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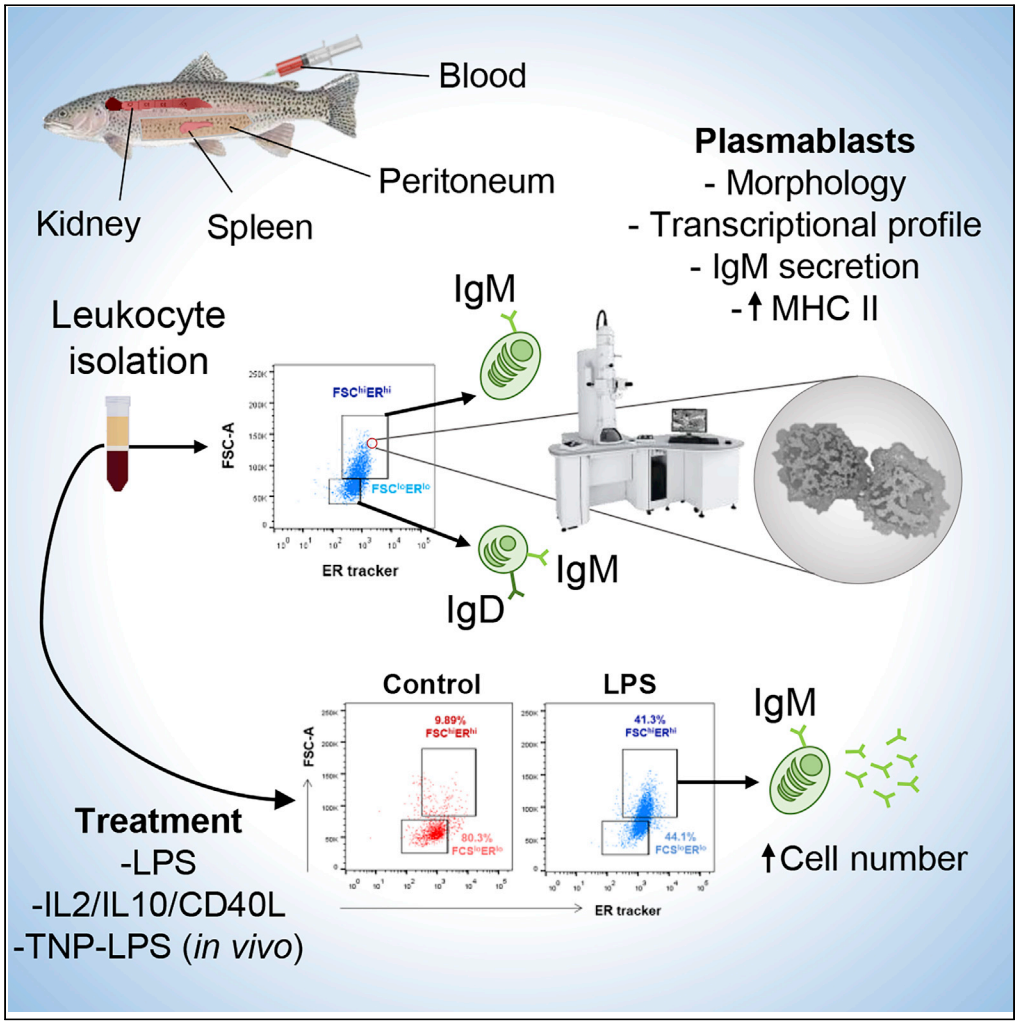

Esther Morel, J. Germán Herranz-Jusdado, Rocío Simón, ..., Milagros Guerra Rodriguez, Patricia Díaz-Rosales, Carolina Tafalla

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Highlights

Fish B cells expand the ER in response to different stimuli in different tissues

Mitochondria content did not vary significantly in activated fish B cells

Transcription of mitochondrial genes decreases during fish B cell differentiation

ER monitorization allows us to differentiate fish naive B cells from plasmablasts

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Endoplasmic reticulum expansion throughout the differentiation of teleost B cells to plasmablasts

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SUMMARY

The differentiation of B cells into antibody-secreting cells is fundamental for the generation of humoral immunity. In mammals, this process involves a series of metabolic and intracellular changes, not studied to date in teleost fish, where a clear distinction between naive B cells and plasmablasts/plasma cells (PCs) is still missing. Thus, in the current study, we have established that upon activation, teleost B cells undergo an expansion of the endoplasmic reticulum (ER) but experience no significant changes in mitochondria content. In parallel, the transcription of genes implicated in B cell differentiation increases, while that of mitochondrial genes decreases. In this context, ER monitoring has allowed us to distinguish between small cells with low amounts of ER ($FSC^{lo}ER^{lo}$ B cells), that correspond to undifferentiated cells, and large cells with expanded ER ($FSC^{hi}ER^{hi}$ B cells), characterized as plasmablasts. The results shed new light on the B cell differentiation process in teleosts and provide us with novel tools to study B cell function in these species.

INTRODUCTION

During the early phases of the humoral immune response, upon antigen recognition, mammalian B cells proliferate in the primary centers of clonal expansion located in specific sites within secondary immune organs, mainly the spleen and lymph nodes. Some of these proliferating B cells have the capacity to differentiate to plasmablasts. These cells, developed extrafollicularly, are usually short lived and secrete antibodies (Abs) of low affinity (usually immunoglobulin M [IgM]), important for pathogen clearance in the early stages of infection. In contrast, other B cells form germinal centers (GCs) in the B cell follicles of these secondary lymphoid tissues, around follicular dendritic cells (DCs). In the GCs, B cells receive help from T cells and undergo somatic hypermutation (SHM) and class switch recombination (CSR), eventually differentiating into long-lived memory B cells or plasma cells (PCs) responsible for the secretion of high-affinity (generally switched) Abs.[1,2] Thus, plasmablasts are short-lived cells that have a high Ab-secreting capacity, while still preserving their dividing capacity and many characteristics of activated B cells. For instance, plasmablasts express immunoglobulins (Igs) and major histocompatibility complex class II (MHC II) molecules on their surface, thereby maintaining the capacity to capture and present antigens to T cells.[3] PCs, on the other hand, also secrete large amounts of Abs but express very low levels of surface Igs and lack MHC II molecules on the plasma membrane, thereby not interacting with T cells anymore.[1] While plasmablasts retain the capacity to proliferate, PCs are considered to be postmitotic and usually migrate to survival niches in the bone marrow where they can persist for more than 20 years.[4]

During this differentiation process, B cells undergo lineage-dependent metabolic reprogramming as the metabolic requirements of B cells vary during the different stages of cell differentiation.[5] These differentiation programs are regulated in last term by the coordinated expression and repression of multiple transcription factors. Thus, transcription factors such as Pax5, Bcl-6, and Bach2 are expressed in mature B cells, suppressing PC differentiation.[6] On the other hand, the transcriptional programs induced by factors such as Blimp1 (encoded by *prdm1*), IRF4, or XBP1 repress B cell-related transcription factors and stimulate differentiation into PCs.[6,7] Interestingly, a strong association between mitochondrial function and the fate of individual B cells has been revealed.[8] Thus, the mitochondrial status stochastically generated during the

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differentiation process predicts the direction of the committed cells toward either CSR (B cells with increased mitochondrial mass and membrane potential) or PC differentiation (cells with decreased mitochondrial mass and potential).[8] Hence, the transcription factor Blimp1 has been shown to reduce mitochondrial mass in B cells, resulting in lower levels of mitochondrial reactive oxygen species (mROS), which antagonize CSR and promote the commitment to the PC lineage. Conversely, CSR occurs specifically in activated B cells with increased mitochondrial mass and membrane potential, which augment mROS, thereby maintaining Bach2 function.[8] In addition, in mammals, it is well known that, to accommodate the biosynthesis and assembly of very large amounts of Abs, both plasmablasts and PCs dramatically expand the secretory pathway to form a highly developed endomembrane transport network. Hence, both plasmablasts and PCs enlarge their cytoplasm increasing the number of layers and size of their endoplasmic reticulum (ER) as well as the Golgi apparatus.[9]

Teleost fish contain B cells along with other elements of the adaptive immune system. Nevertheless, the many structural and functional differences when compared to mammals affect B cell responses in these species. Fish, for example, lack lymph nodes and bone marrow, having the spleen as the main secondary immune organ and the kidney (specially the most anterior section) as the main hematopoietic tissue. Besides, fish B cells do not form conventional GCs. The distribution of fish B and T cells is more diffusely organized both in the spleen and in mucosal surfaces compared to mammals.[10] Because only three Ig classes (IgM, IgD, and IgT) have been described in fish and taking into account that IgT^+ cells constitute an independent B cell linage, no CSR has ever been reported in any species. Finally, recent studies have reported many phenotypical and functional similarities between teleost IgM^+ B cells and mammalian innate B1 cells, responsible for the production of low affinity Abs to antigens detected by innate receptors in the absence of T cell help.[11] These include, for example, the expression of several surface markers, an extended survival in cell culture, a high phagocytic capacity, or a lack of responsiveness to cross-linking of the B cell receptor (BCR).[12,13] In this context, it seems adequate to suggest that teleost B cell responses are analogous to mammalian extrafollicular IgM responses.

Thus far, it has not been easy to discriminate B cell subsets or B cells in different stages of differentiation in teleost fish due to the lack of antibodies against surface marker proteins. It should be noted that the patterns of expression of markers usually employed to discern mammalian B cell subsets are often quite different in fish.[14] Nevertheless, some attempts to differentiate among teleost B cell subsets have been undertaken in the past years. Thus, the expression levels of intracellular transcription factors (Pax5 and EBF1) together with those of surface IgM were used to define diverse B cell stages in the rainbow trout (*Oncorhynchus mykiss*) kidney.[15] In some species such as catfish (*Ictalurus punctatus*) or rainbow trout, the levels of surface IgM and IgD have also been used to define different subsets of B cells.[16,17] In rainbow trout, the loss of surface IgD has been associated with a more differentiated profile of B cells,[18] as occurs in mammals.[19] Very recently, surface expression of CD38 has also been linked to B cell differentiation in rainbow trout.[20] Nevertheless, there are still many aspects of B cell responses that are still not well understood in teleost fish, studies for which the generation of additional immunological tools is essential.

