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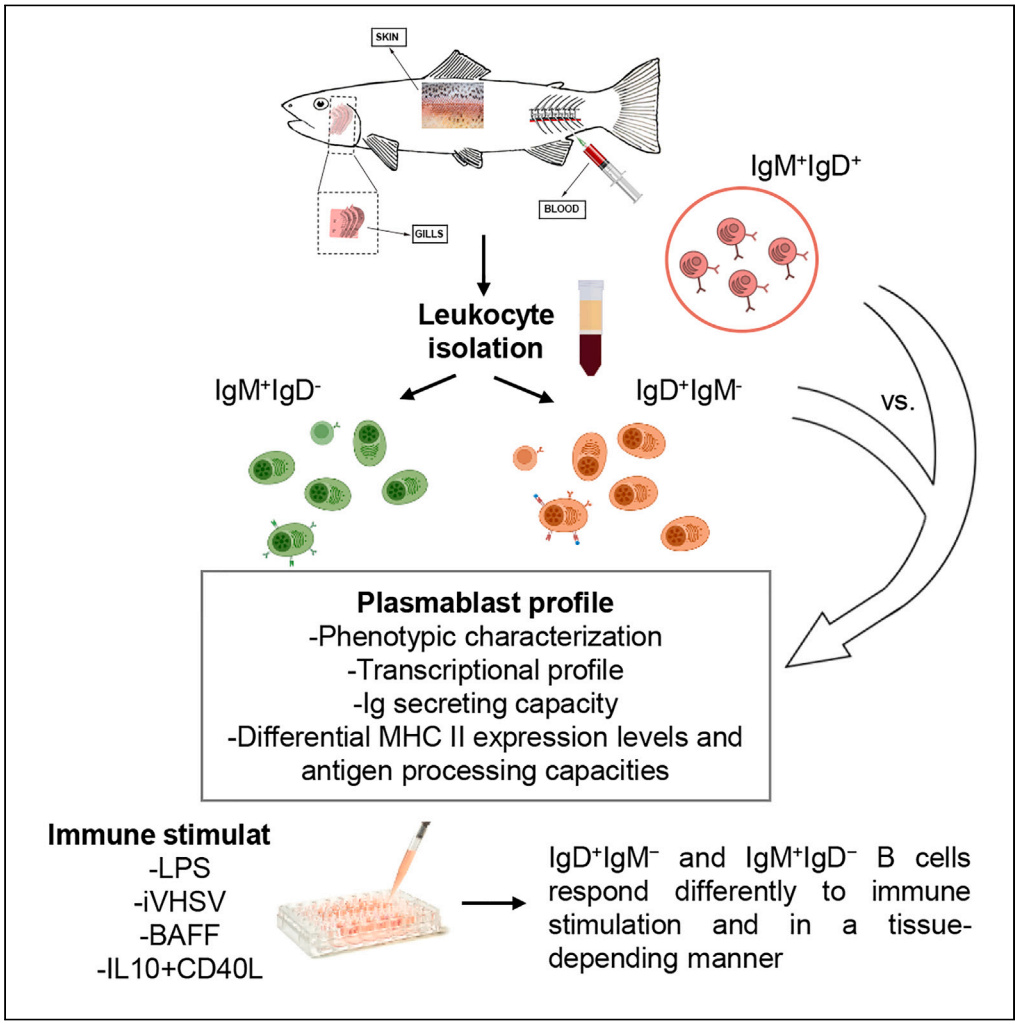

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Highlights

IgD^{+}IgM^{-} B cells are an important non-IgT B cell subset in trout gills and skin

Gills and skin IgD^{+}IgM^{-} cells, as IgM^{+}IgD^{-} cells, have a plasmablast-like phenotype

IgD^{+}IgM^{-} cells have low surface MHC II expression and antigen-processing capacities

IgD^{+}IgM^{-} cells respond differently to immune stimulation than IgM^{+}IgD^{-} cells

Herranz-Jusdado et al., iScience 26, 107434
August 18, 2023
https://doi.org/10.1016/j.isci.2023.107434

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Teleost IgD$^+$IgM$^-$ B cells in gills and skin have a plasmablast profile, but functionally and phenotypically differ from IgM$^+$IgD$^-$ B cells in these sites

J. Germán Herranz-Jusdado,[1,2] Esther Morel,[1,2] Rocío Simón,[1] Patricia Díaz-Rosales,[1] and Carolina Tafalla[1,3,*]

SUMMARY

Although most B cells in teleost systemic compartments co-express IgM and IgD on the surface, cells exclusively expressing either of the two Igs are common in fish mucosal tissues, providing us with a unique opportunity to further characterize IgD$^+$IgM$^-$ B cells, an intriguing B cell subset. Hence, we compared the phenotype of IgD$^+$IgM$^-$ cells to that of IgM$^+$IgD$^-$ B cells in rainbow trout gills and skin, also establishing the response of these subsets to immune stimulation. The transcriptional profile and secreting capacity of IgD$^+$IgM$^-$ B cells corresponded to that of cells that have started a differentiation program toward plasmablasts, similarly to IgM$^+$IgD$^-$ B cells. Yet, IgM$^+$IgD$^-$ B cells retained high levels of surface MHC II and antigen-processing abilities, while these were much lower in IgD$^+$IgM$^-$ cells, suggesting important differences in their antigen-presenting capacities. Our findings contribute to a deeper understanding of the enigmatic role of IgD in mucosal surfaces.

INTRODUCTION

Fish are the oldest animals with an adaptive immune system based on immunoglobulins (Igs). These Igs are either expressed on the B cell surface as B cell receptors (BCR) or free as secreted antibodies.[1] In teleosts, there are only three antibody isotypes, namely IgM, IgD, and the teleost-specific IgT.[2] The heavy chain of IgM and IgD isotypes is generated by alternative splicing, therefore sharing the same variable region on a specific B cell.[3] IgT, on the other hand, is generated by an alternative rearrangement. Because IgT generates antigen recognition diversity through a private V(D)J gene cassette, IgT production is completely independent to that of IgM and IgD.[2,4] In this context, the main subset of B cells in teleost systemic compartments corresponds to B cells that co-express IgM and IgD simultaneously on their surface (IgM$^+$IgD$^+$ B cells).[5,6] These mature naive B cells downregulate IgD after encountering an antigen, starting a differentiation process to plasmablast/plasma cells, becoming IgM$^+$IgD$^-$ B cells,[7] as occurs in mammals.[8] B cells that express IgT on their surface (IgT$^+$ B cells) are also present in most teleost species constituting a distinct linage itself. As the ratio of IgT-to-IgM-expressing cells is higher in mucosal than in systemic tissues, and IgT responses to several parasite models seem confined to mucosal compartments, IgT has been proposed as an Ig with a dedicated mucosal role.[4,9,10] Nevertheless, systemic IgT responses have also been reported in some species.[11,12]

Different B cell subsets exclusively expressing IgD are also present in fish as in mammals.[6,13] First, anergic B cells characterized by elevated surface IgD and reduced IgM expression are found in human peripheral blood and mouse peripheral lymphoid organs.[14] These cells seem to be implicated in establishing tolerance to self-antigens. Additionally, a small subset of mammalian B cells differentiate to IgD$^+$IgM$^-$ plasmablasts after experiencing an unconventional form of class switch recombination (CSR) from IgM to IgD that involves activation-induced deaminase.[15–20] These cells are generated in the lymphoepithelial tissue of aerodigestive organs, including the palatine and pharyngeal tonsils, and from there they can enter the circulation.[17] At these sites, IgD has been shown to bind commensals and pathogens inhabiting the human upper respiratory tract.[17,21] Although human IgD$^+$IgM$^-$ plasmablasts lack the receptors required to home to the intestinal mucosa, IgD$^+$IgM$^-$ plasmablasts have also been identified in the gut mucosa of two independent mice models in which conventional CSR is impaired.[18,20] Interestingly, the differentiation of these intestinal IgD$^+$IgM$^-$ plasmablasts was driven by the microbiota, which was partially coated by secreted IgD. Strikingly, although IgD$^+$IgM$^-$ plasmablasts have never been localized in the human

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https://doi.org/10.1016/j.isci.2023.107434

intestinal mucosa, recent studies point to the involvement of IgD in intestinal hypersensitivity reactions. Hence, secreted IgD generated by food allergens was shown to bind basophils promoting the induction of a Th2 response while attenuating their degranulation, thereby dampening the immunogenicity of these antigens.[22] Thus, although the information regarding IgD functionality is still scarce, many of the studies performed to date point to an important role of IgD in regulating mucosal homeostasis.

