media alone (Control) during 3 d at 20°C. Thereafter, cells were labeled with anti-trout IgM and anti-trout MHCII mAbs and analyzed by flow cytometry. (A) Representative dot plots are shown. (B) Histogram showing MHCII expression levels in IgM⁺ B cells from one representative fish. (C and D) Graphs showing MHCII MFI values for IgM⁺ B (C) and IgM⁻ (D) cells (mean ± SD; n = 7 fish). Individual values are shown as black dots. Asterisks denote significantly different values between cytokine-stimulated cells and controls. *p ≤ 0.05, **p ≤ 0.01.
IgM⁺ B cells as visualized through increased surface MHCII levels and increased phagocytic activity.

**BAFF synergizes with APRIL to increase the survival of blood IgM⁺ B cells**

To date, whether fish BAFF and APRIL signal through the same receptors as those used by mammalian BAFF and APRIL and those used by BALM is currently unknown. To gain some insight on whether all three cytokines signal through the same or different receptors to exert their functions on blood IgM⁺ B cells, we analyzed whether the three cytokines cooperated to increase a higher effect on IgM⁺ B cell survival, IgM secretion, or MHCII surface expression. We found that BAFF cooperated with APRIL to increase the survival of blood IgM⁺ B cells (Fig. 6A, 6B), suggesting that these cytokines signal through at least one common receptor. However, no other cooperative effects between cytokines were found in cell survival or ELISPOT assays (Fig. 6C) or in MHCII surface expression levels (Fig. 6D). These results suggest that BALM does not signal through a receptor different from those already used by BAFF and APRIL.

**BALM binding to blood IgM⁺ B cells**

To investigate the capacity of recombinant BALM to bind rainbow trout IgM⁺ B cells from peripheral blood, we incubated PBLs with a biotinylated version of BALM and investigated its binding capacity through confocal microscopy. We found that PBLs had the ability to bind biotinylated BALM (Fig. 7A–C). Among the total BALM-binding cells, an average 72.2 ± 5.3% were IgM⁺ B cells, whereas the remaining 27.8 ± 5.3% were IgM⁻ cells of unknown nature (Fig. 7D). Among all IgM⁺ B cells, we observed that 14.8 ± 3.3% of blood IgM⁺ B cells had the ability to bind biotinylated BALM (Fig. 7A, 7B, 7E). Thus, it seems that, in blood, BALM preferentially binds IgM⁺ B cells, although this binding is effective on a small subset of IgM⁺ B cells.

To confirm the specificity of the binding, in some experiments, cells were preincubated with nonbiotinylated BALM prior to the addition of biotinylated BALM. This preincubation would occupy BALM-binding places in target cells and consequently significantly decrease the number of PBLs binding BALM (Fig. 7C) as well as the percentage of IgM⁺ B cells that bind BALM (Fig. 7E). Interestingly, this decrease in the percentage of IgM⁺ B cells that bound BALM was also observed when PBLs were preincubated with BAFF or APRIL (Fig. 7E), again suggesting that, in blood IgM⁺ B cells, BALM shares receptor(s) with BAFF and APRIL.

**Discussion**

Agnathans (jawless fish) are the first animal group in which innate responses are supplemented with some adaptive immune elements, such as variable lymphocyte receptors, whereas it was when jawed fish (both cartilaginous and teleost fish) emerged that most of the elements of the adaptive immune system such as MHC and BCRs and TCRs appeared (30). However, many structural and functional differences exist between the basic adaptive system of teleost fish (both cartilaginous and teleost fish) and that of mammals, in which a much higher degree of specialization has been acquired. These differences strongly condition how these animal groups react to an initial and a secondary encounter with an Ag. In what concerns B cell function, the main differential traits of the teleost adaptive immune system include the following: the lack of lymph nodes and cognate germinal centers, the kidney being the main hematopoietic tissue and the main site for B cell lymphopoiesis, the limited repertoire of Ig isotypes that results in no class switch recombination during the B cell differentiation process, and the fact that IgM⁺ B cells phenotypically and functionally
resemble mammalian innate B1 cells (20). In this context, it is interesting to point out that some authors have hypothesized that the divergence of the TNFSF and TNFRSF families parallels the evolution of the adaptive immune system (31). Under this hypothesis, studying how these cytokines and their receptors regulate B cell responses in different species has proven a fascinating research topic to better understand how B cells have evolved from fish to mammals.