In this context, in the current study, we have studied the ER and mitochondria content during the differentiation of teleost B cells, using the rainbow trout as a model. Although the ER clearly expanded upon activation, no significant changes were found in mitochondria content when compared to unstimulated cells. Nonetheless, the transcription of mitochondrial genes significantly decreased in B cells upon activation. Taking advantage of these changes, we have used the monitoring of ER expansion as a tool to differentiate naive B cells from plasmablasts in fish. Thus, while small IgM^+ cells with low amounts of ER ($FSC^{lo}ER^{lo}$ IgM^+ B cells) correspond to nonactivated B cells, large IgM^+ cells with an expanded ER ($FSC^{hi}ER^{hi}$ IgM^+ cells) correspond to differentiated plasmablasts as confirmed by transmission electron microscopy (TEM), transcriptional analysis, and determination of their secretory capacity. This protocol was used to study the expansion of the differentiated population of $FSC^{hi}ER^{hi}$ IgM^+ cells in response to different stimuli, in different tissues and upon an *in vivo* stimulation. The findings offer important insights into the B cell differentiation process in teleosts and provide us with new tools to study B cell responses in these species.

RESULTS

LPS-stimulated IgM^+ B cells undergo an expansion of the ER

As an initial step, we decided to evaluate if lipopolysaccharide (LPS) stimulation affected the amount of ER and mitochondria of trout blood IgM^+ B cells by flow cytometry. LPS is a potent B cell mitogen that in

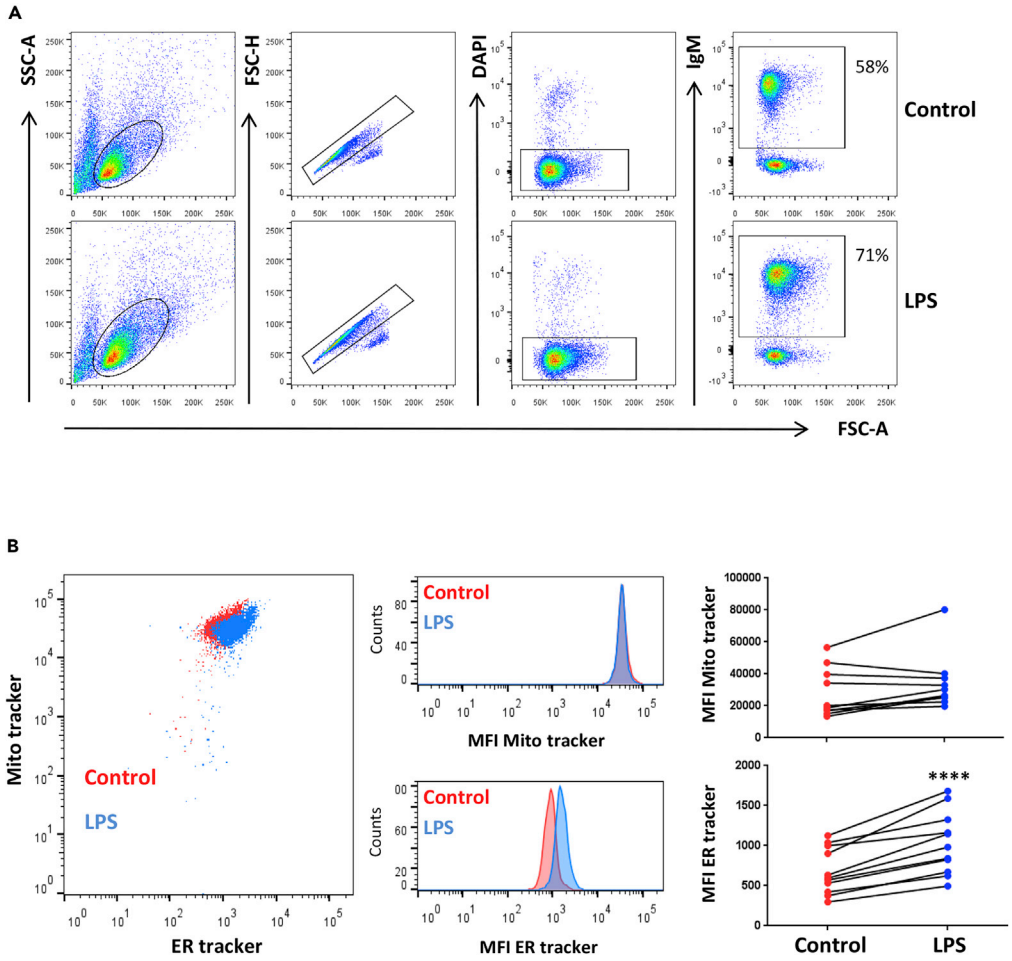

Figure 1. Mitochondria and ER tracking after LPS stimulation of rainbow trout B cells

Rainbow trout blood leukocytes were incubated with LPS (100 μg/mL) or media alone during 3 days at 20°C.

(A) Dot plots show the gating strategy for the analysis of IgM$^+$ B cells in stimulated (LPS) or non-stimulated cultures (Control). The percentage of live IgM$^+$ B cells evaluated by flow cytometry is shown.

(B) Representative dot plot showing Mitotracker and ER tracker staining on IgM$^+$ B cells from control (red) and LPS-treated (blue) cultures. Representative histograms and graphs show mean fluorescence intensity (MFI) of Mitotracker (upper panels) and ER tracker (lower panels) on IgM$^+$ B cells from control (red) and LPS-treated (blue) cultures (n = 11). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences between LPS-treated and control cultures (****$p \leq 0.001$).

rainbow trout has been shown to increase the survival, IgM secretion, and MHC II surface expression of IgM$^+$ B cells and to promote their proliferation.[21] Thus, to study whether ER or mitochondria content of B cells were affected by LPS stimulation, we incubated isolated peripheral blood leukocytes (PBLs) with or without 100 μg/mL of LPS for 3 days in standard culture conditions and then used MitoTracker and ER Tracker to quantify the amount of mitochondria and ER in IgM$^+$ B cells by flow cytometry. This time point was selected after conducting a preliminary time course experiment in which we established that this was the point where LPS stimulatory effects were the highest (Figure S1).

As described before,[21] a significant increase in the percentage of IgM$^+$ B cells was visible when PBLs were exposed to LPS (Figure 1A). In addition, a significant expansion of the ER in IgM$^+$ B cells stimulated with LPS compared to control cells was evident as the mean fluorescence intensity (MFI) of the ER signal significantly increased in the presence of LPS (Figure 1B). In contrast, no significant changes were detected in the mitochondria mass between LPS-stimulated and control IgM$^+$ B cells (Figure 1B). No differences were evident in flow cytometry, nor when cells were stained with MitoTracker and visualized under a confocal microscope (Figure S2).

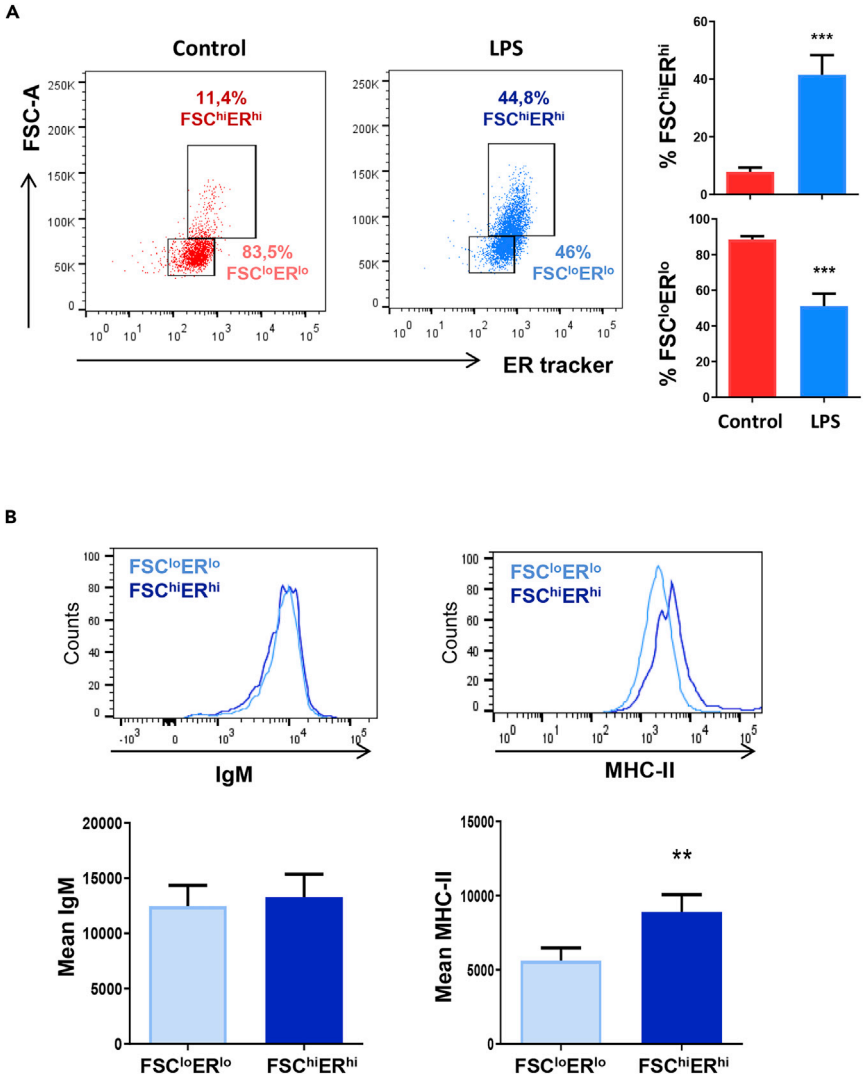

Figure 2. Expansion and characterization of blood FSC^{hi}ER^{hi} B cell populations in response to LPS

Rainbow trout blood leukocytes were incubated with LPS (100 μg/mL) or media alone during 3 days at 20°C.

(A) Representative dot plots show percentage of FSC^{hi}ER^{hi} and FSC^{lo}ER^{lo} IgM^{+} B cells in non-stimulated (Control, red) and LPS-stimulated (LPS, blue) cultures. Graphs show mean percentages +SD (n = 11). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences between the percentages obtained in control and LPS-treated cultures (***p ≤ 0.005).

(B) Representative histograms showing IgM and MHC II expression levels on FSC^{hi}ER^{hi} (dark blue) and FSC^{lo}ER^{lo} (light blue) IgM^{+} B cells from LPS-treated cultures obtained from a representative fish are shown together with a quantification of IgM and MHC II MFI values, shown as mean +SEM (n = 11). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences in MFI values between FSC^{hi}ER^{hi} and FSC^{lo}ER^{lo} IgM^{+} B cells (**p ≤ 0.01).