In teleost fish, IgD^+IgM^- B cells were first described in catfish (*Ictalurus punctatus*) peripheral blood.[23] In rainbow trout (*Oncorhynchus mykiss*), these cells were shown to be more frequently detected in mucosal tissues such as gut and gills,[6,24] often being IgD^+IgM^- B cells the main B cell subset among non-IgT B cells in these sites. A comparative analysis of the IgD repertoire in gills and gut to that of spleen recently demonstrated that mucosal IgD was partially mutated and highly expanded when compared splenic IgD,[6] strongly suggesting that mucosal IgD^+IgM^- B cells are differentiated to plasmablasts. Yet, this hypothesis was never confirmed through alternative methods. Finally, also in rainbow trout, secreted IgD was found to establish a mutualistic relation with intestinal commensals.[6]

In this context, the presence of IgD^+IgM^- B cells in rainbow trout mucosal surfaces provides us with a unique opportunity to further characterize phenotypically and functionally this enigmatic B cell subset. To this aim, we have undertaken an extensive characterization of gills and skin IgD^+IgM^- B cells, comparing them to the IgM^+IgD^- B cell subset, also frequently identified in these tissues. We have studied their transcriptomic profile, size, Ig-secreting capacity, level of MHC II surface expression as well as their capacity to process antigens. At a more functional level, we have determined their response to a wide range of immune stimuli known to regulate splenic B cells in rainbow trout, such as bacterial lipopolysaccharide (LPS), inactivated viral hemorrhagic septicemia virus (iVHSV), B cell activating factor (BAFF), or a combination of interleukin 10 (IL10) and CD40 ligand (CD40L).[25–27] The results obtained highlight the differences among IgM^+IgD^- and IgD^+IgM^- B cells in these two mucosal surfaces, suggesting unique roles for IgD in teleost mucosal immunity.

RESULTS

IgM^+IgD^- and IgD^+IgM^- cells constitute the main B cell subsets in rainbow trout gills and skin

Contrary to what happens in systemic tissues such as spleen or blood,[5] the percentage of IgM^+IgD^+ B cells found in gills and skin was almost non-existent (~0.3%), while IgM^+IgD^- and IgD^+IgM^- B cell subsets made up for most of the non-IgT B cell population (Figure 1A). Specifically, we found that the percentages of IgM^+IgD^- and IgD^+IgM^- B cell subsets among isolated leukocytes were very similar and also between organs as no significant differences were found between the percentage of IgM^+IgD^- B cells in gills (~3.5%) and skin (~4%) or between the percentage of IgD^+IgM^- B cells found in gills (~2.5%) and skin (~3%). Doublets and dead cells were excluded from the flow cytometry analysis following the gating strategy described in Figure S1. Isotype controls were also included in all the flow cytometry analysis performed (Figure S2A) and in some cases different antibody concentrations were also compared (Figure S2B).

We further investigated the presence of IgM^+IgD^+, IgM^+IgD^-, and IgD^+IgM^- B cell subsets in gills and skin leukocytes by immunofluorescence. As shown in Figure 1B, all subpopulations were detected by confocal microscopy, although again the frequency of IgM^+IgD^+ B cells was much lower than that of IgM^+IgD^- and IgD^+IgM^- B cells in both tissues. Also in this case, isotype controls were included for all antibodies to confirm the specificity of the signal obtained (Figure S3).

Rainbow trout gills and skin IgM^+IgD^- and IgD^+IgM^- cells are differentiated to plasmablasts

We next investigated the transcriptional profile of IgM^+IgD^- and IgD^+IgM^- B cells in gills and skin and compared them to the transcriptional profile of blood IgM^+IgD^+ B cells, commonly identified as naive B cells.[7] We studied the levels of transcription of several genes associated with B cell functionality at specific differentiation stages.[8] The results showed that IgM^+IgD^- B cells from both gills and skin had significantly higher levels of transcription of *irf4*, *il1b*, all genes coding for Blimp1 isoforms (*prdm1a-1*, *prdm1a-2*, *prdm1c-1*, and *prdm1c-2*), and secreted IgM when compared to IgM^+IgD^+ B cells from blood (Figure 2), strongly suggesting that these cells correspond to B cells that have started a differentiation process toward plasmablasts/plasma cells. Interestingly, the levels of transcription of *bcma*, also commonly associated with B cell differentiation,[8] were only significantly higher than those of blood IgM^+IgD^+ B cells in IgM^+IgD^- B cells from gills but not in those from skin (Figure 2). Also surprising was the fact that *pax5* transcription levels in mucosal IgM^+IgD^- B cells were not significantly lower than those of blood IgM^+IgD^+ B cells (Figure 2).

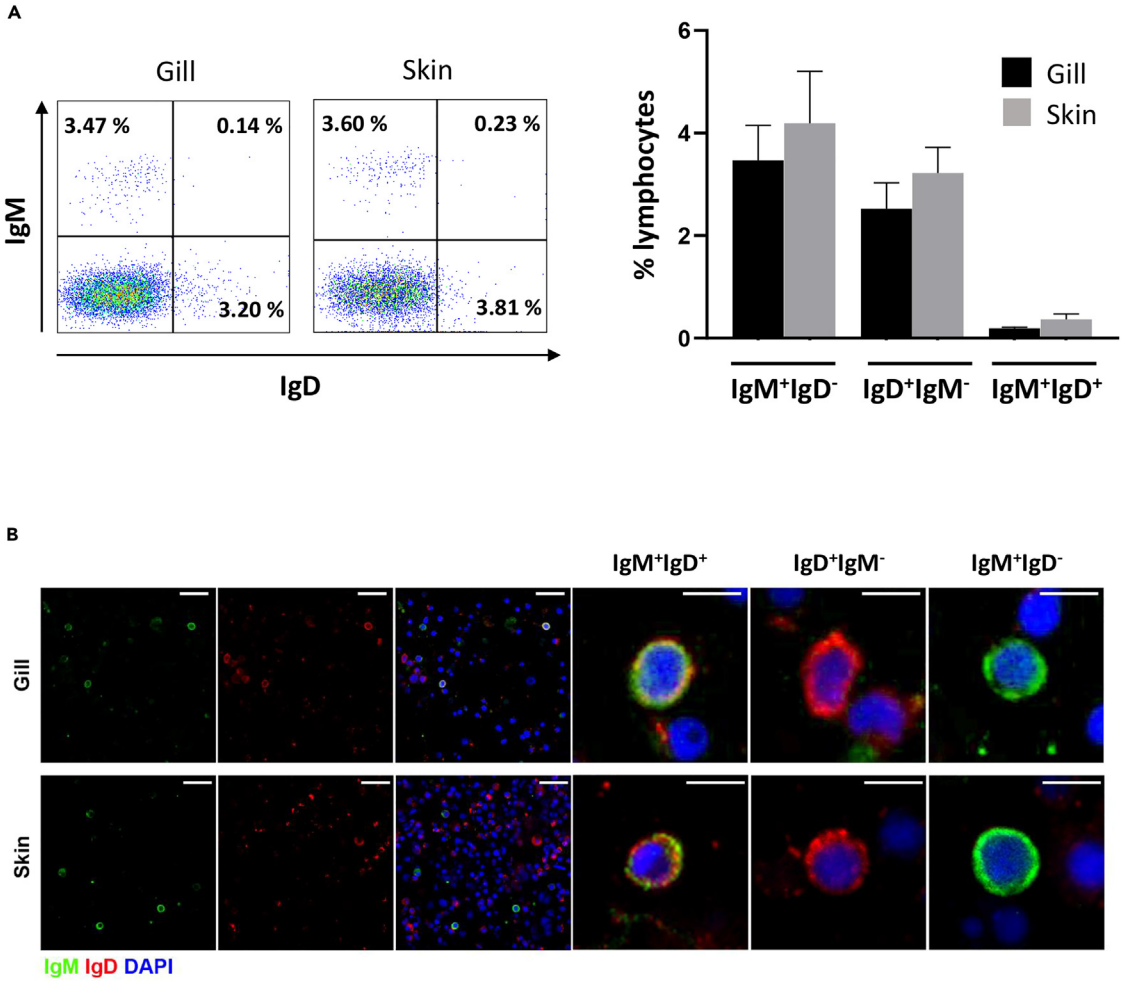

Figure 1. Rainbow trout IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ cells are the main non-IgT B cell subsets in gills and skin

The percentages of live IgM$^+$IgD$^-$, IgD$^+$IgM$^-$, and IgM$^+$IgD$^+$ B cells among cells in the lymphocyte gate were determined by flow cytometry.

(A) Representative dot plots are included along with a graph showing the mean percentages of each subset in gills and skin (mean +SEM; n = 12 independent fish).

