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Yersinia ruckeri infection activates local skin and gill B cell responses in rainbow trout

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A B S T R A C T

Teleost fish lack organized structures in mucosal tissues such as those of mammals, but instead contain dispersed B and T cells with the capacity to respond to external stimuli. Nonetheless, there is still a great lack of knowledge regarding how B cells differentiate to plasmablasts/plasma cells in these mucosal surfaces. To contribute to a further understanding of the mechanisms through which fish mucosal B cells are activated, in the current study, we have studied the B cell responses in the skin and gills of rainbow trout (*Oncorhynchus mykiss*) exposed to *Yersinia ruckeri*. We have first analyzed the transcription levels of genes related to B cell function in both mucosal surfaces, and in spleen and kidney for comparative purposes. In a second experiment, we have evaluated how the infection affects the presence and size of B cells in both skin and gills, as well as the presence of plasmablasts secreting total or specific IgMs. The results obtained in both experiments support the local differentiation of B cells to plasmablasts/plasma cells in the skin and gills of rainbow trout in response to *Y. ruckeri*. Interestingly, these plasmablasts/plasma cells were shown to secrete specific IgMs as soon as 5 days after the exposure. These findings contribute to a further understanding of how B cells in the periphery respond to immune stimulation in teleost fish.

1. Introduction

Aquatic organisms such as fish are in constant contact with water which is a microbe-rich environment. In this sense, aquatic organisms are more exposed to microbial threats than terrestrial animals evidencing the importance of fish mucosal epithelial barriers such as the skin, the gills, the intestine or the nasopharynx [1]. These mucosal barriers represent the first line of defense against environmental threats, not only acting as a physical barrier, but also playing an important immunological role through the action of their local immune elements. Hence, mucosal surfaces contain scattered myeloid and lymphoid cells either in the epithelium or in the lamina propria, which include innate immune cells such as macrophages, neutrophils, natural killer (NK) cells, mast cells or dendritic cells (DCs), as well as adaptive immune cells, namely populations of CD4 and CD8 T cells, as well as B cells [2,3]. Because the lymphoid elements in fish mucosae are scattered throughout the tissue and lack the organization of mammalian mucosal structures, some authors consider that these associated immune elements cannot be considered a true mucosa-associated lymphoid tissue (MALT) [1,3]. Nevertheless, most authors still refer to these teleost immune elements as MALTs for simplification, that depending on the mucosal surface can be further designated as skin-associated lymphoid tissue (SALT), gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT), nasopharynx-associated lymphoid tissue (NALT), and the recently described, buccal and pharyngeal MALT [4,5]. Nonetheless, it should be noted that the composition of these MALTs varies widely between fish species.

Fish B cells can be classified into different subsets according to the expression of one or more of the three immunoglobulin (Ig) isotypes present in teleost, namely IgM, IgD and IgT. Hence, the main subset of B cells in systemic immune tissues are cells that co-express IgM and IgD simultaneously on the cell surface (IgM^+IgD^+ B cells). These naïve B cells, as occurs in mammals [6,7], downregulate surface IgD upon antigen encounter, starting a differentiation program to plasmablast/plasma cells, thereby becoming IgM^+IgD^- B cells [8,9]. Additionally, IgD^+IgM^- B cells have been described in catfish (*Ictalurus punctatus*) peripheral blood [10] and more recently, in mucosal tissues of rainbow trout (*Oncorhynchus mykiss*) such as gut, gills and skin [11–13]. Finally, B cells that express IgT on their surface (IgT^+ B cells), present in most fish species, represent an independent lineage to that of

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IgM/D-expressing B cells, and have been proposed as a B cell subset with a preferential role in mucosal immunity, given that IgT and IgT$^+$ B cells were prevailing in mucosal surfaces such as skin, gut or gills when compared to IgM responses in response to different parasites [14–16]. Nonetheless, IgM/D-bearing populations are also present in mucosal surfaces, possibly contributing to maintaining mucosal homeostasis and contributing to pathogen clearance. Hence, a recent work undertaken by our group demonstrated that IgM$^+$IgD$^-$ and IgD$^+$IgM$^-$ B cells constituted the main IgM/D-bearing B cell populations in rainbow trout skin and gills [13]. Interestingly, although both B cell subsets corresponded to cells that have started a differentiation program towards plasmablasts, IgM$^+$IgD$^-$ B cells retained high levels of surface MHC II and antigen-processing abilities, while these were much lower in IgD$^+$IgM$^-$ cells [13], suggesting important differences in the function that both subsets play in mucosal immunity.