LPS stimulation expands an IgM^{+} B cell subpopulation characterized by increased size and ER

Having established that LPS provokes a global expansion of the ER in stimulated cells, we focused our efforts on optimizing a method based on this that would help us differentiate between cells that have been in fact activated and those that have not despite the presence of the stimulus. For this, we combined the quantification of ER through the ER tracker with a measurement of the size (forward scatter, FSC) of the cells as cellular enlargement is another well-defined phenotypical trait of B cell differentiated stages (plasmablasts/PCs) both in mammals and fish.[18,22] Taking these two parameters into account, we could classify IgM^{+} B cells in FSC^{lo}ER^{lo} B cells (small cells with low amounts of ER) and FSC^{hi}ER^{hi} B cells

(large cells with high amounts of ER) (Figure 2A). Upon stimulation with LPS, the percentage of $FSC^{hi}ER^{hi}$ B cells significantly increased, whereas that of $FSC^{lo}ER^{lo}$ B cells decreased (Figure 2A).

To further characterize these $FSC^{lo}ER^{lo}$ and the $FSC^{hi}ER^{hi}$ B cell subpopulations, we compared their levels of IgM and MHC II surface expression in LPS-stimulated PBLs. Interestingly, the $FSC^{hi}ER^{hi}$ subset had significantly higher MHC II surface levels than the $FSC^{lo}ER^{lo}$ subpopulation, whereas no changes were observed in the levels of surface IgM between both populations (Figure 2B). In concordance, the capacity to process antigens was also higher in the $FSC^{hi}ER^{hi}$ B cell subset when compared to that of the $FSC^{lo}ER^{lo}$ subpopulations (either stimulated or unstimulated) (Figure S3). When the pattern of MHC II and IgM surface expression was studied in $FSC^{lo}ER^{lo}$ and $FSC^{hi}ER^{hi}$ B cells from non-stimulated PBL cultures, we found that in this case $FSC^{hi}ER^{hi}$ B cells not only had higher levels of surface MHC II expression but also had higher levels of surface IgM (Figure S4).

CD40L, IL-10, and IL-2B stimulation also expands the $FSC^{hi}ER^{hi}$ IgM^+ B cell subset

In rainbow trout, the combination of CD40L, IL-10, and IL-2B has been identified as a highly stimulatory signal to promote the survival of IgM^+ B cells and their differentiation.[23] Thus, we decided to use this combination of stimuli to assess if the $FSC^{hi}ER^{hi}$ IgM^+ B cell subset was expanded in response to stimulations different from LPS. When PBLs were stimulated with recombinant rainbow trout CD40L, IL-10, and IL-2B, we found a significant increase in the percentage of $FSC^{hi}ER^{hi}$ IgM^+ B cells accompanied by a significant decrease in the percentage of the $FSC^{lo}ER^{lo}$ IgM^+ B cells in comparison with those found in unstimulated cultures (Figure 3A). As occurred in LPS-stimulated cultures, the $FSC^{hi}ER^{hi}$ IgM^+subset had significantly higher MHC II surface levels than the $FSC^{lo}ER^{lo}$ IgM^+ subpopulation, whereas no changes were observed in the levels of surface IgM between both populations (Figure 3B).

LPS expands the $FSC^{hi}ER^{hi}$ B cell population from kidney and spleen leukocytes

Next, we wanted to confirm that the expansion of $FSC^{hi}ER^{hi}$ IgM^+ B cells observed after the stimulation of leukocytes is not exclusive of PBLs and takes place in other lymphoid tissues such as kidney and spleen. Similar to what occurred with PBLs, in response to LPS, a significant increase in the percentage of the $FSC^{hi}ER^{hi}$ IgM^+ subpopulation along with a significant reduction in the percentage of $FSC^{lo}ER^{lo}$ IgM^+ B cells was found in comparison to control cultures in kidney (Figure 4A) and spleen (Figure 4B) leukocyte cultures. These results point to a general expansion of the ER in activated B cell populations regardless of their origin.

LPS-stimulated $FSC^{hi}ER^{hi}$ B cells possess phenotypical and functional plasmablast characteristics

Once established that stimulation of trout B cells from different sources or by means of different stimuli significantly expands the population of $FSC^{hi}ER^{hi}$ IgM^+ B cells, we wanted to unequivocally establish that these cells in fact correspond to differentiated B cells. To this end, we first analyzed the morphological and ultrastructural features of $FSC^{hi}ER^{hi}$ IgM^+ B cells from LPS-stimulated cultures by TEM and compared them to those of $FSC^{lo}ER^{lo}$ IgM^+ B cells from LPS-stimulated cultures, as well as to those of $FSC^{lo}ER^{lo}$ B cells from unstimulated cultures. We did not include $FSC^{hi}ER^{hi}$ B cells from unstimulated cultures in these studies because this population was variable from fish to fish and often very small. Unstimulated $FSC^{lo}ER^{lo}$ IgM^+ B cells exhibited a characteristic round morphology with small cytoplasmic regions and abundant microvilli-like protrusions at the cell surface (Figure 5A). In contrast, LPS-activated $FSC^{lo}ER^{lo}$ IgM^+ B cells (Figure 5B), and especially LPS-treated $FSC^{hi}ER^{hi}$ IgM^+ B cells (Figures 5B and 5C), showed increased size and less spherical shapes. In addition, both LPS-treated B cell subsets displayed an eccentric nucleus surrounded by large cytoplasmic areas and large, rounded plasma membrane protrusions. Interestingly, quantitative TEM analysis showed a significant increase in the percentage of cytoplasmic area of LPS-treated $FSC^{hi}ER^{hi}$ IgM^+ B cells (57.4%, SD = 7.7) when compared to LPS-treated (46.3%, SD = 8.2) and non-treated (41.4%, SD = 8.4) $FSC^{lo}ER^{lo}$ IgM^+ B cells (Figure 5B). This indicates that the enlarged cytoplasm observed in LPS-activated $FSC^{hi}ER^{hi}$ IgM^+ B cells is not just a mere consequence of increased cell size. Finally, when LPS-treated $FSC^{hi}ER^{hi}$ IgM^+ B cells were examined at higher magnification, their cytoplasmic areas showed abundant rough ER (Figures 5D and 5E) and a prominent Golgi complex (Figure 5F), typical phenotypic traits of differentiated B cell stages. Although mitochondria were also clearly visualized in the cytoplasm of LPS-treated $FSC^{hi}ER^{hi}$ IgM^+ B cells, in close relation to the ER (Figures 5D–5F), an increase in mitochondrial content was not evident through this technique either.

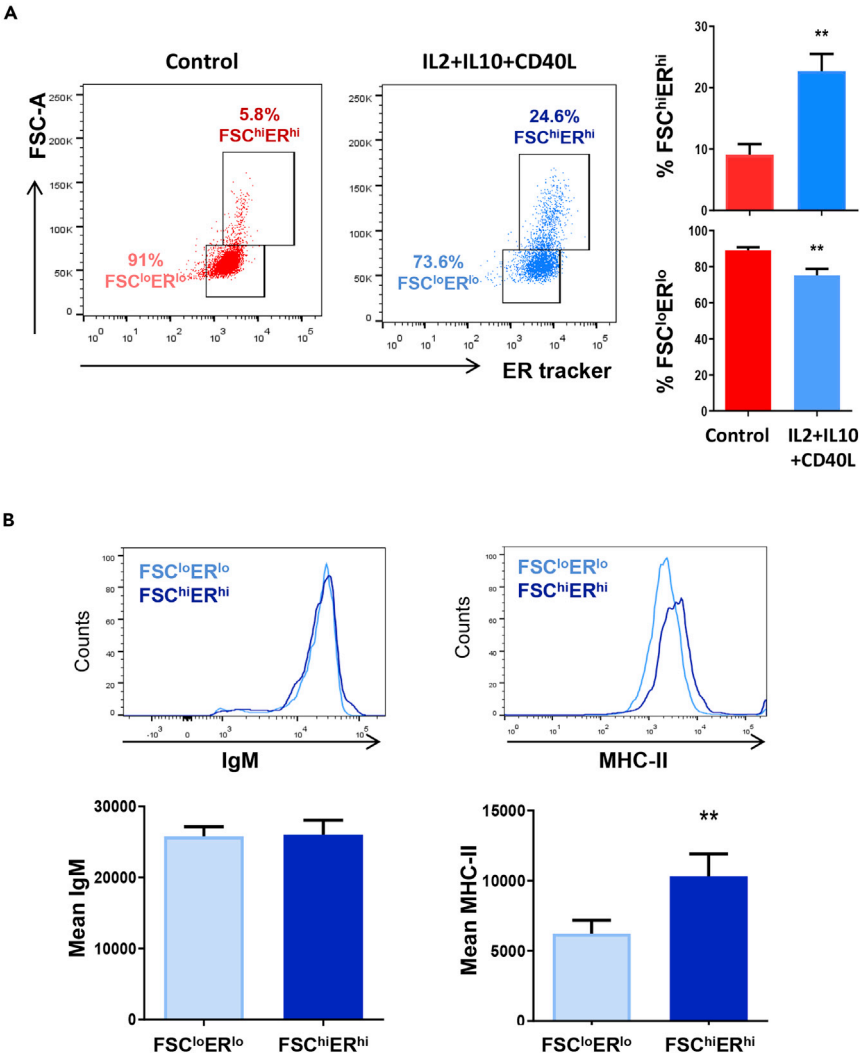

Figure 3. Expansion and characterization of blood FSC^{hi}ER^{hi} B cell populations in response to a combination of CD40L, IL-2B, and IL-10

Rainbow trout blood leukocytes were incubated with IL-2B (200 ng/mL) together with IL-10 (200 ng/mL) and CD40L (5 μg/mL) or media alone during 3 days at 20°C.

(A) Representative dot plots show percentage of FSC^{hi}ER^{hi} and FSC^{lo}ER^{lo} IgM$^+$ B cells in non-stimulated (Control, red) or stimulated (IL-2B + IL-10 + CD40L, blue) cultures. Graphs show mean percentages +SD (n = 11). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences between the percentages obtained in control and LPS-treated cultures (**p ≤ 0.01).