(B) Visualization of gill and skin IgM$^+$IgD$^+$, IgD$^+$IgM$^-$, and IgM$^+$IgD$^-$ B cells under the confocal microscope. Examples of each subset are shown. Scale bars in left-hand images: 20 μm; scale bars in right-hand images: 5 μm. See also Figures S1–S3.

IgD$^+$IgM$^-$ B cells also had a transcriptional profile that seemed to correspond to that of cells that had initiated a differentiation process toward plasmablasts/plasma cells, but in this case, there were some important differences in the transcriptional profile of these cells in gills and skin. Hence, IgD$^+$IgM$^-$ B cells from gills and skin had higher *irf4* and lower *pax5* transcription levels than those found in blood IgM$^+$IgD$^+$ B cells (Figure 4), but the *irf4* levels observed in skin IgD$^+$IgM$^-$ B cells were significantly higher than those of their counterpart in gills (Figure 2). Concerning the different genes that code for Blimp1 isoforms, only *prdm1c-1* and *prdm1c-2* mRNA levels were consistently higher in IgD$^+$IgM$^-$ cells when compared to blood IgM$^+$IgD$^+$ B cells (Figure 2). In the case of skin IgD$^+$IgM$^-$ cells, *prdm1a*-1 was also elevated when compared to blood naive B cells (Figure 2). Similarly, *prdm1c-2* mRNA levels reached by this B cell subset were significantly higher in skin than in gills (Figure 2). As observed for IgM$^+$IgD$^-$ B cells, gill IgD$^+$IgM$^-$ B cells also had higher *il1b* transcription levels that those of naive blood cells (Figure 2).

It has been previously established that as trout B cells differentiate toward a plasmablast/plasma cell profile, they increase their size.[7] In order to verify whether this was true for IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells from trout gills and skin, we analyzed the size of these subsets by flow cytometry (referred to as forward scatter) and compared it to the size of blood IgM$^+$IgD$^+$ B cells. As shown in Figure S4, IgM$^+$IgD$^-$ and

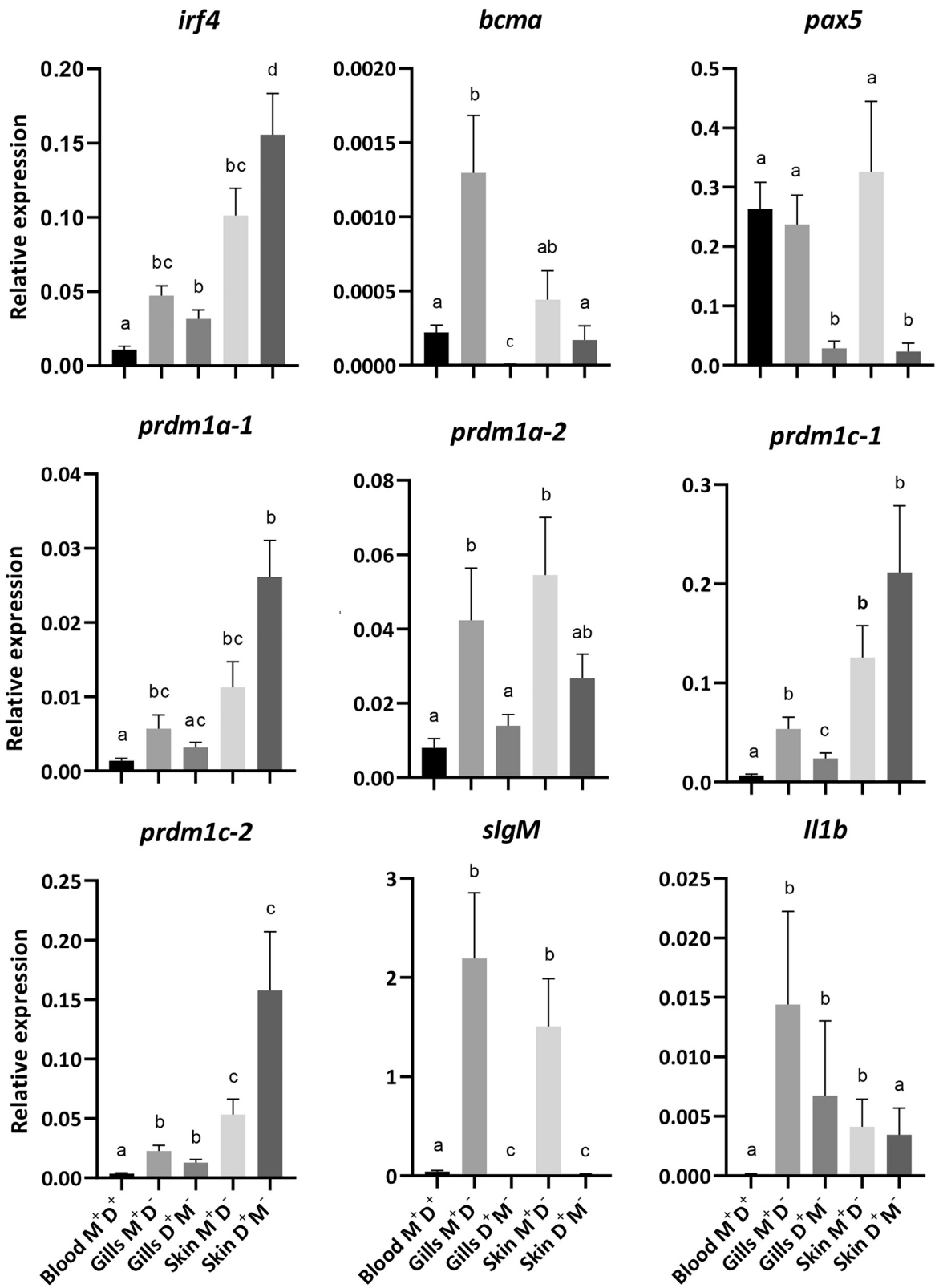
irf4
bcma
pax5
prdm1a-1
prdm1a-2
prdm1c-1
prdm1c-2
sIgM
Il1b
Relative expression
Blood M+D+
Gills M+D-
Gills D+M-
Skin M+D-
Skin D+M-

Figure 2. Rainbow trout gill and skin IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ cells have a differentiated plasmablast-like transcriptional profile

Gill and skin IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cell populations were sorted along with blood IgM$^+$IgD$^+$ B cells to determine the levels of transcription of *irf4*, *bcma*, *pax5*, *prdm1a-1*, *prdm1a-2*, *prdm1c-1*, *prdm1c-2*, secreted IgM (sIgM), and *il1b* by real-time PCR. Results are shown as relative expression values to endogenous control *ef1a* in the different subpopulations (mean +SEM, n = 8 independent fish). Statistical differences were evaluated by a paired two-tailed Student's *t* test when data were normally distributed, whereas non-normally distributed data were analyzed with the non-parametric Wilcoxon matched-pairs signed-rank test. Different lower case letters indicate significant differences between groups ($p \leq 0.05$). See also Figure S4.

IgD$^+$IgM$^-$ B cells from both gills and skin were all significantly larger than blood IgM$^+$IgD$^+$ B cells. Nonetheless, significant differences were found regarding the size of both B cell subsets within a specific tissue. Hence, while in gills, IgD$^+$IgM$^-$ cells were significantly larger than IgM$^+$IgD$^-$ cells, in the skin, IgD$^+$IgM$^-$ cells were significantly smaller than IgM$^+$IgD$^-$ cells (Figure S4).

To unequivocally confirm that mucosal IgD$^+$IgM$^-$ and IgM$^+$IgD$^-$ B cells have a differentiated plasmablast/plasma cell phenotype, we determined the Ig-secreting capacity of both populations by ELISA. For this, IgD$^+$IgM$^-$ and IgM$^+$IgD$^-$ B cells from both skin and gills were sorted and cultivated in complete media for 48 h. At that point, culture supernatants were collected and the concentration of IgM and IgD was estimated by ELISA. As expected, IgM$^+$IgD$^-$ B cells secreted significantly higher amounts of IgM than IgD$^+$IgM$^-$ cells (Figure 3), which on the other hand secreted significant amounts of IgD to the culture media (Figure 3).

Mucosal IgD$^+$IgM$^-$ B cells have lower surface MHC II expression than mucosal IgM$^+$IgD$^-$ B cells

B cells have the ability to present antigens internalized through the BCR in an MHC II context.[28] Hence, to further characterize the B cell subsets found in gills and skin, we analyzed their levels of MHC II surface expression by flow cytometry (Figure 4). As shown in the corresponding histograms and graphs, the mean fluorescence intensity of surface MHC II was significantly lower in IgD$^+$IgM$^-$ B cells compared to that of IgM$^+$IgD$^-$ B cells in both tissues (Figures 4 and S5). When compared to the levels of surface MHC II detected in blood IgM$^+$IgD$^+$ B cells, the levels of MHC II surface expression skin IgM$^+$IgD$^-$ B cells were similar, but those of gill IgM$^+$IgD$^-$ B cells were lower (Figure 4).