Yersinia ruckeri is a Gram-negative rod-shaped enterobacterium and the causative agent of enteric redmouth disease (ERM), a fatal disease affecting salmonids and other fish species of commercial importance worldwide [17]. The bacterium enters the fish via the secondary gill lamellae and from there it spreads to the blood and internal organs. To date, information regarding the response of mucosal B cells to this bacterial pathogen is still scarce. Hence, we used *Y. ruckeri* as a model pathogen to study B cell responses in the skin and the gills of rainbow trout. First, we analyzed the levels of transcription of a series of genes related to B cell function in fish exposed to inactivated or live *Y. ruckeri* and compared them to those of control fish exposed to culture media alone. We included *il1b* as an indicator of global inflammation, the secreted and membrane forms of all Ig isotypes, genes related to B cell differentiation (*prdm1a-1*, *prdm1a-2*, *prdm1c-1* and *prdm1c-2*, *irf4 and pax5*) as well as cytokines involved in B cell activation (*baff*, *april* and *balm*) and their receptors (*baff-r*, *taci* and *bcma*). *prdm1a* genes code for Blimp1 (B lymphocyte-induced maturation protein 1), a key transcriptional factor that controls the differentiation of B cells, together with *irf4* [18], downregulating the expression of transcription factors responsible for maintaining a mature resting B cell profile, including *pax5* [19]. In rainbow trout, four different homologues of *prdm1a* genes have been identified [20]. Although the four genes seem to be implicated the differentiation process in fish, they seem to play different roles in different B cell subsets [20]. This transcriptional study was undertaken in skin and gills, and in kidney and spleen for comparative purposes. Additionally, we studied B cells in skin and gills in *Y. ruckeri* infected and control fish by flow cytometry. Finally, to determine how the bacteria affected local IgM secretion, we determined the number of cells secreting total and *Y. ruckeri*-specific IgM in the skin and gills of the infected fish. Our results demonstrate that there is an early local differentiation of B cells in both skin and gills in response to the bacterial infection, providing further insight on how fish B cells in the periphery respond to pathogens.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The motile strain Yr 026-01/15 of *Yersinia ruckeri* (serotype O1), isolated from diseased rainbow trout and provided by Acuipharma Aquaculture Health S.L was used for the *in vivo* assays. This serotype is commonly associated with mortalities in outbreaks reported in cultured salmonids [21]. The bacteria was cultured in Tryptic Soy Agar or Broth (TSA or TSB, respectively) (Pronadisa, Spain) at 24 °C for 24 h with gentle agitation (100 rpm) and stored in Luria Broth (LB) plus 20% glycerol at −80 °C.

2.2. Challenge experiments

In the current study, two different challenge experiments were undertaken. The first one was performed with both live and inactivated *Y. ruckeri* cells to analyze the transcriptomic response of different immune tissues, while the second one was performed exclusively with live *Y. ruckeri* cells to determine the effects of the infection on skin and gill leukocyte populations.

For the first experiment, rainbow trout (*Oncorhynchus mykiss*) fingerlings of 0.5 g purchased from a commercial hatchery (Felechosa, Austurias, Spain) were taken to the fish facilities of the Central Service for Experimental Research (SCSIE) of the University of Valencia (Spain). Fish were maintained there for three months until the experiment was conducted. No clinical signs or mortalities were observed during this period. During this time, different bacterial concentrations were tested in a pre-challenge bath experiment in order to validate a suitable infective dose (lethal dose 50, LD_{50}) for the challenge following the protocol described by Reed and Muench [22]. For this, rainbow trout were randomly distributed into three 200 l tanks (30 fish per tank). At this point, the average weight of the fish was approximately 11 g. Fish were then exposed to the previously established LD_{50} *Y. ruckeri* dose by bath (2.5×10^7 cfu/ml), to the same dose of heat inactivated *Y. ruckeri* cells (60 °C for 1 h) or to the same volume of TSB. After 1 h, fish were moved to clean 200 l tanks. At days 2, 4 and 7 post-infection, 10 fish per group were sampled. To do so, fish were euthanized by MS-222 (Sigma, USA) overdose. Blood was collected from the caudal vein with heparinized syringes and spleen, kidney (complete kidney), skin and gills sampled and preserved in RNA later (Ambion, USA) until later used for RNA extraction. A similar gill arch and a specific skin section (above the lateral line and close to the gills) was consistently sampled in all fish.

In the second experiment, rainbow trout of approximately 50 g were obtained from a commercial fish farm (Piscifactoria Cifuentes, Guadalajara, Spain) and maintained at the animal facilities of the Animal Health Research Center (CISA-INIA-CSIC). Prior to the experimental procedure, fish were acclimatized to laboratory conditions for 2 weeks, and during this period, no clinical signs or mortalities were ever observed. Fish (14 fish per group) were infected with *Y. ruckeri* by bath (2.8×10^7 cfu/ml) or mock-infected with the same volume of TSB. After 1 h, fish were moved to clean 100 l tanks. At days 2, 4 and 7 post-infection, 10 fish per group were sampled. For this, fish were euthanized by MS-222 overdose. Blood was collected from the caudal vein with heparinized syringes and skin and gills sampled for leukocyte isolation.