(B) Representative histograms showing IgM and MHC-II expression levels on FSC^{hi}ER^{hi} (dark blue) and FSC^{lo}ER^{lo} (light blue) IgM$^+$ B cells from stimulated cultures obtained from a representative fish are shown together with a quantification of IgM and MHC-II MFI values, shown as mean +SEM (n = 9). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences in MFI values between FSC^{hi}ER^{hi} and FSC^{lo}ER^{lo} IgM$^+$ B cells (**p ≤ 0.01).

Another trait of plasmablasts/PCs is their higher IgM-secreting capacity. Thus, we sorted FSC^{hi}ER^{hi} IgM$^+$ B cells from LPS-stimulated cultures as well as FSC^{lo}ER^{lo} IgM$^+$ B cells from LPS-stimulated cultures and FSC^{lo}ER^{lo} IgM$^+$ B cells from unstimulated cultures, incubated them for 48 h, and analyzed the IgM content on the supernatants by ELISA. Our results demonstrated the higher capacity of FSC^{hi}ER^{hi} IgM$^+$ B cells (LPS-stimulated) to secrete IgM in comparison to FSC^{lo}ER^{lo} IgM$^+$ B cells from either LPS-stimulated or non-stimulated cultures (Figure 5H).

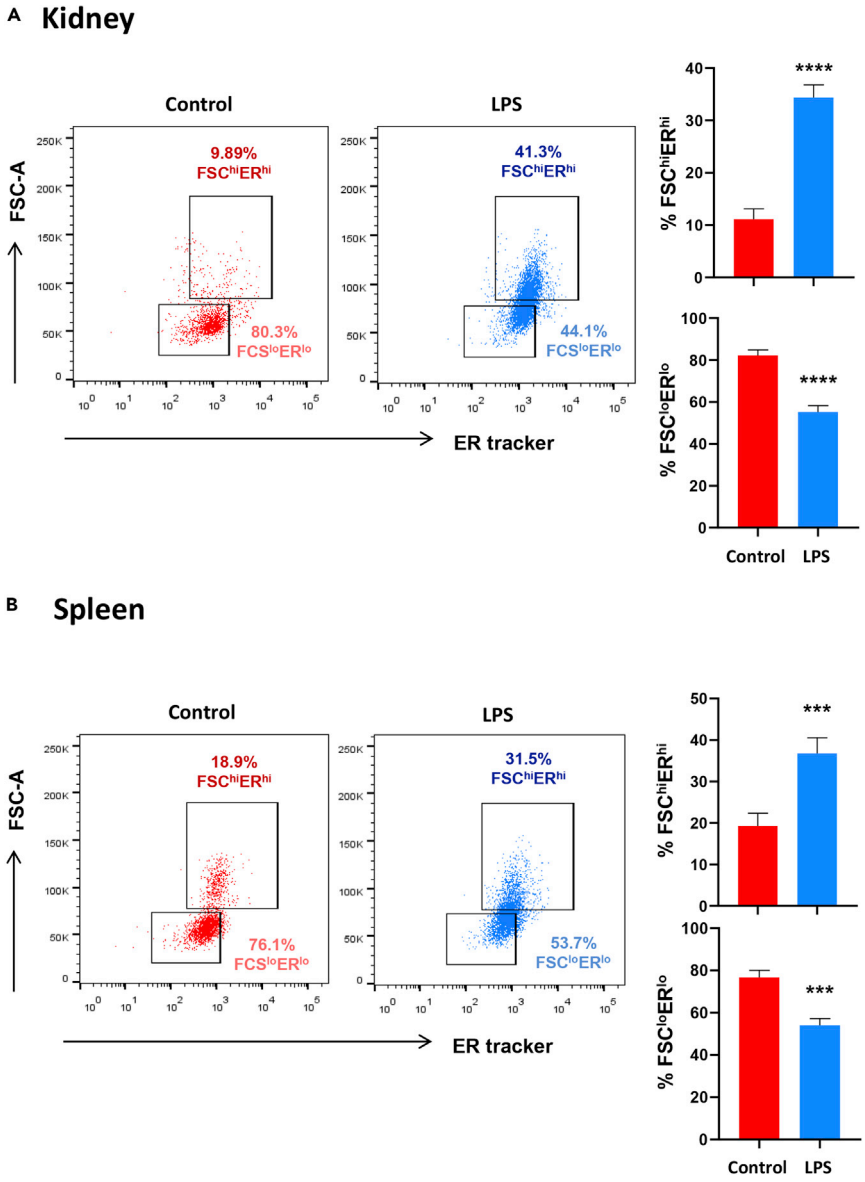

Figure 4. Expansion and characterization of kidney and spleen FSC^{hi}ER^{hi} B cell populations in response to LPS

(A and B)Rainbow trout kidney and spleen leukocytes were incubated with LPS (100 μg/mL) or media alone during 3 days at 20°C. Representative dot plots show percentage of FSC^{hi}ER^{hi} and FSC^{lo}ER^{lo} IgM^{+} B cells in non-stimulated (Control, red) or stimulated (LPS, blue) kidney (A) or spleen (B) cultures. Graphs show mean +SD (n = 11). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences in stimulated cultures compared to controls, where **p ≤ 0.01, ***p ≤ 0.005 and ****p ≤ 0.001.

In addition, we evaluated the levels of transcription of several differentiation B cell markers on the three sorted subpopulations (control FSC^{lo}ER^{lo} IgM^{+} B cells and LPS-stimulated FSC^{lo}ER^{lo} IgM^{+} and FSC^{hi}ER^{hi} IgM^{+} B cells) by real-time PCR. We detected a significant increase in the mRNA levels of *prdm1a-2*, *irf4*, *bcma*, secreted *igmh*, and *il1b* in the FSC^{hi}ER^{hi} IgM^{+} subpopulation in comparison with both the control and LPS-stimulated FSC^{lo}ER^{lo} IgM^{+} subpopulations (Figure 6). There was also a significant downregulation in the transcription of membrane *igdh* and *pax5* in our FSC^{hi}ER^{hi} IgM^{+} population compared to the other two (Figure 6). All these transcriptional changes point to the acquisition of a plasmablast/PC phenotype by the FSC^{hi}ER^{hi} IgM^{+} subpopulation. Thus, we have demonstrated by different means (TEM, ELISA, and real-time PCR) that the FSC^{hi}ER^{hi} IgM^{+} subpopulation that expands in response to stimulation corresponds to B cells with a differentiated antibody secreting cell (ASC) profile.

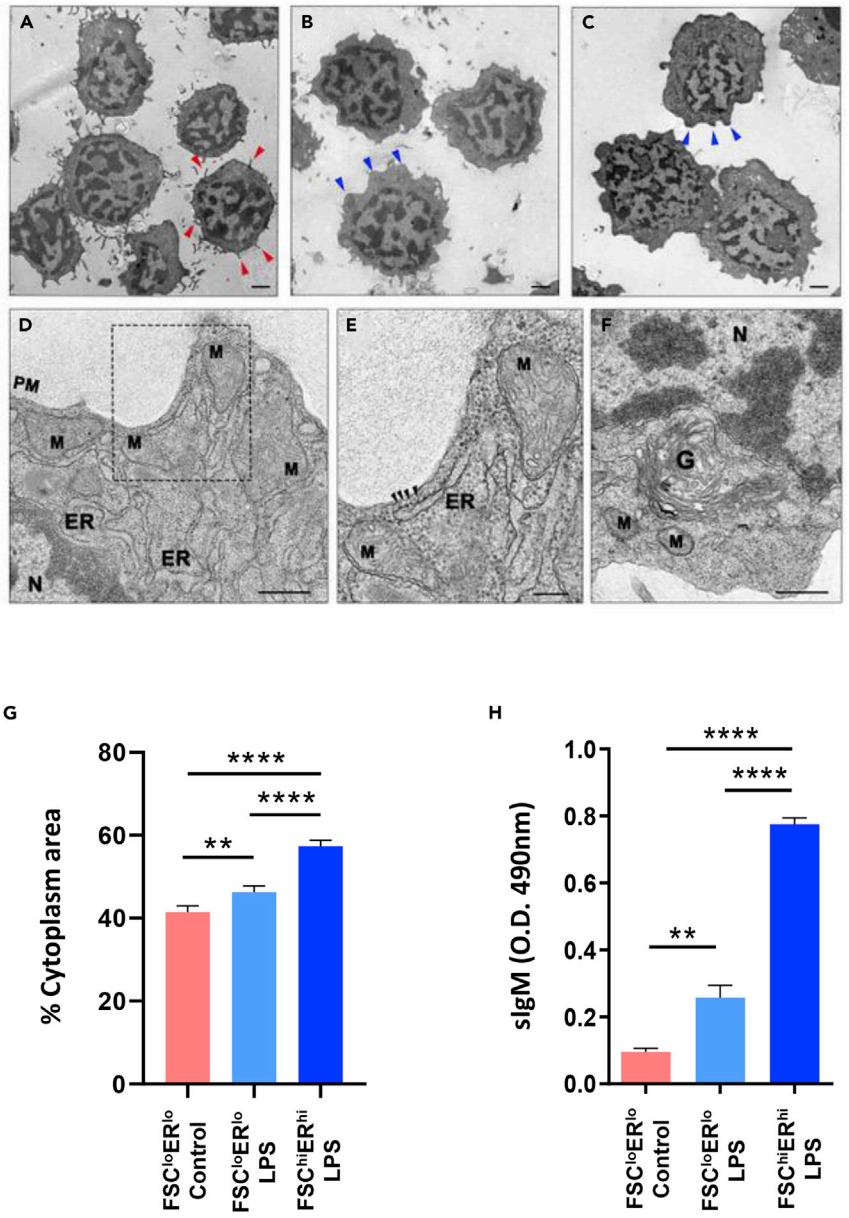

Figure 5. Rainbow trout FSC^{hi}ER^{hi} B cells have a plasmablast phenotype and a higher IgM-secreting capacity

Blood rainbow trout leukocytes were incubated in the presence or absence of LPS (100 μg/mL) for 3 days at 20°C. Thereafter, control and treated FSC^{lo}ER^{lo} and treated FSC^{hi}ER^{hi} IgM^{+} B cells were sorted.