Gill IgD$^+$IgM$^-$ B cells have lower antigen-processing capacities than gill IgM$^+$IgD$^-$ B cells

The fact that the levels of surface MHC II expression were so different between IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells suggested that the antigen-processing capacity of these two B cell populations could also vary between the two subsets. To test this, we studied the ability of the different B cell subsets to degrade DQ-CASEIN upon endocytosis. In this case, the low number of leukocytes obtained from rainbow trout skin did not allow us to obtain reliable results for this technique and therefore this experiment was only conducted in gills. In concordance with their lower surface MHC II expression levels, the capacity of gill IgD$^+$IgM$^-$ B cells to process antigens was very low, and significantly lower than that of gill IgM$^+$IgD$^-$ B cells (Figure 5).

Effects of different stimuli on rainbow trout IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cell subpopulations

Finally, we studied how these B cell subsets in gills and skin leukocyte cultures responded to a wide range of immune stimuli known to have strong effects on systemic B cells.[25–27] In gills, LPS stimulation significantly increased the percentages of IgM$^+$IgD$^-$ B cells in cultures, whereas it decreased the percentage of IgD$^+$IgM$^-$ B cells (Figure 6). Stimulation with iVHSV also decreased the percentage of IgD$^+$IgM$^-$ B cells in gill leukocyte cultures, but had no effect on the IgM$^+$IgD$^-$ B cell subset (Figure 6). Although BAFF has been previously shown to regulate the survival of trout splenic and blood B cells as well as their IgM-secreting capacities,[26,29] this cytokine had no effect on the frequency of these B cell subsets in gill leukocyte cultures (Figure 6). No variations in the frequency of IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells were detected in gill cultures in response to the combination of IL10 and CD40L either (Figure 6).

The response to the different stimuli was quite different in skin leukocyte cultures. In this case, LPS provoked an increase in the percentage of both IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells (Figure 7). iVHSV had no effect on the frequency of these B cell subsets in skin leukocyte cultures (Figure 7). In contrast to what occurred in gill cultures, both BAFF and the combination of IL10 and CD40L had an effect on the frequency of IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells in skin cultures. While BAFF provoked a significant upregulation of the percentage of IgM$^+$IgD$^-$ B cells, and decreased the percentage of IgD$^+$IgM$^-$ B cells (Figure 7), stimulating skin

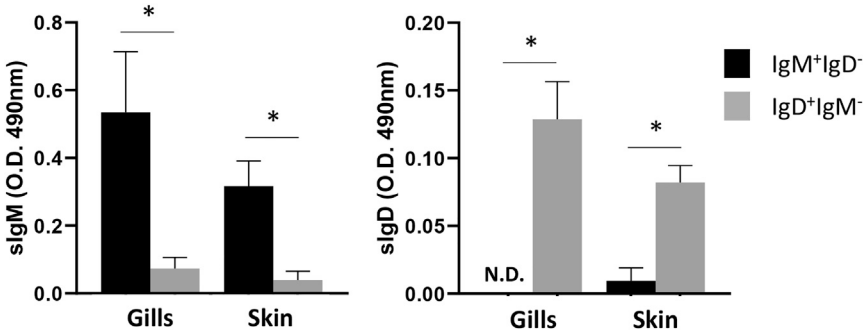

Figure 3. Mucosal IgM$^+$IgD$^-$ and IgD$^+$IgM– B cells secrete IgM and IgD, respectively

Sorted gill and skin IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells were collected in culture media and incubated for 48 h at 20°C. After that time, culture supernatants were collected and the amount of secreted IgM (left graph) and IgD (right graph) was estimated by ELISA. Graphs show the mean absorbance values at 490 nm + SEM obtained in supernatants from different sorted subpopulations (n = 3 independent fish). Statistical differences were evaluated by a paired two-tailed Student's *t* test, and asterisks denote significant differences between subpopulations as indicated (*p $\leq$ 0.05).

leukocyte cultures with IL10 and CD40L significantly decreased the frequency of the IgM$^+$IgD$^-$ B cells and increased that of IgD$^+$IgM$^-$ B cells (Figure 7).

DISCUSSION

The fact that IgD has been conserved throughout evolution[30,31] implies that this ancient Ig plays an important but yet undefined role in immunity. More specifically, several recent reports in both mammals and fish suggest that IgD could be participating in preserving mucosal homeostasis and regulating immune responses at some specific mucosal sites. In mammals, these sites seem to include the upper respiratory tract and the intestine.[17,18,20] In rainbow trout, cells exclusively expressing IgD have been identified in gills,[24] and later on in the intestinal mucosa.[6] Although in rainbow trout, IgD$^+$IgM$^-$ B cells are rare in peripheral blood, in catfish, it was in blood where the presence of these cells was reported.[23] Interestingly, the number of IgD$^+$IgM$^-$ B cells varied considerably at an individual level in this species and while some individuals had little or no IgD$^+$IgM$^-$ B cells in peripheral blood, in others the IgD$^+$IgM$^-$ population could make up for up to 72% of all blood leukocytes.[23] This suggests that the distribution of IgD$^+$IgM$^-$ B cells is dependent on still unknown factors, also differing significantly among teleost species. In the case of mucosal tissues, the way in which these tissues are processed to isolate leukocytes is also relevant. Thus, for example, in previous experiments performed by our group, the presence of IgM$^+$IgD$^+$ B cells seemed more pronounced in gills.[6] However, by optimizing the protocol to isolate rainbow trout gill leukocytes, we have now reduced to a minimum the cross contamination with blood B cells. With this optimized protocol, we have demonstrated that the vast majority of non-IgT cells in rainbow trout gills or the skin correspond to cells that only express one Ig isotype, namely IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells.

The fact that B cells in these mucosal tissues express only one Ig isotype strongly suggests that these cells are differentiated to plasmablasts/plasma cells. The previous discovery that IgM and IgD sequences in gut and gills were mutated and expanded in comparison to their systemic counterparts also seemed indicative that in these mucosal tissues B cells had experienced a differentiation process.[6] In this context, the main aim of the current study was to confirm this hypothesis. For this, we first performed a transcriptomic analysis of IgD$^+$IgM$^-$ and IgM$^+$IgD$^-$ cells in both gills and skin in comparison to blood IgM$^+$IgD$^+$ B cells, which have been shown to correspond to naive mature B cells.[7] Although there are many aspects of the B cell differentiation process that are still unclear in teleost, a strong association between *irf4* transcription and a differentiated B cell profile has been previously established in rainbow trout,[32] as in mammals.[33] In the current study, *irf4* transcription was found consistently upregulated in IgD$^+$IgM$^-$ and IgM$^+$IgD$^-$ cells from gills and skin, when compared to naive blood B cells. Blimp1 encoded by the *prdm1* gene is another key transcriptional factor that drives the maturation of B cells into plasma cells.[8] In rainbow trout, four homologs of the mammalian *prdm1* gene were recently identified.[34] In this work, we detected important differences in the levels of transcription of these four genes, suggesting that specific B cell subsets might use different Blimp1 homologs during their differentiation, something previously expected given that, in the peritoneum, IgM$^+$IgD$^-$ cells with a plasmablast profile showed higher levels of transcription of *prdm1a-2*, *prdm1c-1*,