In 2007, Glenney and Wiens (15) identified in rainbow trout a sequence with high similarity with BAFF that contains a D–E loop characteristic of APRIL, which was designated as BALM. This new TNFSF ligand was also found in other teleost species, such as fugu and three-spined stickleback, whereas it was selectively deleted in other fish species, such as zebrafish (15). The fact that BALM is absent in all tetrapods points to BALM as an ancestral BAFF-like ligand that appeared early in evolution and was then lost, maybe as BAFF and APRIL took over its functions. In this sense, it is very interesting to establish the role that this primitive cytokine has on the regulation of B cells, comparing its effects to those of the related cytokines BAFF and APRIL. To date, only a few studies had faintly addressed the functional role of BALM on teleost B cells. Thus, BALM was shown to increase the survival of rainbow trout peritoneal (17) and kidney (18) IgM\(^+\) B cells, showing proliferative effects in the case of the kidney. However, to our knowledge, a thorough study of the effects that this cytokine has on naive IgM\(^+\) B cells had not been conducted until now, and this is what we have addressed in this paper. In this case, we chose to examine the effects on PBLs, as the blood is where the higher percentage of IgM\(^+\) B cells

**FIGURE 6.** APRIL cooperates with BAFF to promote IgM\(^+\) B cell survival. Blood leukocytes were cultured in the presence of APRIL (1 \(\mu g/ml\)), BAFF (3 \(\mu g/ml\)), BALM (10 \(\mu g/ml\)), or media alone (Control) or combinations of cytokines, as indicated (APRIL–BAFF, APRIL–BALM, or BAFF–BALM) during 3 d at 20°C. Thereafter, different experiments were performed as previously reported. (A and B) IgM\(^+\) B cell survival analysis. Representative dot plots are shown for each experimental condition (A) together with a graph showing the quantification of average IgM\(^+\) B cells in cultures relativized to the number of cells in untreated control cultures (B) (mean ± SD) (\(n = 8\) fish). (C) ELISPOT analysis. Graph showing the quantification of spot-forming cells relativized to number of spots in control cultures (mean ± SD) (\(n = 6\) fish). (D) Analysis of MHCII surface expression. Graph showing MHCII MFI values for IgM\(^+\) B cells relativized to values obtained in control cultures (mean ± SD; \(n = 7\) fish). Individual values are shown as black dots. Asterisks denote significantly different values between indicated groups. ***\(p \leq 0.005\).
cells are found in rainbow trout (≈44%) (24). Including BAFF and APRIL in these studies allowed us not only to compare the effects of BALM to those of the related cytokines but also to establish whether the effects that BAFF and APRIL exerted on blood IgM⁺ B cells were similar to those previously reported using splenic IgM⁺ B cells (11, 13). It should also be noted that our experiments were performed with complete PBL cultures, afterward examining the effects of the cytokines on IgM⁺ B cells. Therefore, we cannot

FIGURE 7. Rainbow trout BALM binds IgM⁺ B cells. To analyze the specific binding of BALM to IgM⁺ B cells, freshly isolated PBLs were incubated with 10 µg/ml biotinylated BALM (BALM*bio) or left unstimulated (Control) for 30 min at 20°C. Thereafter, the cells were plated onto poly-L-lysine-coated glass slides, fixed, and labeled with allophycocyanin-anti-IgM (shown as red) and FITC-streptavidin (shown as green), then counterstained with DAPI (blue) and analyzed by fluorescence microscopy. (A) Representative overviews of control cells and cells incubated with BALM*bio are shown (scale bar, 20 µm). (B) Amplification detail of a single IgM⁺BALM⁺ cell and an IgM⁺BALM⁻ cell from the sample incubated with BALM*bio shown in (A) (scale bar, 5 µm). (C) Quantification of the total number of BALM-binding cells per field in cells incubated with BALM*bio. Some of these cells were previously incubated for 30 min at 20°C with nonbiotinylated APRIL (1 µg/ml), BAFF (3 µg/ml), or BALM (10 µg/ml) proteins as indicated. Cell images were analyzed in 15 digital fields for each condition from at least four different individuals. Results are shown as mean number of cells per field + SD (n = 4). (D) Pie chart depicting the percentage of IgM⁺ and IgM⁻ cells among BALM-binding cells in cultures incubated with BALM*bio (E) Graph showing percentages of IgM⁺BALM⁺ cells within the total IgM⁺ cell population in cultures incubated with BALM*bio alone or cultures previously incubated with nonbiotinylated BALM, BAFF, or APRIL (mean + SD; n = 4). Individual values are shown as black dots. Asterisks denote significantly different values in indicated groups compared with values obtained in cultures only incubated with BALM*bio. *p ≤ 0.05.
rule out that some other cell types effected by the cytokines secrete factors that indirectly affect the functionality of IgM⁺ B cells. However, it is expected that these effects would be minor, as IgM⁺ B cells preferentially express all the receptors used by these cytokines, whereas they are only weakly expressed by other leukocyte types present in cultures obtained from unstimulated fish (data not shown). Furthermore, through binding assays, we have established that 72% of the cells that bind BALM among PBLs are IgM⁺ B cells.