(A–G) TEM of control FSC^{lo}ER^{lo} (A) and LPS-treated FSC^{lo}ER^{lo} (B) or FSC^{hi}ER^{hi} (C-F) IgM^{+} B cells. Note that control FSC^{lo}ER^{lo} IgM^{+} cells (A) look smaller and more spherical than LPS-treated FSC^{lo}ER^{lo} (B) and FSC^{hi}ER^{hi} (C) IgM^{+} B cells. Also, control cells display abundant microvilli-like structures (red arrowheads in A) whereas LPS-treated cells display rounded membrane protrusions (blue arrowheads in B and C) at the cell surface. (D-F) Cytoplasmic details of LPS-treated FSC^{hi}ER^{hi} IgM^{+} B cells showing abundant endoplasmic reticulum (ER), pleomorphic mitochondria (M), and a prominent Golgi (Go) complex. (E) Enlarged detail of the area delimited in panel d showing ER membranes decorated with abundant ribosomes (arrowheads). Bars, 1,000 nm (A-C), 500 nm (D-F) and 200 nm (E). (G) Quantitative TEM analysis. Graph shows mean percentage of cytoplasmic area +SEM in the three different sorted subpopulations (30 different cell profiles per condition were analyzed at 1,200X magnification). An unpaired Student's *t*-test was used to compare the mean percentage of the cytoplasmic areas between the different cell types (**$p \leq 0.01$ and ****$p \leq 0.001$).

Figure 5. *Continued*

(H) Sorted control FSC^{lo}ER^{lo} and LPS-treated FSC^{lo}ER^{lo} and FSC^{hi}ER^{hi} IgM^{+} B cells were cultured for 48 h at 20°C. After that time, supernatants were collected, and the amount of secreted IgM was determined by ELISA. Graph shows mean absorbance values at 405 nm + SEM obtained in supernatants from different sorted subpopulations (n = 7). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences between different conditions, where **$p \leq 0.01$ and ****$p \leq 0.001$.

LPS stimulation represses the transcription of mitochondrial genes in B cells

We also analyzed the levels of transcription of four mitochondrial genes on the three sorted subpopulations mentioned above (control FSC^{lo}ER^{lo} IgM^{+} B cells and LPS-stimulated FSC^{lo}ER^{lo} IgM^{+} and FSC^{hi}ER^{hi} IgM^{+} B cells). We found a significant down-regulation in the transcription of *cox2*, *r16s*, and *pink1* in both LPS-stimulated subpopulations, FSC^{lo}ER^{lo} IgM^{+} and FSC^{hi}ER^{hi} IgM^{+} B cells, compared to control FSC^{lo}ER^{lo} IgM^{+} B cells (Figure 7). In the case of *cox1*, its transcription levels were significantly lower in LPS-stimulated FSC^{hi}ER^{hi} IgM^{+} B cells than in control FSC^{lo}ER^{lo} IgM^{+} B cells (Figure 7).

Flow cytometry analysis of the FSC^{hi}ER^{hi} subpopulation in TNP-LPS-immunized fish

Finally, we wanted to establish whether we could use ER expansion and size to study B cell responses after an *in vivo* stimulation. For this, we immunized trout with 2,4,6-Trinitrophenyl hapten conjugated to lipopolysaccharide (TNP-LPS) intraperitoneally and analyzed the percentage of FSC^{hi}ER^{hi} IgM^{+} B cells in different organs (blood, spleen, kidney, and peritoneum) at day 7 after immunization. A significant increase in the percentage of FSC^{hi}ER^{hi} IgM^{+} B cells was observed in the spleen and peritoneum of immunized fish compared to that of controls (Figure 8). In contrast, in the kidney, a significant decrease in the percentage of FSC^{hi}ER^{hi} IgM^{+} B cells was detected in response to TNP-LPS (Figure 8).

DISCUSSION

In mammals, it is well known that upon differentiation, B cells adapt their morphology and reprogram their metabolism to adjust to the novel functionalities.[24] Thus, resting mature conventional B cells do not secrete Abs and possess a small cytoplasm, with little ER cisternae. Upon antigen encounter or stimulation with mitogens such as LPS, B cells proliferate and differentiate into ASCs that require the reorganization of all their cellular machinery to secrete high amounts of Abs (up to thousands per second). The ER plays a crucial role in this cellular secretory pathway as it is responsible for protein folding, disulfide bond formation, and protein oligomerization.[25] Thus, for instance, when the mouse CH12 B cell line is stimulated with LPS, an expansion of the ER and the Golgi complex takes place. In this process, the area, volume, and density of membrane-bound ribosomes are significantly increased in the rough endoplasmic reticulum (RER). In addition, the area and volume of the Golgi apparatus are also augmented, thus enabling the CH12 cell line to increase its Ab secretory activity.[26] Taking into account that teleost IgM^{+} B cells share many functional and phenotypical traits of mammalian B1 cells that secrete natural IgMs in homeostasis,[12,13] it could have been possible that this ER expansion was not as noticeable in teleosts as in mammals. However, we have confirmed by both flow cytometry and TEM that this expansion is also significant for fish IgM^{+} B cells.

In contrast, no changes in the mitochondrial load were found between LPS-stimulated and control IgM^{+} B cells. Although the TEM analysis evidenced the presence of mitochondria in close association with the ER, a significant increase in the number of mitochondria in FSC^{hi}ER^{hi} IgM^{+} B cells in comparison to FSC^{lo}ER^{lo} IgM^{+} B cells was not clear through this technique, nor through the use of MitoTracker in flow cytometry or through confocal microscopy (comparing non-stimulated and stimulated populations). Interestingly, as previously mentioned, in mammals, the mitochondrial mass has been shown to condition the B cell destiny. Thus, while activated B cells with more mitochondria and membrane potential increase mROS and are more prone to experience CSR, B cells with less mitochondrial load and potential and lower mROS are predisposed to suffer PC differentiation along with an ER expansion.[8] Furthermore, Blimp1 has been shown to reduce the mitochondrial mass and thereby reduce mROS, thus promoting PC differentiation.[8] Thus, the fact that fish IgM^{+} B cells do not undergo CSR upon activation but instead differentiate to plasmablasts through the activation of several Blimp1 homologs could explain why mitochondria mass is not consistently increased in rainbow trout B cells in response to LPS. In addition, it should be taken into account that FSC^{hi}ER^{hi} IgM^{+} B cells were shown to express higher levels of surface MHC II and a greater antigen-processing capacity than nonactivated cells, strongly suggesting that, in fish, plasmablasts maintain functions of naive mature B cells such as the capacity to present antigens. On the other hand, the transcriptional analysis performed revealed that LPS significantly downregulated the expression of different mitochondrial genes, such as *cox2*, *r16s* and *pink1* (in both FSC^{lo}ER^{lo} IgM^{+} B cells and

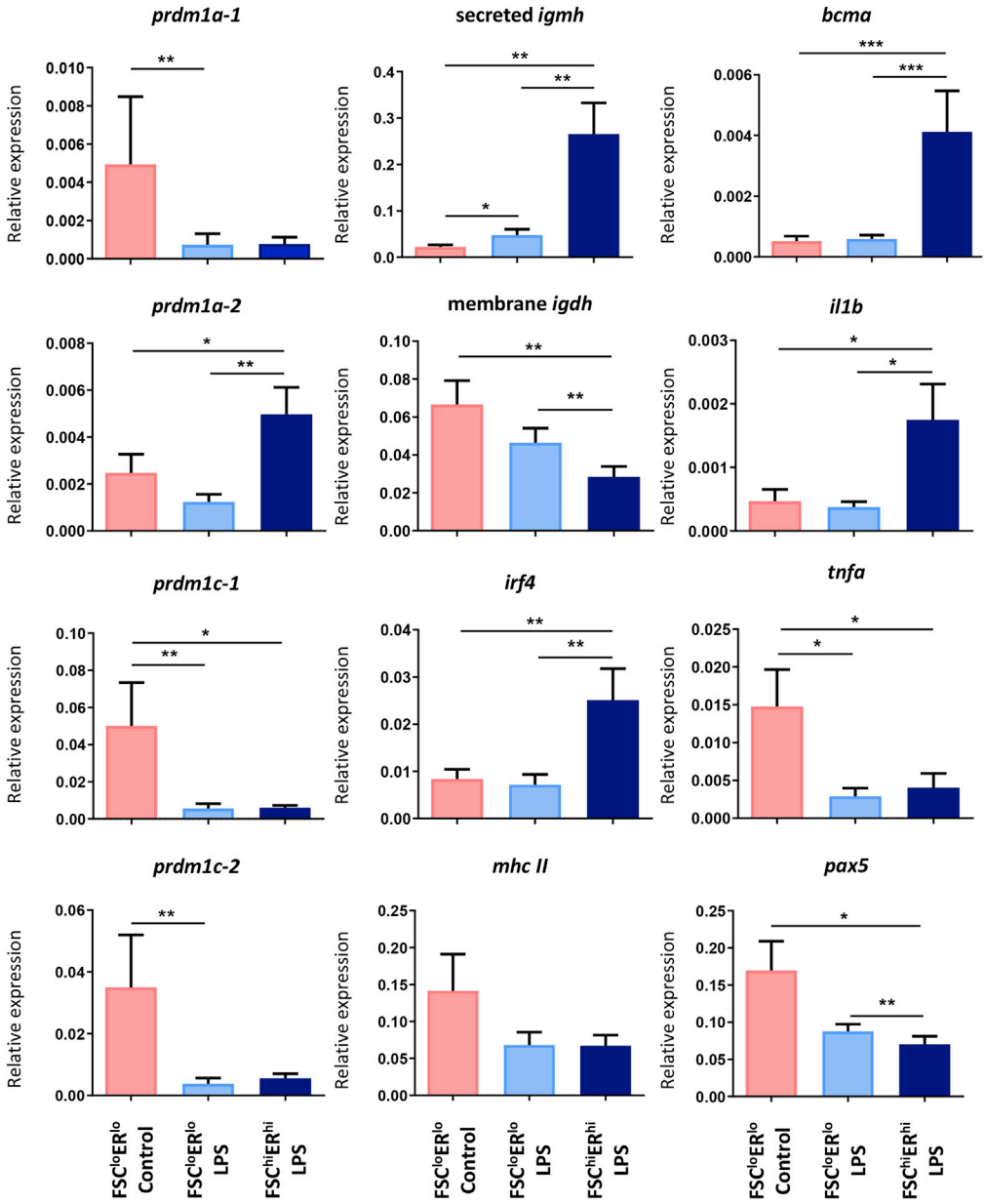

Figure 6. Transcriptional analysis of sorted rainbow trout FSC^{lo}ER^{lo} and FSC^{hi}ER^{hi} B cells

Blood rainbow trout leukocytes were incubated in the presence or absence of LPS (100 μg/mL) for 3 days at 20°C. After that time, control FSC^{lo}ER^{lo} and LPS-treated FSC^{lo}ER^{lo} or FSC^{hi}ER^{hi} IgM^{+} B cells were sorted for real-time PCR analysis. Graphs show levels of transcription of *prdm1a-1*, *prdm1a-2*, *prdm1c-1*, *prdm1c-2*, secreted *igmh*, membrane *igdh*, *irf4*, *mhcII*, *bcma*, *il1b*, *tnfa*, and *pax5* in each sorted subpopulation. Results are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD (n = 10–13). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences among different IgM^{+} B cell subpopulations, where *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.005.