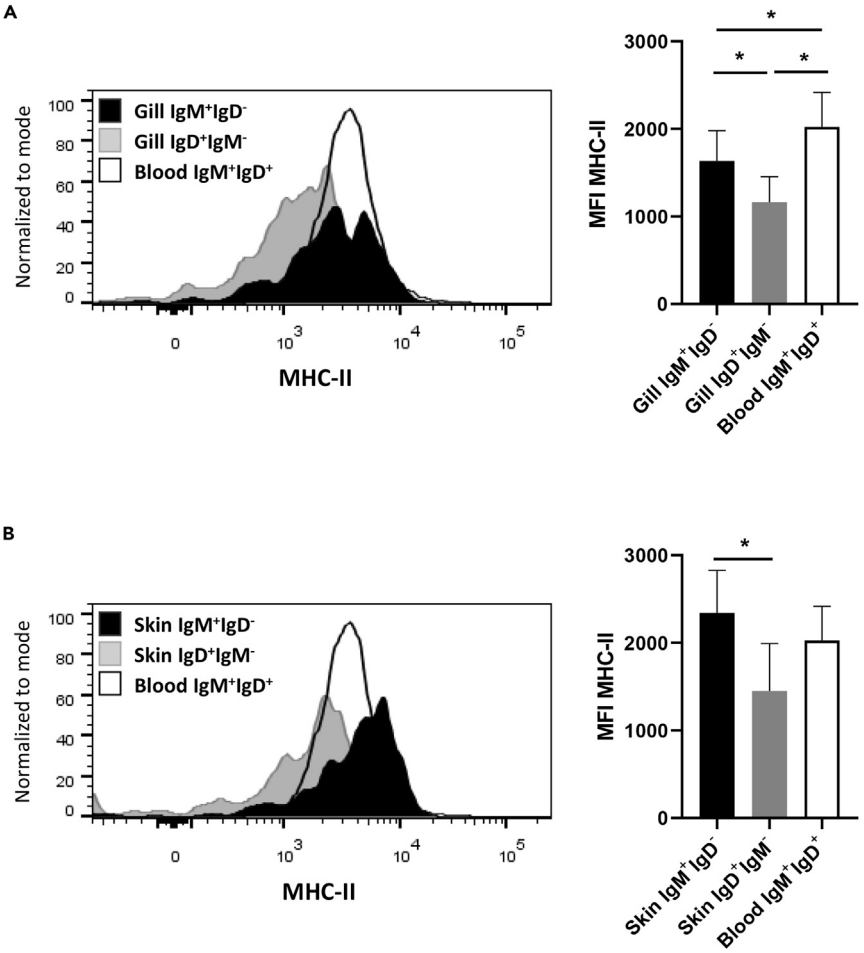

Figure 4. Mucosal IgD$^+$IgM$^-$ B cells have low surface MHC II expression compared to mucosal IgM$^+$IgD$^-$ B cells

Gill, skin, and blood leukocytes were isolated and stained with anti-IgM, anti-IgD, and anti-MHC II monoclonal antibodies to evaluate the levels of surface MHC II expression on mucosal IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells and blood IgM$^+$IgD$^+$ B cells by flow cytometry.

(A and B) Representative histogram showing expression of MHC II in each B cell subset along with a graph showing the mean fluorescence intensity (MFI) values of MHC II on IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells from gills (mean +SEM, n = 7 independent fish) (A) and skin (mean +SEM, n = 10 independent fish) (B) in comparison to blood IgM$^+$IgD$^+$ B cells. Statistical differences were evaluated by a paired two-tailed Student's *t* test and asterisks denote significantly different values between groups as indicated (*$p < 0.05$, **$p < 0.01$). See also Figure S5.

and *prdm1c-2* when compared to naive B cells, but no significant differences in *prmd1a-1* levels.[34] In the case of gills and skin IgM$^+$IgD$^-$ cells, although all four genes were upregulated when compared to blood naive B cells, *prdm1a-2* was the one that showed the greatest differences. The pattern of expression of these genes was much more variable in the case of IgD$^+$IgM$^-$ B cells, with important differences between the two tissues. Hence, the fact that the transcription of *prdm1a-1*, *prdm1c-1*, and *prdm1c-2* as well as that of *irf4* was much higher in IgD$^+$IgM$^-$ B cells from skin than gills might suggest that this population is closer to terminal differentiation in skin than in gills. Another interesting difference between IgD$^+$IgM$^-$ and IgM$^+$IgD$^-$ cells was that only IgD$^+$IgM$^-$ B cells showed a reduced *pax5* transcription when compared to blood naive B cells. In mammals, reduced expression of *pax5* is also considered a hallmark of B cell differentiation, as it is the reduction of *pax5* expression that allows the upregulation of Blimp1.[35] In rainbow trout, our results seem to indicate that this reduction could be more drastic in some types of plasmablasts than others, yet more studies should be performed in the future to confirm this hypothesis. Finally, we observed that all four mucosal subsets transcribed *il1b* at much higher levels than blood naive B cells. The specific reason for this is currently unknown, but might suggest that in fish mucosal surfaces B cells

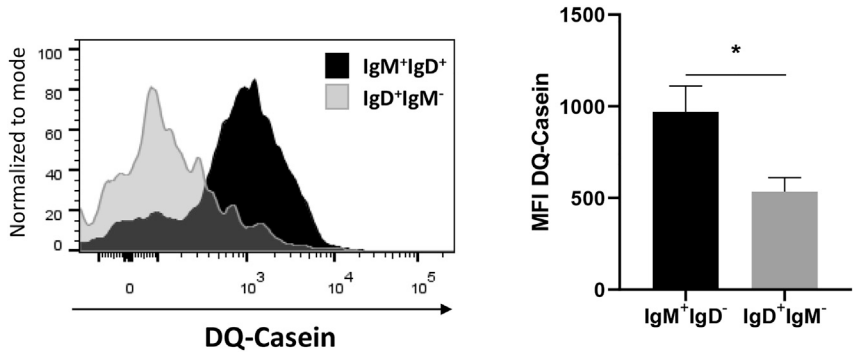

Figure 5. Gill IgD$^+$IgM$^-$ B cells have lower antigen-processing capacities than gill IgM$^+$IgD$^-$ B cells

Gill leukocytes were isolated and incubated with DQ-casein (5 μg/mL) for 1 h at 20°C. After this incubation, cells were labeled with anti-IgM and anti-IgD monoclonal antibodies and analyzed by flow cytometry. A representative histogram is shown along with a graph showing DQ-casein mean fluorescence intensity (MFI) values of IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cell subsets from different individual fish (mean +SEM, n = 7). Statistical differences were evaluated by a paired two-tailed Student's *t* test and asterisks denote significantly different values between groups as indicated (*$p < 0.05$).

participate in the initiation of the inflammatory response. Interestingly, murine IgM-expressing plasma cells were shown to transcribe *il1b* at high levels, while IgG2a and IgG2b expressing plasma cells do not.[36] Additionally, the fact that IgD$^+$IgM$^-$ and IgM$^+$IgD$^-$ cells from these mucosal surfaces are significantly bigger than blood naive B cells further supports that these mucosal B cells have started a differentiation program toward plasmablasts/plasma cells. Nonetheless, to fully confirm this differentiation, we established by ELISA that rainbow trout IgM$^+$IgD$^-$ B cells from gills and skin secrete IgM, whereas IgD$^+$IgM$^-$ B cells have the capacity to secrete IgD into the culture supernatant.

Traditionally, B cells that differentiated to plasmablasts/plasma cells were thought to lose their antigen presentation machinery.[37] However, recent results in mammals have revealed that this is not always true. For example, Pelletier and colleagues demonstrated that plasma cells differentiated in response to thymus-independent antigens maintained MHC II expression.[38] Posterior studies confirmed that effector murine plasma cells secreting different Ig isotypes also retain the MHC II machinery, thereby still participating in the activation of T helper cells.[36] In the current study, we found that gill and skin IgM$^+$IgD$^-$ B cells retained high MHC II surface levels and the ability to process antigens, despite having a transcriptional profile of differentiated B cells. Interestingly, the surface MHC II expression levels of gill IgM$^+$IgD$^-$ B cells were lower than those of blood IgM$^+$IgD$^+$ B cells. In contrast, the surface MHC II expression levels of skin IgM$^+$IgD$^-$ B cells were not significantly different than those of blood IgM$^+$IgD$^+$ B cells. Similarly, IgM$^+$IgD$^-$ B cells from the rainbow trout adipose tissue had a differentiated profile and still retained high MHC II levels, in fact higher than those of blood naive B cells.[39] Nevertheless, this is not a general rule for teleost, given that rainbow trout B cells differentiated in response to IL6 decreased surface MHC II levels.[25] Interestingly, IgD$^+$IgM$^-$ plasmablasts had significantly lower surface MHC II levels than mucosal IgM$^+$IgD$^-$ B cells and had a significantly lower capacity to process antigens, strongly suggesting that rainbow trout IgD$^+$IgM$^-$ plasmablasts no longer participate in antigen presentation or cognate T cell activation, while IgM-secreting plasmablasts still do.