Our results have demonstrated that BALM, similar to BAFF and APRIL, increase the survival of blood IgM⁺ B cells, exerting proliferative effects on these cells, effects that were further increased when coupled to cross-linking of the BCR. Interestingly, BAFF has no lymphoproliferative effects on splenic IgM⁺ B cells [(11), this paper], but instead, it is capable of inducing the proliferation of peripheral blood IgM⁺ B cells, confirming that the effects that these cytokines have on different subpopulations of B cells diverge. Similarly, although BAFF increased the phagocytic capacity of blood IgM⁺ B cells, it had no such effect on splenic IgM⁺ B cells (11). One of the possible explanations for this differential response could be that the range of receptors expressed in B cells from different sources is different. Along this line, in mammals, the expression profile of BAFF and APRIL receptors significantly varies, depending on the B cell subset, the anatomical location, or the stage of differentiation (4, 5), thus conditioning the response of these cells to these cytokines. In rainbow trout, we analyzed the transcription levels of BAFF-R, TACI, and BCMA in sorted IgM⁺ B cells from the blood and spleen, and no statistically significant differences were observed (data not shown). However, it should be pointed out that these were transcriptional studies, and posttranscriptional regulation of these receptors has been reported in mammals (32).

As in mammals, IgM secretion is increased by BAFF through the increased survival of these IgM-secreting cells in splenocyte cultures (11). Additionally, APRIL increases IgM secretion in splenocyte cultures and increases the size of splenic IgM⁺ B cells (13). In the case of rainbow trout blood IgM⁺ cells, the increased IgM secretion went along with a slight decrease in surface IgD expression (data not shown) and an increase in cell size that strongly suggested a differentiation of blood IgM⁺ B cells to plasmablasts/plasma cells in response to the three cytokines. Of course, to clearly establish that this is true, additional studies would need to be undertaken once the molecular signatures of different subsets of Ab-secreting cells are clearly defined in teleost fish.

The Ag-presenting capacity of teleost IgM⁺ B cells is presumed to be higher than that of mammalian B cells, as their strong phagocytic capacity would enable them to present not only Ags acquired through the BCR but also engulfed particulate Ags (29). As mammalian conventional B cells have lost their phagocytic capacity, it seemed interesting to establish whether this ancient cytokine that was later lost through evolution affected this innate trait maintained by teleost B cells (28) and only exhibited in mammals in innate B1 cells (33). Interestingly, all three cytokines increased the number of phagocytic IgM⁺ B cells in the cultures as well as the capacity of these cells to engulf microparticles. Consequently, it would also be important to establish whether BAFF and APRIL can influence the phagocytic capacity of mammalian B1 cells. Finally, the Ag-presenting capacities of rainbow trout IgM⁺ B cells would probably benefit from the increased levels of MHCII expressed on the cell surface in response to the three cytokines. Similarly, BAFF and APRIL had been shown to upregulate membrane MHCII expression on splenic B cells and, in case of BAFF, also in peritoneal B cells (11, 13, 14).

In conclusion, we have demonstrated that BALM, an ancient member of the TNFSF not present in tetrapods, regulates a plethora of immune functions of rainbow trout IgM⁺ B cells, significantly promoting the survival, proliferation, IgM secretion, differentiation, MHCII surface expression, and phagocytic potential of this B cell subset that constitutes the predominant B cell type in all teleost central immune tissues. Interestingly, all of these effects provoked by BALM on blood IgM⁺ B cells were similarly regulated by BAFF and APRIL and seemed to be executed through the binding of similar receptors, given that no cooperative effects between BALM and the other two cytokines were found. This hypothesis was supported by competitive binding assays, in which we established that the preincubation of PBL cultures with nonbiotinylated BAFF or APRIL significantly decreased the binding of biotinylated BALM to blood IgM⁺ B cells. These results might explain why BALM disappeared in tetrapods as its functions were overtaken by BAFF and APRIL.

Our results help us to establish how different members of the TNFSF regulate B cell functionality in teleosts while at the same time providing us with new information to understand how this family of cytokines evolved.

Acknowledgments
We thank Lucia Gonzalez and Diana Martin for technical support.

Disclosures
The authors have no financial conflicts of interest.

References