FSC^{hi}ER^{hi} IgM^{+} B cells), and *cox1* (only in FSC^{hi}ER^{hi} IgM^{+} B cells). Thus, it is possible that during the process of differentiation of these cells toward a mature stage, mitochondrial functions undergo significant changes that include the modulation of mitochondrial gene transcription, with no significant changes in mitochondrial quantity. Interestingly, a study performed in mice in which mitochondrial content was increased in B cells upon activation reported no changes in mitochondrial DNA transcription, suggesting that B cells generated more mitochondria without replicating their mitochondrial DNA.[24] Also in mammals, *cox2* and *pink1* mRNA induction

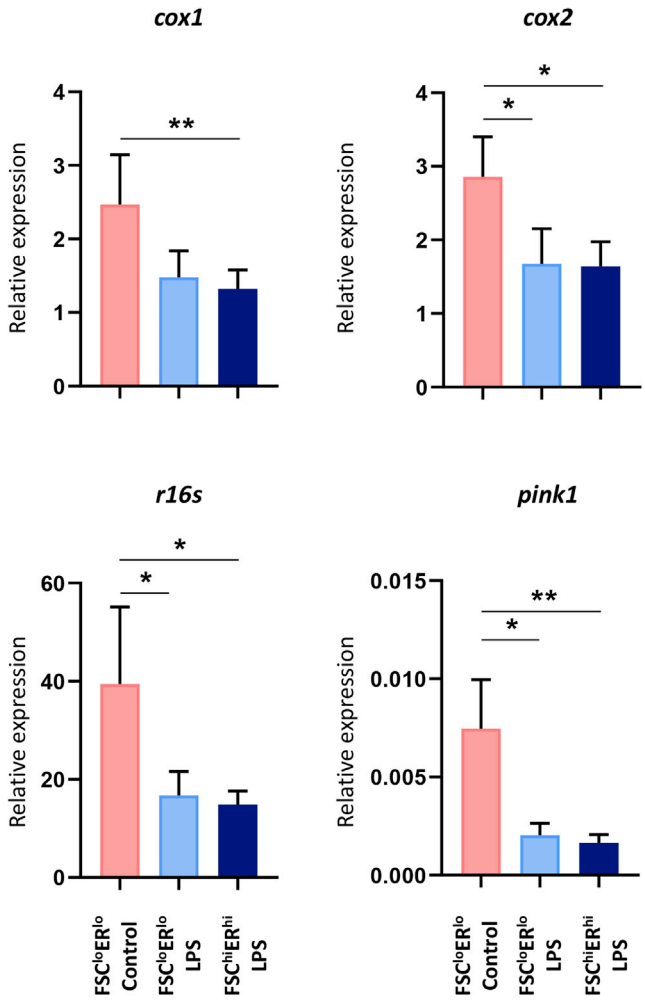

Figure 7. Transcriptional analysis of sorted rainbow trout $FSC^{lo}ER^{lo}$ and $FSC^{hi}ER^{hi}$ B cells

Blood rainbow trout leukocytes were incubated in the presence or absence of LPS (100 μg/mL) for 3 days at 20°C. After that time, control $FSC^{lo}ER^{lo}$ and LPS-treated $FSC^{lo}ER^{lo}$ or $FSC^{hi}ER^{hi}$ IgM^+ B cells were sorted for real-time PCR analysis. Graphs show levels of transcription of *cox1*, *cox2*, *r16s*, *and pink1* in each sorted subpopulation. Results are shown as the mean gene expression relative to the expression of an endogenous control (EF-1α) ± SD (n = 10–13). Statistical differences were evaluated by a paired two-tailed Student's *t*-test, and asterisks denote significant differences among different IgM^+ B cell subpopulations, where *$p \leq 0.05$ and **$p \leq 0.01$.

have been correlated with an increase in mROS production (Kiritoshi S et al 2003; Yu et al., 2021). Thus, at least in fish blood LPS-stimulated $FSC^{hi}ER^{hi}$ IgM^+ B cells, where the transcriptional expression of Blimp1 homolog (*prdm1a-2*) is significantly increased compared to control cells, a presumed lower mROS could be inhibiting the expression of *cox* and *pink1* genes. Nonetheless, the quantification by qPCR of 16s rRNA gene encoded by mitochondrial DNA provides us with an idea of mitochondrial DNA replication during B cell maturation.[27] Interestingly, in mammals, it has been shown that impaired mitochondrial DNA replication in activated B cells shifts metabolism to glycolysis;[27] therefore, it would be interesting to study in the future whether this metabolic shift takes place in activated fish B cells.

By studying ER expansion through flow cytometry, we have been able to differentiate rainbow trout B cells that are in fact activated in stimulated cultures ($FSC^{hi}ER^{hi}$ IgM^+ B cells) from those that are not ($FSC^{lo}ER^{lo}$ IgM^+ B cells). Targeting the response of this population offers an advantage in comparison to studying the response of all B cells in the culture regardless of their ER content or size, given that $FSC^{hi}ER^{hi}$ IgM^+ B cells from stimulated cultures secrete significantly higher amounts of IgM than $FSC^{lo}ER^{lo}$ IgM^+ B cells and have a more differentiated transcriptional profile. Thus, it seems that not all IgM^+ B cells in a stimulated culture respond equally to the stimulus,

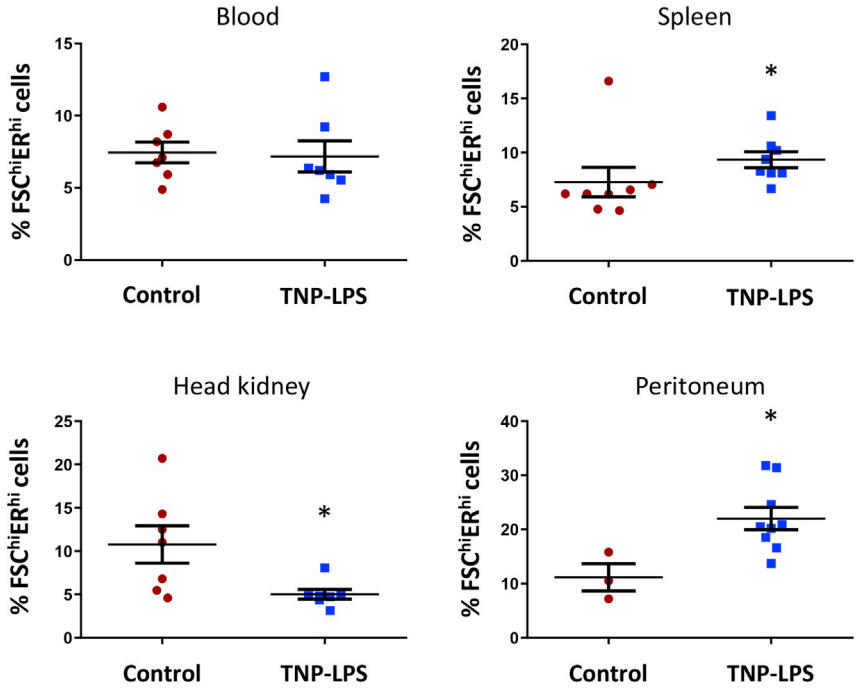

Figure 8. Analysis of FSC^{hi}ER^{hi} IgM^{+} B cells in blood, spleen, kidney, and peritoneum of rainbow trout immunized with TNP-LPS

Rainbow trout were intraperitoneally injected with 100 μL of saline solution (0.9% NaCl) containing 50 μg of TNP-LPS (TNP-LPS) or with the same volume of saline solution alone (Control). After 7 days, leukocytes from blood, spleen, kidney, and peritoneum were isolated for flow cytometry analysis. Graphs show percentages of FSC^{hi}ER^{hi} IgM^{+} B cells, shown as mean ± SD (n = 3–9). Statistical differences were evaluated by an unpaired two-tailed Student's *t*-test. Asterisks denote significant differences in the percentage of FSC^{hi}ER^{hi} IgM^{+} B cells between groups (*p ≤ 0.05).

and we have demonstrated that through the analysis of ER content we can identify those cells that are most responsive. Whether this implies the presence of two distinct B cell subsets with a differential response to stimulation requires further investigation. We have confirmed that rainbow trout FSC^{hi}ER^{hi} IgM^{+} B cells correspond in fact to cells that have started a commitment to plasmablasts/PCs through TEM analysis (that confirmed a plasmablast/PC morphology), transcriptional studies (that also revealed a pattern of gene expression consequent with a differentiated B cell), and ELISA (establishing the increased capacity to secrete IgM). Interestingly, these rainbow trout FSC^{hi}ER^{hi} plasmablasts were shown to have higher levels of surface MHC II expression than FSC^{lo}ER^{lo} IgM^{+} B cells and also a higher antigen-processing capacity. Although reduced surface MHC II expression was generally seen as a hallmark of B cell differentiation, mammalian studies later demonstrated that when PCs are differentiated in response to thymus-independent (TI) antigens such as LPS, MHC II expression is maintained.[3] Interestingly, the authors postulated that such PCs are not exclusively specialized in antibody secretion but retain roles in antigen presentation and pathogen clearance through a functional antigen-presenting machinery. In the current study, FSC^{hi}ER^{hi} plasmablasts differentiated in response to a combination of CD40L, IL-2B, and IL-10 also had significantly higher MHC II levels than inactivated cells, suggesting that in fish, the maintenance of surface MHC II during the differentiation of B cells is more common than what occurs in mammals. Nevertheless, there are some cases in which differentiation of rainbow trout B cells goes along with decreased MHC II levels. For example, previous studies from our group demonstrated that when rainbow trout splenic B cells are stimulated with IL-6, a reduction of MHC II levels is observed.[21] Thus, it would be interesting to investigate in future studies ER and mitochondria levels in these IL-6-stimulated B cells. Similarly, in the peritoneal cavity, a subpopulation of B cells with a plasmablast phenotype expressed lower surface MHC II levels than peritoneal naive B cells (Granja and Tafalla, 2019). Therefore, it seems possible that whether plasmablasts retain or even increase their antigen-processing capacities is dependent on the stimulus that triggered the differentiation and also on the source of B cells. Likewise, it seems probable that the use of certain Blimp1 isoforms by plasmablasts is not a general feature and might be dependent on how these cells were generated, their source, or even the stage of differentiation. Thus, while FSC^{hi}ER^{hi} blood plasmablasts have an increased *prdm1a-2* transcription, rainbow trout peritoneal plasmablasts were shown to have increased transcription levels of *prdm1a-2*, *prdm1c-1*, and *prdm1c-2* when compared to

peritoneal naive B cells (Perdiguero et al., 2020). Hence, although it seems clear that B cell differentiation in rainbow trout implies the transcriptional upregulation of at least one of these factors, how they interact with each other or if each of them is specialized in a defined B cell subset or on a specific differentiation stage is something that requires further investigation.