Finally, to provide further insights regarding the immune role of IgD$^+$IgM$^-$ B cells in mucosal surfaces, we determined how immune stimulation affected the frequency of the different B cell subsets in gills and skin leukocyte cultures. To this purpose, we stimulated both gill and skin leukocyte cultures with a wide range of immune stimuli known to have strong effects on systemic B cells, such as LPS, iVHSV, BAFF, and a combination of IL10 and CD40L.[25–27] We found that the response to the different stimuli was quite different between the two B cell subsets and also between tissues. Hence, in gill cultures, IgM$^+$IgD$^-$ B cells only increased in response to LPS stimulation, whereas LPS and iVHSV provoked in these cultures a decrease in the percentage of IgD$^+$IgM$^-$ B cells. In contrast, IgM$^+$IgD$^-$ B cells increased in skin leukocyte cultures in response to LPS or BAFF and decreased in response to IL10 and CD40L. In these cultures, the percentage of IgD$^+$IgM$^-$ B cells increased in response to LPS and the combination of IL10 and CD40L, but decreased in response to BAFF. Overall, these results seem to suggest that IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells play different roles in these mucosal surfaces and point to the fact that these B cell subpopulations are more

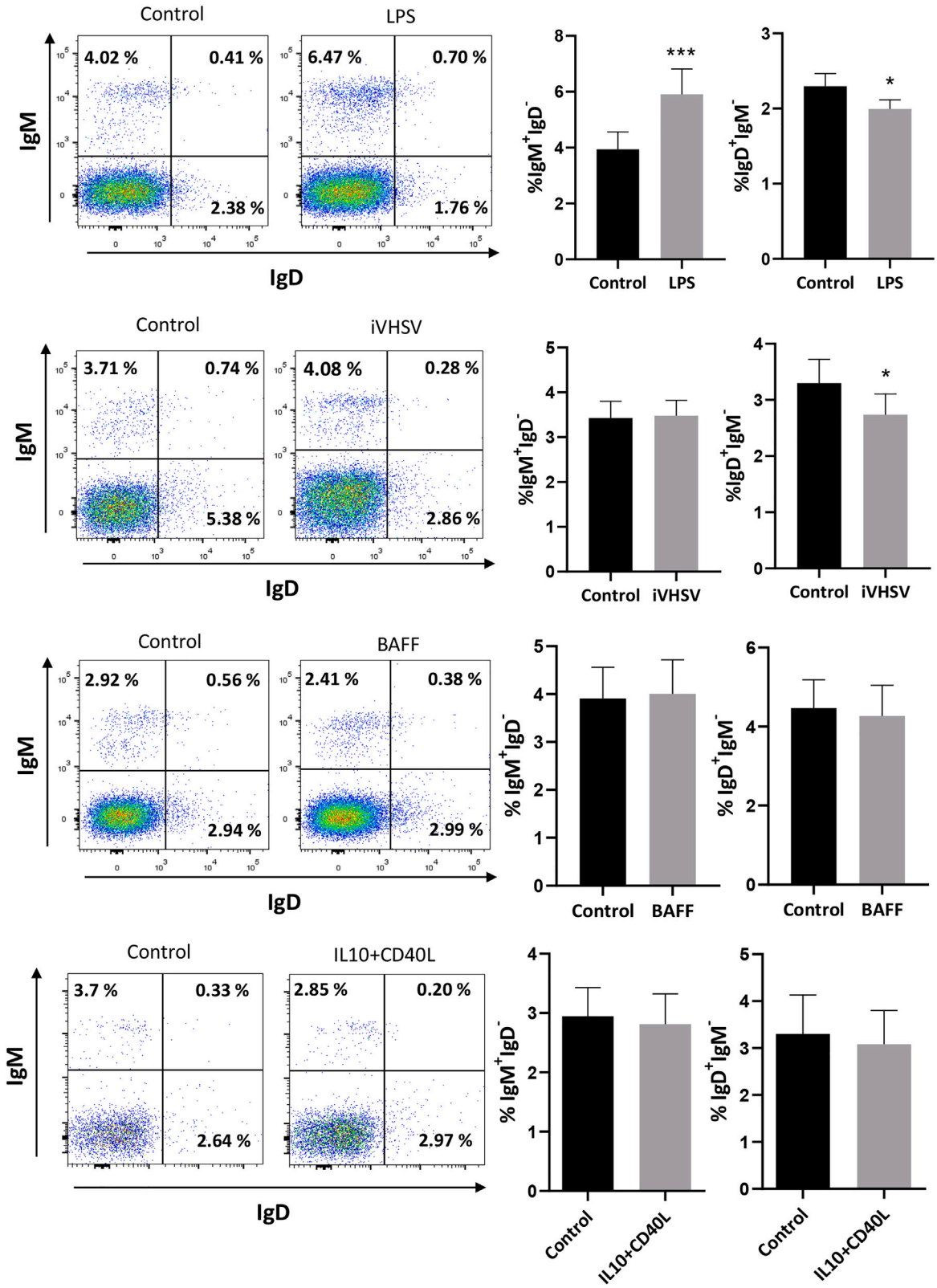
Control
LPS
4.02 %
0.41 %
2.38 %
6.47 %
0.70 %
1.76 %
IgM
IgD
%IgM+IgD-
%IgD+IgM-
***
*
Control
LPS
Control
iVHSV
3.71 %
0.74 %
5.38 %
4.08 %
0.28 %
2.86 %
iVHSV
Control
BAFF
2.92 %
0.56 %
2.94 %
2.41 %
0.38 %
2.99 %
BAFF
Control
IL10+CD40L
3.7 %
0.33 %
2.64 %
2.85 %
0.20 %
2.97 %
IL10+CD40L

Figure 6. Effect of stimulation on the frequency of IgM^+IgD^- and IgD^+IgM^- B cells in gill leukocyte cultures

Leukocyte populations obtained from rainbow trout gills were stimulated with LPS (100 μg/mL), iVHSV (2.5×10^7 virus/ml), recombinant rainbow trout BAFF (3 μg/mL), or a combination of IL10 (200 ng/mL) and CD40L (5 μg/mL). Cells treated with media alone were included as controls. After 48 h of incubation at 20°C, the percentages of live IgM^+IgD^- and IgD^+IgM^- B cells among cells in the lymphocyte gate were determined by flow cytometry. Representative dot plots are shown along with graphs presenting the percentages of IgM^+IgD^- and IgD^+IgM^- B cells in cultures (mean +SEM; n = 15 independent fish). Statistical differences were evaluated by a paired two-tailed Student's *t* test and asterisks denote significantly different values in treated groups compared to controls (*$p < 0.05$ and ***$p < 0.005$).

responsive to external stimuli in the skin than in the gills. Nonetheless, whether increased or decreased numbers of these different B cell subsets were a consequence of B cell proliferation or increased B cell survival, or could be affected by changes in other leukocyte populations, it is unknown at this point. Thus, future experiments are warranted to further elucidate the exact effects of the stimuli on these populations.

In conclusion, in the current study, we have confirmed that gills and skin IgD^+IgM^- B cells are cells that have initiated a differentiation program to plasmablasts/plasma cells. This has been undertaken studying their transcriptomic profile, size, and Ig-secreting capacity. Their MHC II surface expression and antigen-processing capacities have also been studied and compared to those of mucosal IgM^+IgD^- plasmablasts. Our data reveal important differences among IgM^+IgD^- and IgD^+IgM^- B cell subpopulations in the two mucosal surfaces studied that further point to IgD as an Ig with unique yet unrevealed roles in specific teleost mucosal surfaces.

Limitations of the study

In this study, we have confirmed that IgD^+IgM^- B cells in rainbow trout gills and skin are cells with a plasmablast profile, similarly to IgM^+IgD^- cells present in these surfaces. Yet, there are still important differences between these two B cell subsets that point to a specific role of IgD^+IgM^- B cells in teleost mucosal immunity. This characterization was mainly carried out through a transcriptomic analysis, determination of size, MHC II surface levels, and Ig-secreting capacities. Of course, in mammals, where surface markers clearly define B cells in different differentiation status, such a study would have been complemented with a flow cytometry analysis of plasmablast/plasma cell markers. However, in teleost fish, where the antibodies that we have available to discriminate B cell subsets are quite limited, such a study cannot be carried out.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107434.

ACKNOWLEDGMENTS

The authors want to thank Lucía González for technical assistance. Dr. Tiehui Wang is also greatly acknowledged for providing recombinant rainbow trout IL10. This work was supported by the European Research

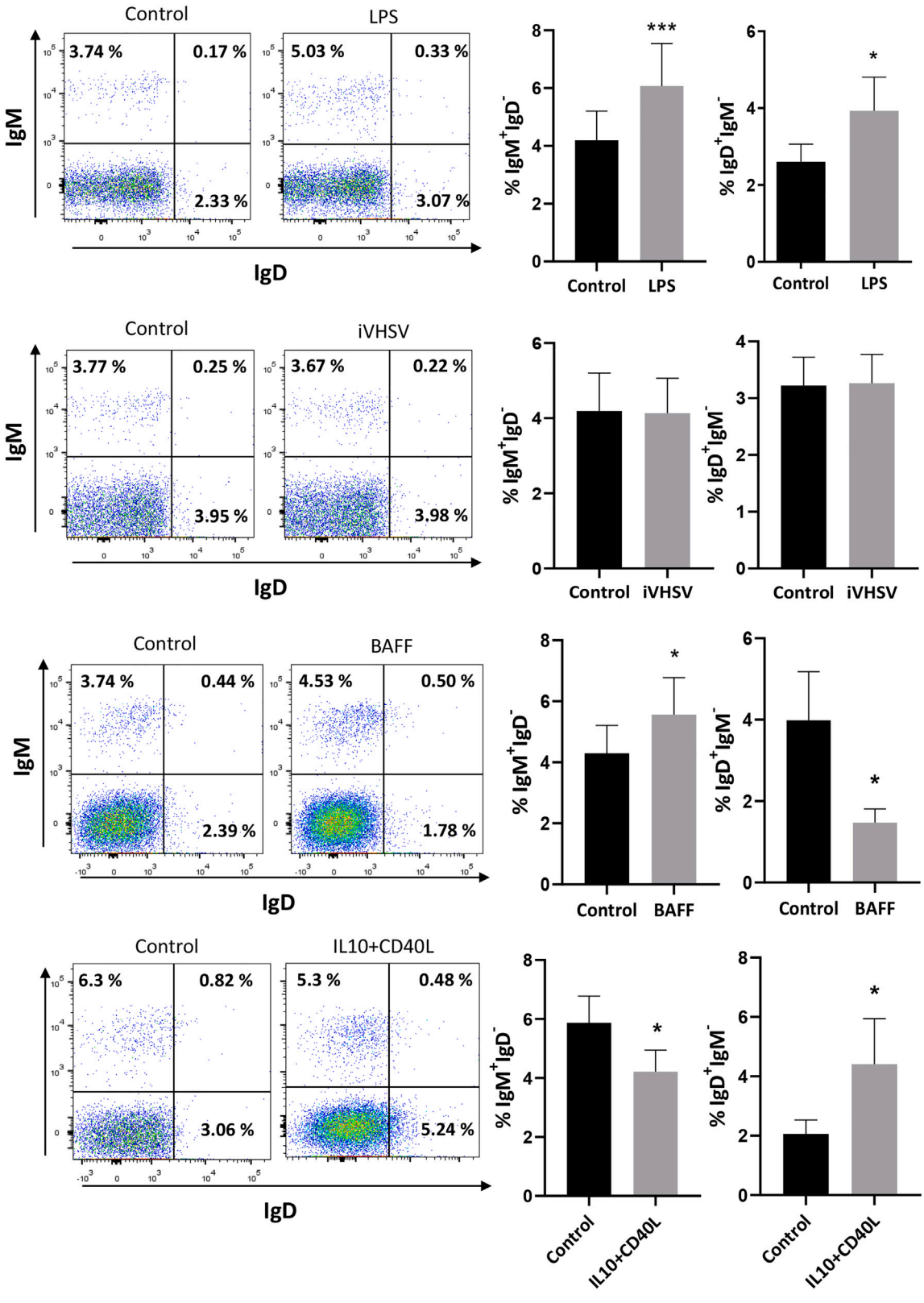
Control
LPS
3.74 %
0.17 %
2.33 %
5.03 %
0.33 %
3.07 %
IgM
IgD
***
*
% IgM+IgD-
% IgD+IgM-
Control
LPS
Control
iVHSV
3.77 %
0.25 %
3.95 %
3.67 %
0.22 %
3.98 %
Control
iVHSV
Control
BAFF
3.74 %
0.44 %
2.39 %
4.53 %
0.50 %
1.78 %
Control
BAFF
Control
IL10+CD40L
6.3 %
0.82 %
3.06 %
5.3 %
0.48 %
5.24 %
Control
IL10+CD40L

Figure 7. Effect of stimulation on the frequency of IgM^+IgD^- and IgD^+IgM^- B cells in skin leukocyte cultures

Leukocyte populations obtained from rainbow trout skin were stimulated with LPS (100 μg/mL), iVHSV (2.5×10^7 virus/ml), recombinant rainbow trout BAFF (3 μg/mL), or a combination of IL10 (200 ng/mL) and CD40L (5 μg/mL). Cells treated with media alone were included as controls. After 48 h of incubation at 20°C, the percentages of live IgM^+IgD^- and IgD^+IgM^- B cells among cells in the lymphocyte gate were determined by flow cytometry. Representative dot plots are shown along with graphs presenting the percentages of IgM^+IgD^- and IgD^+IgM^- B cells in cultures (mean +SEM; n = 15 independent fish). Statistical differences were evaluated by a paired two-tailed Student's *t* test and asterisks denote significantly different values in treated groups compared to controls (*$p < 0.05$ and ***$p < 0.005$).

Council (ERC Consolidator Grant 725061) and by the Spanish Ministry of Science and Innovation (project PID2020-113268RB-I00). J.G.H.-J. was supported by a Juan de la Cierva contract (FJC2019-041967-I) from the Spanish Ministry of Science and Innovation.

AUTHOR CONTRIBUTIONS

C.T. conceived the study. J.G.H.-J. and E.M. carried out all the experimental work aimed at the characterization of B cell subsets in rainbow trout gills and skin with the supervision of P.D.R. E.M. also provided technical assistance in flow cytometry and cell sorting. R.S. performed the IFA. C.T., J.G.H.-J., E.M., and R.S. wrote the manuscript with contributions from the other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: February 1, 2023
Revised: June 15, 2023
Accepted: July 17, 2023
Published: July 20, 2023

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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	Granja et al. 2015[40]	NA
mouse anti-trout IgM (1.14 mAb)	Deluca et al. 1983[41]	NA
mouse anti-trout IgM (4C10 mAb)	Thuvander et al. 1990[42]	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
Trypan blue solution	Sigma-Aldrich	Cat#T8154
Lipopolysaccharides from *Escherichia coli* O26:B6	Merck Millipore	Cat#L8274
CD40L	Granja et al. 2019[7]	NA
IL-2B	Wang et al. 2018[43]	NA
IL-10	Abos et al. 2020[27]	NA
Hydroxyurea	Sigma-Aldrich	Cat#H8627
ER-Tracker™ Green dye	Invitrogen	Cat#E34251
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
Paraformaldehyde	Merck	Cat#1.04005.1000
Glutaraldehyde	Electron Microscopy Sciences	Cat#16220

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
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/
Image J	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 STUDY PARTICIPANT DETAILS

Healthy rainbow trout (*Oncorhynchus mykiss*) adults of approximately 100 g were obtained from *Piscifactoria Cifuentes* (Cifuentes, Guadalajara, Spain) and maintained at the animal facilities of the Animal Health Research Center (CISA-INIA, Spain) in an aerated recirculating water system at 15°C, with a 12:12 h light:dark photoperiod. All animals used were females and the influence of sex was not considered in the analysis of the data. Fish were fed twice a day with a commercial diet (Skretting) and were acclimatized to laboratory conditions for at least 2 weeks prior to any experimental procedure. During this period, no clinical signs were ever observed. All the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for use of laboratory animals and were approved by the Ethics Committee from INIA (Code PROEX 065.3/21). All efforts were made to minimize suffering.

METHOD DETAILS

Tissue sampling and leukocyte isolation

Fish were euthanized by anesthetic overdose using a water bath with approximately 150 ppm of benzocaine (Sigma). Blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with Leibovitz medium (L-15, Life Technologies) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (P/S), 10 units/ml heparin and 5% fetal calf serum (FCS) (all supplements also obtained from Life Technologies). Blood suspensions were layered onto 51% Percoll cushions and centrifuged at 400 × *g* for 30 min at 4°C, without brake. Leukocytes were collected from the interface and washed in L-15 containing P/S and 5% FCS.

Gills and skin were always sampled after extraction of blood to avoid the contamination of mucosal samples with blood leukocytes. For this, gill arches were individualized and then transferred to a tube containing L-15 supplemented with P/S, 2% FCS and 10 U/ml heparin. Each gill arch was washed individually and transferred to a new tube with a clean medium of equal composition. Gill suspensions were then obtained by pressing samples through a 100 μm nylon cell strainer (BD Biosciences). Due to excessive debris in the cell suspensions, samples were washed by centrifugation (400 × *g* for 10 min) to remove cell debris. Clarified gill cell suspensions were placed onto 30/51% discontinuous Percoll (GE Healthcare) density gradients, and centrifuged at 400 × *g* for 30 min at 4°C, without brake. Cells at the interface, corresponding to leukocytes, were collected and washed in L-15 containing P/S and 5% FCS. Counting and cell viability were then determined by trypan blue (Sigma) exclusion.