Even though some studies have used transcription markers (Pax5, EBF1) together with other cellular markers (RAG1, surface and secreted IgM) to identify B cell developmental and activated states in the trout,[28,29] the protocols used were methodologically complicated, and the analysis of the B cell subpopulations required a careful interpretation. However, the protocol provided in the current study was very simple and proved to be effective to study the activation of B cells from different sources in response to different stimuli administered either *in vitro* or *in vivo*. Through it, we were able to establish that upon an intraperitoneal TNP-LPS stimulation, activated B cells are mostly found in the spleen and peritoneum after 7 days, while at this point, activated cells decrease in the head kidney. Likewise, after TNP-LPS intraperitoneal immunization of trout, the highest number of ASCs was found in the spleen during the primary response, changing to the head kidney during the secondary immune response.[30]

In summary, we have investigated for the first time in teleost fish the cellular changes that B cells undergo upon activation, revealing an expansion of the ER but no significant changes in mitochondria content. In parallel, the transcription of mitochondrial genes was shown to decrease in B cells upon activation. Our studies have led us to unequivocally establish that $FSC^{hi}ER^{hi}$ IgM^{+} B cells correspond to cells that have started a differentiation program to plasmablasts/PCs as they have an increased IgM-secreting capacity and a transcriptional profile that agrees with a differentiated profile (increased *prdm1a-2*, *irf4*, *il1β*, and *bcma* transcription and decreased IgD and *pax5* mRNA levels) when compared to $FSC^{lo}ER^{lo}$ IgM^{+} B cells, even from stimulated cultures. However, these cells maintain high MHC II levels and antigen-processing capacities, suggesting that in addition to Ab secretion they still play an important role in antigen presentation. Nevertheless, the protocol optimized constitutes a simple and easy tool to identify differentiated B cells in different tissues, upon stimulation with various stimuli or even after *in vivo* immunization.

Limitation of the study

In this study, we have used ER expansion and size to identify a subset of B cells that have differentiated to IgM-secreting plasmablasts. Of course, in mammals, where antibodies are available against many surface markers that define different B cell subsets, such a study would not be a great advantage. However, in teleost fish, where the antibodies that we have available to discriminate B cell subsets are quite limited, the defined protocol is of great use. Nevertheless, we could use this work as an initial step and use the $FSC^{hi}ER^{hi}$ IgM^{+} B cell population to perform extensive transcriptomic and proteomic analysis that would allow the identification of specific surface activation markers in teleost B cells.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105854.

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AUTHOR CONTRIBUTIONS

CT and PP conceived and supervised the study. EM carried out all experimental procedures with help from JGH-J, BA, EM-A, RS, AM-M, and PD-R. PP also collaborated in the transcriptional analysis performed in the sorted B cell populations. MG and GA performed the transmission electron microscopy experiments and analyzed the data. BA and EM wrote an initial manuscript draft. CT wrote the final manuscript with inputs from all other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
mouse anti-trout major histocompatibility complex (MHC) II β-chain	[37]	NA
mouse anti-trout IgM (1.14 mAb)	[38]	NA
mouse anti-trout IgM (4C10 mAb)	[39]	NA
Biological samples		
Healthy rainbow trout (*Oncorhynchus mykiss*),	Piscifactoria Cifuentes (Cifuentes, Guadalajara, Spain)	NA
Chemicals, peptides, and recombinant proteins		
Benzocaine	Sigma-Aldrich	Cat#E1501
Heparin	Sigma-Aldrich	Cat#H3149
Leibovitz's medium (L-15)	Gibco	Cat#11415–049
Penicillin Streptomycin solution	Gibco	Cat#11548876
Fetal calf serum	Gibco	Cat#10270–106
Percoll	GE Healthcare	Cat#17-0891-01
APC Lightning-Link labeling kits	Innova Biosciences	Cat#705–0010
RPE Lightning-Link labeling kits	Innova Biosciences	Cat#703–0010
Biotin Conjugation Kit (Fast, Type A) - Lightning-Link	Innova Biosciences	Cat#370–0010
DAPI	Sigma-Aldrich	Cat#D9542
7-AAD	BD Biosciences	Cat#51–2359KC
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation	Invitrogen	Cat#L10119
EnzChek® Protease Assay Kit green fluorescence	Invitrogen	Cat#E6638
Trypan blue solution	Sigma-Aldrich	Cat#T8154
Lipopolysaccharides from *Escherichia coli* O26:B6	Merck Millipore	Cat#L8274
CD40L	[40]	NA
IL-2B	[41]	NA
IL-10	[23]	NA
Hydroxyurea	Sigma-Aldrich	Cat#H8627
ER-Tracker™ Green dye	Invitrogen	Cat#E34251
ER-Tracker™ Blue-White DPX	Invitrogen	Cat#E12353
MitoTracker® Deep RedFM	Invitrogen	Cat#M22426
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat#A2153
Tween-20	Sigma-Aldrich	Cat#P7949
Phosphate Buffered Saline	Lonza	Cat#17–516F
Streptavidin-HRP	Thermo Scientific	Cat#21126
OPD (O-Phenylenediamine Dihydrochloride) substrate	Sigma-Aldrich	Cat#P8287
H_2SO_4	Sigma-Aldrich	Cat#339741
Power SYBR Green Cells-to-Ct Kit	Invitrogen	Cat#4402954
2,4,6-Trinitrophenyl hapten conjugated to lipopolysaccharide (TNP-LPS)	Biosearch Technologies	Cat#T-5065-5

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Paraformaldehyde	Merck	Cat#1.04005.1000
Glutaraldehyde	Electron Microscopy Sciences	Cat#16220
Phosphate buffer –Sodium hydrogen phosphate -Sodium dihydrogen phosphate	-Merck -Carlo Erba	-Cat#1.06586.0500 -Cat#10049-21-5
Bovine skin gelatin	Sigma-Aldrich	Cat#G-9382
OsO_4	Sigma-Aldrich	Cat#20816-12-0
K_3 Fe(CN)$_6$	Sigma-Aldrich	Cat#244023.100
Acetone	Merck	Cat#1.00014.1000
Epoxy –812 resin -MNA -DDSA -BDMA	-TAAB Laboratories -TAAB Laboratories -TAAB Laboratories -TAAB Laboratories	-T026 -M012 -D027 -B036
Uranyl acetate	Electron Microscopy Sciences	Cat#22400
Lead citrate –Lead nitrate -sodium citrate dihydrate -sodium hydroxide	-Merck -Merck -Merck	-C156298.100 –1.06448.1000 –1.06498.1000
Oligonucleotides		
Primers for real time PCR, see Table S1	See Table S1	NA
Software and algorithms		
BD FACSDiva™ software	BD Biosciences	https://www.bd.com/
FlowJo® v.10	FlowJo LLC, Tree Star	https://www.flowjo.com/
GraphPad Prism 6 software	GraphPad Software	https://www.graphpad.com/
ImageJ	ImageJ	https://imagej.nih.gov/ij/
Adobe Photoshop CS6 software	Adobe Systems	https://www.adove.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Carolina Tafalla (tafalla@inia.csic.es).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Healthy specimens of female rainbow trout (*Oncorhynchus mykiss*) of ~50 g were obtained from Piscifactoria Cifuentes (Guadalajara, Spain) and maintained at the animal facilities of the Animal Health Research Center (CISA-INIA-CSIC) at 14°C with a recirculating water system and 12:12 h light/dark photoperiod. Fish were fed twice a day with a commercial diet (Skretting). Prior to any experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks, and during this period, no clinical signs were ever observed. All of 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 Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) Ethics Committee (CEEA PROEX002/17).

Leukocyte isolation and stimulation

Fish were sacrificed by benzocaine (Sigma) overdose and total leukocyte populations isolated from peripheral blood, spleen, peritoneum and kidney. Blood was extracted from the caudal vein using a heparinized needle and diluted 10 times with Leibovitz medium (L-15; GIBCO) supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin (P/S, Life Technologies), 10 IU/mL heparin, and 2% fetal calf serum (FCS) (all supplements obtained from Life Technologies). Spleen and kidney cell suspensions were obtained by passing the tissues through 100 μm nylon strainers (BD Biosciences) using L-15 containing P/S, 10 U/mL heparin and 2% FCS. Peritoneal leukocytes were obtained by recovering cells from the peritoneal cavity in L-15 medium containing P/S, 10 U/mL heparin and 2% FCS. Kidney, spleen and peritoneum cell suspensions were placed onto 30/51% Percoll (GE Healthcare) gradients whereas blood suspensions were layered onto 51% Percoll cushions. All suspensions were then centrifuged at 500 × *g* for 30 min at 4°C, without brake. Cells at the interface were collected and washed twice in L-15 medium containing P/S and 5% FCS. The viable cell concentration was determined by Trypan blue (Sigma-Aldrich) exclusion and cell suspensions were adjusted to 2×10^6 cells/mL in L-15 medium containing P/S and 5% FCS.

To stimulate the differentiation of B cells, lipopolysaccharide (LPS) from *Escherichia coli* O26:B6 was used (Merck Millipore) at a final concentration of 100 μg/mL for 3 days. In other experiments, recombinant rainbow trout CD40L (5 μg/mL), IL-2B (200 ng/mL) and IL-10 (200 ng/mL), produced as described before,[23,40,41] were combined for 3 days to stimulate the differentiation of B cells as previously described.[23] Non-stimulated controls were always included in the experiments.