For the isolation of skin leukocytes, a piece of skin of approximately 10 cm^2 was carefully collected from each side of the fish and placed on a Petri dish with 2 ml of L-15 supplemented with P/S and 2% FCS. There, all muscle tissue was carefully removed and the skin was cut into small pieces and transferred to a tube containing L-15 with P/S, 5% FCS and 2 mg/ml dispase (Gibco) and incubated for 2 h at 4°C with continuous agitation. Subsequently, the samples were pressed through a 100 μm nylon cell strainer and the suspension washed as previously described for gills. Likewise, skin leukocytes were separated using a discontinuous Percoll gradient (30/51%) and leukocytes collected, washed and counted as described above.

Flow cytometry

To characterize B cell populations from gills and skin, isolated leukocytes were stained with anti-trout IgM [1.14 mAb mouse IgG1 coupled to R-phycoerythrin (R-PE); 0.25 μg/ml] and anti-trout IgD [mAb mouse IgG1 coupled to allophycocyanin (APC); 5 μg/ml] diluted in staining buffer (phenol red-free L-15 medium supplemented with 2% FCS) for 1 h in darkness at 4°C. In some experiments, leukocytes were also stained with a specific antibody against trout MHC II β-chain [mAb mouse IgG1 coupled to fluorescein isothiocyanate (FITC); 1.5 μg/ml]. All antibodies have been previously characterized[40,41,44] and were fluorescently labeled using R-PE, FITC or APC Lightning-Link labeling kits (Innova Biosciences) following the manufacturer's instructions. In all cases, cells incubated with FITC, R-PE or APC-conjugated mouse IgG1 isotypes (clone MOPC-21, Biolegend) were used as controls, to confirm the specificity of the mAbs used. After the staining, cells were washed three times in staining buffer and resuspended in staining buffer for their analysis in a FACS Celesta flow cytometer (BD Biosciences) with a BD FACSDiva software (BD Biosciences). The data obtained were analyzed using the FlowJo® v.10 software (FlowJo LLC, Tree Star). In all cases, cell viability was checked using 4′,6-diamine-2′-phenylindole dihydrochlorid (DAPI) at 0.2 μg/ml.

Confocal microscopy

Gill and skin leukocytes were seeded on a poly-L-lysine (0.01% solution)-coated slide and incubated at room temperature (RT) for 1 h in a humidified chamber. Thereafter, the samples were fixed in 4% paraformaldehyde solution for 30 min at RT and incubated for 1 h with blocking solution (TBS with 5% BSA and 0.5% saponin). Samples were then incubated with anti-trout IgM coupled to FITC (15 μg/ml) and anti-trout IgD coupled to APC (50 μg/ml) for 1 h at RT. Samples incubated with FITC or APC-conjugated mouse IgG1 isotypes at the same concentrations were also included to confirm the specificity of the antibody signal. Slides were counterstained with 1 μg/ml of DAPI (Sigma) for 10 min, then rinsed with PBS 1× and mounted with Fluoromount for microscopy. All images were obtained using a laser scanning confocal microscope (Zeiss Axiovert LSM 880) and they were further processed with Adobe Photoshop CS6 software.

Cell sorting

FACS sorting of IgM^+IgD^- and IgD^+IgM^- B cells from gills and skin, and IgM^+IgD^+ B cells from blood was performed on a FACSAria™ III flow cytometer (BD Biosciences) equipped with BD FACSDiva™ software based on the fluorescence from anti-trout IgM coupled to R-PE and anti-trout IgD coupled to APC after staining the cells with the specific antibodies as described above. Approximately 70000 cells from each subset were collected in staining buffer for subsequent RNA isolation. An equal number of IgM^+IgD^- and IgD^+IgM^- B cells were also sorted from each fish (10000–50000 cells) to compare the Ig secreting capacity of these B cell subsets.

Real-time PCR analysis

Total RNA was isolated from sorted IgM^+IgD^- and IgD^+IgM^- B cells from gills and from blood IgM^+IgD^+ B cells using the Power SYBR Green Cells-to-Ct Kit (Invitrogen) following manufacturer's instructions. RNA was treated with DNase during the process to remove genomic DNA that might interfere with the reactions. Reverse transcription was also performed using the Power SYBR Green Cells-to-Ct Kit 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 (Roche) using SYBR Green PCR core Reagents (Applied Biosystems) and specific primers previously described (Table S1). Each sample was measured under the following conditions: 10 min at 95°C, followed by 40 amplification cycles (15 s at 95°C and 1 min at 60°C). A melting curve for each primer set was obtained by reading fluorescence every degree between 60°C and 95°C to ensure only a single product had been amplified. The expression of individual genes was normalized to the relative expression of trout housekeeping gene elongation factor 1α (EF-1α) and the expression levels were calculated using the $2^{-\Delta Ct}$ method, where ΔCt is determined by subtracting the EF-1α value from the target Ct. EF-1α was selected as reference gene according to the MIQE guidelines[45] given that no statistical differences were detected among Ct values obtained for EF-1α in the different samples. Negative controls with no template and *minus*-reverse transcriptase (-RT) controls were included in all experiments.

Determination of Ig secreting capacity

Sorted gill and skin IgM^+IgD^- and IgD^+IgM^- B cells were collected in L-15 medium supplemented with P/S and 10% FCS and cultured for 48 h at 20°C. At this point, supernatants were collected from B cell cultures to evaluate the levels of secreted IgM and IgD by ELISA. For this, 96-well ELISA plates were coated overnight with 100 μl of 2 μg/ml mouse anti-trout IgM or anti-trout IgD mAbs diluted in 0.05 M carbonate tampon pH 9.7. After 3 washes in 0.05% Tween-20 PBS (PBST), wells were blocked with 100 μl of 1% BSA in PBST for 1 h at RT. Plates were then washed 3 times with PBST and supernatants diluted 1:5 in PBS containing 1% BSA added to the wells. Samples were incubated 1 h at RT and washed 3 times in PBST. Thereafter, 50 μl of biotinylated anti-trout IgM or anti-trout IgD mAb (1 μg/ml) diluted in blocking buffer were added to the wells. After a 1 h incubation at RT, the wells were washed 3 times with PBST and then incubated with 50 μl of Streptavidin-HRP (diluted 1:1000 in PBS supplemented with 1% BSA) for 1 h at RT. Wells were washed again 3 times and then 50 μl of OPD (O-Phenylenediamine Dihydrochloride) (Sigma) added (1 mg/ml). The reaction was stopped by adding 50 μl of 2.5 M H_2SO_4 and absorbance at 490 nm measured in a FLUO Star Omega Microplate Reader (BMG Labtech). Positive and negative controls were included in all the plates.

Antigen-processing assay

The antigen-processing capacity of different B cell subsets was measured using the EnzChek protease Assay kit (Invitrogen). Briefly, gill leukocytes, seeded in 96-well plates at a concentration of 2×10^6 cells/ml (100 μl/well) were incubated with green fluorescent BODIPY DQ-casein at 5 μg/ml during 1 h. BODIPY DQ-casein is a self-quenched form of fluorescently labeled casein commonly used to study protease-mediated antigen processing due to the fact that it exhibits bright green fluorescence upon proteolytic processing due to the released dye molecules.[46] Thereafter, the cells were washed with FACS staining buffer three times and labeled with anti-trout IgM coupled to R-PE and anti-trout IgD coupled to APC and analyzed by flow cytometry as described above.

In vitro stimulation

Gill and skin leukocytes obtained as described above were cultured in L-15 medium supplemented with P/S and 5% FCS and one of the following stimuli: *Escherichia coli* O111:B4 lipopolysaccharide (LPS) (Sigma, 100 μg/ml), viral hemorrhagic septicemia virus (VHSV) previously inactivated for 30 min at 56°C (2.5 ×

10^7 virus/ml), recombinant rainbow trout BAFF (3 μg/ml)[26] or a combination of recombinant rainbow trout IL10 (200 ng/ml) and CD40L (5 μg/ml).[27] Controls incubated with media alone were also included. After 48 h at 20°C, cells were stained with anti-IgM and anti-IgD and the percentage of IgM^+IgD^- and IgD^+IgM^- B cells established through flow cytometry as described above.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed using GraphPad Software (GraphPad Prism v8.0.1, La Jolla California, USA). Data were presented as mean +SEM and significance was assigned at $p \leq 0$ 0.05. Nonetheless, in some experiments differences between the mean values were considered significant on different degrees, where * means $p \leq 0.05$, ** means $p \leq 0.01$ and *** means $p \leq 0.005$. Data were checked for normality using the Shapiro-Wilk test and q-q plots. Two-tailed Student's *t*-test was used for normally distributed data, whereas non-normally distributed data were tested with non-parametric Wilcoxon matched-pairs signed-rank test.