Flow cytometry

The anti-trout IgM [1.14 mAb mouse IgG1 coupled to R-phycoerythrin (R-PE), 1 μg/mL] and the anti-trout major histocompatibility complex (MHC) II β-chain [mAb mouse IgG1 coupled to APC, 2 μg/mL] used in this study have been previously characterized.[37,38] Antibodies were fluorescently labeled using R-PE or APC Lightning-Link labeling kits (Innova Biosciences) following the manufacturer's instructions. ER-Tracker™ Green dye and MitoTracker® Deep Red^{FM} were purchased from Invitrogen. 4′,6-diamine-2′-phenylindole dihydrochloride (DAPI) was purchased from Sigma and 7-AAD from BD Biosciences.

Leukocytes were incubated for 30 min on ice in the dark with anti-trout IgM-RPE and/or anti-MHC II-APC in FACS staining buffer (L-15 without phenol red containing 2% FCS). Following two washing steps, cells were then stained with ER-Tracker™ Green dye (330 nM for 5 min) and/or MitoTracker® Deep Red FM (10 nM for 10 sec) and then resuspended in staining buffer for two more washing steps. Cells were finally counterstained with 0.2 μg/mL DAPI or 2.5 μg/mL 7-AAD to remove dead cells.

All samples were analyzed on a FACS Celesta^{TM} flow cytometer (BD Biosciences) equipped with FACS DIVA software following the gating strategy described in Figure 1A. In all cases, a minimum of 20,000 events (live single cells) were acquired per sample. Flow cytometry analysis was performed with FlowJo V10 (TreeStar).

Antigen-processing assay

The antigen-processing capacity of different B cell subsets cells was measured using the EnzChek protease Assay kit (Invitrogen). Briefly, blood leukocytes were incubated in the presence or absence of LPS (100 μg/mL) as described above. After 3 days, the cells were incubated with green fluorescent BODIPY DQ-CASEIN at 5 μg/mL for 1 h. BODIPY DQ-CASEIN is a self-quenched form of fluorescently labeled CASEIN, commonly used to study protease-mediated antigen processing given that it exhibits bright green fluorescence upon proteolytic processing due to the released dye molecules (Jones et al., 1997). At this point, cells were washed with FACS staining buffer and labeled with anti-trout IgM coupled to R-phycoerythrin (R-PE) as described above. Following two washing steps, cells were incubated with 5μM of ER-Tracker™ Blue-White DPX (Invitrogen) for 5 min. Cells were counterstained with LIVE/DEAD™ Fixable Near-IR Stain (Invitrogen) to discard dead cells and analyzed by flow cytometry as described above.

Immunofluorescence and confocal microscopy

Blood leukocyte suspensions were collected as described above and seeded on a poly-L-lysine (0.01% solution, Sigma)-coated slide and incubated at RT for 1 h in a humidified chamber. The cells were then incubated with MitoTracker® Deep Red FM (200 nM for 20 min), which passively diffuse across

the plasma membrane and accumulate in active mitochondria. After that, cells were washed twice with PBS and fixed with 4% paraformaldehyde solution for 30 min at RT. The fixed samples were incubated for 1 h at RT with blocking solution (PBS, pH 7.5 containing 5% BSA and 0.5% saponin) to minimize non-specific adsorption of the antibodies to the coverslip. Next, cells were incubated with anti-trout IgM antibody (coupled to FITC, 17 μg/mL) for 1 h at RT in a humidified chamber and counterstained with 1 μg /mL DAPI (Sigma-Aldrich) for 10 min at RT. Finally, cells were rinsed with PBS 1× and mounted with Fluoromount (Sigma-Aldrich) for microscopy. Laser scanning confocal microscopy images were acquired with an inverted Zeiss Axiovert LSM 880 microscope with Zeiss Zen software and subsequently processed using Adobe Photoshop CS6 software package. The mean cell area, the MFI and total fluorescence (area x MFI) of MitoTracker of IgM^+ cells was measured and quantified using Fiji imaging software in 10 digital fields (63x magnification) from 6 different individuals from each experimental condition (control and LPS).

Cell sorting

FACS sorting of control $FSC^{lo}ER^{lo}$ IgM^+ cells and LPS-stimulated $FSC^{hi}ER^{hi}$ and $FSC^{lo}ER^{lo}$ IgM^+ cells was performed on a FACSAria™ III flow cytometer (BD Biosciences) equipped with BD FACSDiva™ software based on their FSC/SSC profile and then on the basis of the fluorescence emitted by the anti-IgM coupled to R-phycoerythrin (R-PE) antibodies and the ER-Tracker™ Green dye. $FSC^{lo}ER^{lo}$ IgM^+ B cells from non-stimulated cultures and $FSC^{hi}ER^{hi}$ and $FSC^{lo}ER^{lo}$ IgM^+ B cells from LPS-stimulated cultures were sorted. Approximately 70,000 from each subset were collected in PBS for subsequent RNA isolation, whereas 50,000 cells from each subset were collected for TEM analysis and determination of the IgM secreting capacity. The purity of the sorted samples was always verified and was $\geq$95% in all cases.

Transmission electron microscopy

For ultrastructural analysis, sorted $FSC^{lo}ER^{lo}$ IgM^+ B cells from non-stimulated cultures and $FSC^{hi}ER^{hi}$ and $FSC^{lo}ER^{lo}$ IgM^+ B cells from LPS-stimulated cultures were sedimented at 300 × *g* for 5 min and fixed with 4% paraformaldehyde (PFA) (Merck) and 2% glutaraldehyde (GLA) (Electron Microscopy Sciences) in 0.1 M phosphate buffer (PB, pH 7.4) (Merk) for 2 h at RT. Cell pellets were then embedded in 10% bovine skin gelatin (Sigma) and post-fixed with 1% OsO_4 (Sigma), 0.8% K_3 $Fe(CN)_6$ (Sigma) in water at 4°C for 1 h. Samples were dehydrated with ethanol (Merck) and embedded in epoxy resin (TAAB 812 resin; TAAB Laboratories) according to standard procedures. After polymerization, 80-nm-thick (ultrathin) sections were obtained and stained with uranyl acetate (Electron Microscopy Sciences) and lead citrate (Merck) according to standard procedures. Samples were examined in a JEOL JEM1400Flash electron microscope operating at 100 kV. Images were recorded with an OneView 16-Megapixel CMOS Camera from Gatan. Quantification of cytoplasmic areas in the different B cell subsets was performed using Image J software. At least 30 different cell profiles per condition were analyzed at 1,200X magnification.

ELISA

Sorted blood IgM^+ B cell subpopulations (control or LPS-treated $FSC^{lo}ER^{lo}$ IgM^+ and LPS-treated $FSC^{hi}ER^{hi}$ IgM^+ B cells) were cultured during 2 days at 20°C and supernatants collected to evaluate the levels of secreted IgM by ELISA. For this, 96-well ELISA plates were coated overnight with 100 μL of 2 μg/mL mouse anti-trout Ig mAb 4C10.[39] Wells were then blocked with 100 μl of 1% BSA in 1% Tween-20 PBS for 1 h at RT. Plates were washed 3 times with PBS-1% Tween-20 and serum samples were diluted 1:100 in PBS-1% BSA and added to the wells. Samples were incubated 1 h at RT and washed 3 times in PBS-1% Tween-20. Then, 50 μL of biotinylated 4C10 mAb (1 μg/mL) diluted in blocking buffer were added to the wells and samples incubated for 1 h at RT. After three washing steps, plates were incubated with 50 μL of Streptavidin-HRP (1:2000 in PBS-1% BSA) for 1 h at RT. Wells were washed again 3 times and then 50 μL of OPD (O-Phenylenediamine Dihydrochloride) substrate (Sigma) was added. The reaction was stopped by adding 50 μL of 2.5 M H_2SO_4 and absorbance at OD_{490} nm was measured in a FLUO Star Omega Microplate Reader (BMG Labtech). Positive and negative controls were included in all the plates.

Transcriptional analysis of sorted B cell populations

To evaluate the levels of transcription of different immune genes in sorted leukocyte populations, DNase I-treated total RNA was reverse transcribed directly from FACS sorted subpopulations (control $FSC^{lo}ER^{lo}$ and LPS-treated $FSC^{lo}ER^{lo}$ or $FSC^{hi}ER^{hi}$ IgM^+ B cells) using the Power SYBR Green Cells-to-Ct Kit (Invitrogen) following the manufacturer's instructions. To evaluate the levels of transcription of the different genes,

real-time PCR was performed with a LightCycler 96 System instrument using SYBR Green PCR Core Reagents (Applied Biosystems) and specific primers (Table SI). 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). The expression of individual genes was normalized to relative expression of trout EF-1α, and the expression levels were calculated using the $2^{-\Delta Ct}$ method, in which Δ threshold cycle (Ct) is determined by subtracting the EF-1α value from the target Ct. Negative controls with no template were included in all the experiments. A melting curve for each PCR was determined by reading fluorescence every degree between 60 and 95°C to ensure only a single product had been amplified.

Intraperitoneal immunization procedure and sampling

Rainbow trout received 50 μg of 2,4,6-Trinitrophenyl hapten conjugated to lipopolysaccharide (TNP-LPS), obtained from Biosearch Technologies, in 200 μL of saline solution (0.9% NaCl) by means of an intraperitoneal injection. A mock-immunized group (control) received 200 μL of saline solution intraperitoneally. Prior to the immunization, fish were starved for three days and sedated using benzocaine at 10 mg/L (Sigma Aldrich). Sampling was performed after 7 days, collecting 9 rainbow trout from each group. For this, rainbow trout were killed by benzocaine overdose (50 mg/L) and leukocytes isolated from blood, peritoneum, kidney and spleen as described above.

Statistical analysis

Data were analyzed using GraphPad Prism 6 software. Statistical analyses for flow cytometry and ELISA studies were performed by using a two-tailed Student's *t*-test with Welch's correction. A Student's unpaired *t*-test was used to compare the mean percentage of the cytoplasmic areas between the different lymphocyte groups for ultrastructural analysis and to compare the results from the *in vivo* experiment. The differences between the mean values were considered significant on different degrees, where * means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.005$ and **** means $p \leq 0.001$.