In both cases, fish were fed twice a day (2% body weight per day) with a commercial diet (Skretting, Norway) and maintained in aerated fresh water at 17.5 °C ± 0.5 under natural photoperiod (12 h light/12 h dark). All of the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) and the Spanish law ("Real Decreto 53/2013") for the protection of animals used in scientific experiments. The first experiment was approved by the Ethics and Animal Welfare Committee of University of Valencia and the local authority (Consellería de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural. Generalitat Valenciana) (project license 2021/VSC/PEA/0150). The second experiment was approved by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) Ethics Committee (project license PROEX002/17).

2.3. RNA isolation and transcriptional analysis

Tissue samples obtained from the first experiment and preserved in RNAlater were used to isolate total RNA using TRI Reagent Solution (Thermo Fisher Scientific, USA) following the manufacturer's instructions. The RNA pellet was washed with 75% ethanol, dissolved in RNAse-free water and stored at −80 °C until use. One μg of RNA was treated with DNAse I (Thermo Fisher Scientific) to remove any genomic DNA traces that might interfere with the PCR reactions and then used to obtain cDNA using the RevertAid reverse transcriptase (Thermo Fisher Scientific) and oligo$(dT)_{23}$VN, following the manufacturer's instructions. The cDNA was diluted in a 1:10 proportion with RNAse-free water and stored at −20 °C until use.

To evaluate immune gene transcription levels, real-time PCR was performed in a LightCycler96 System (Roche, Switzerland) using Fast-Start Essential DNA Green Master reagents (Roche) and specific primers (Table S1). Each sample was exposed to the following conditions: 10 min at 95 °C, followed by 40 amplification cycles (10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C). A dissociation curve 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 [23] 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.

The bacterial load was also estimated in the first experiment, detecting the levels of *Y. ruckeri* 16S RNA by real-time PCR in all tissue samples obtained from infected fish after 4 and 7 days. To do so, one μg of RNA was used to obtain cDNA using the RevertAid Reverse Transcriptase and random primers (Thermo Fisher Scientific), following the manufacturer's instructions. These cDNAs were then used to perform real-time PCRs using specific primers (Table S1) and the FastStart Essential DNA Green Master reagents as described above. Expression levels were also normalized to the relative expression of trout EF-1α as described for the B cell related genes.

2.4. Isolation of skin and gill leukocytes

To isolate skin leukocytes, a piece of skin of approximately 6 cm^2 was carefully collected from each side of the fish and placed on a Petri dish with 2 ml of Leibovitz medium (L-15, Invitrogen, USA) supplemented with an antibiotic mixture consisting of 100 I.U./ml penicillin and 100 μg/ml streptomycin (P/S, Life Technologies, USA) and 5% fetal calf serum (FCS, Life Technologies). All muscle tissue was carefully removed, and the skin cut into small pieces and transferred to a tube containing L-15 medium supplemented with P/S, 5% FCS and 2 mg/ml dispase (Thermo Fisher Scientific). After 2 h at 4 °C in continuous agitation, the samples were pressed 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 15 min). Clarified skin cell suspensions were placed onto 30/51% discontinuous Percoll (Merck, Germany) 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 (Merck) exclusion.

To isolate gill leukocytes, each gill arch was cut and placed in a tube containing L-15 supplemented with P/S and 2% FCS. Gills were carefully washed and transferred to a new tube with fresh medium of the same composition, where they were thoroughly pressed through a 100 μm nylon cell strainer to obtain the cells suspension. The suspension obtained was washed as previously described for skin leukocytes to eliminate excessive debris. Likewise, gill leukocytes were separated using a discontinuous Percoll gradient (30/51%) and the layers containing leukocytes obtained after centrifugation collected, washed and counted as described above.

2.5. Flow cytometry

For the characterization of gill and skin B cells, isolated leukocytes were stored in continuous agitation at 4 °C overnight in L-15 medium containing 5% FBS and P/S and stained the day after with anti-trout IgD (mAb mouse IgG1 coupled to APC; 5 μg/ml) and anti-trout IgM (1.14 mAb mouse IgG1 coupled to R-phycoerythrin (R-PE); 0.25 μg/ml) diluted in staining buffer (phenol red-free L-15 medium supplemented with 2% FBS and P/S) for 1 h in darkness at 4 °C. All antibodies were fluorescently labeled using R-PE or APC Lightning-Link labeling kits (Innova Biosciences, United Kingdom) following the manufacturer's instructions. Finally, cells were washed and resuspended in staining buffer for their analysis in a FACS Celesta flow cytometer (BD Biosciences, USA) using a BD FACSDiva software (BD Biosciences). Data from the flow cytometer were analyzed using the software FlowJo® v.10 (FlowJo LLC, Tree Star). In all cases, cell viability was checked using 4′,6-diamine-2′-phenylindole dihydrochlorid (DAPI) at 0.2 μg/ml.

2.6. Analysis of total and specific IgM secreting cells in skin and gills

The number of total and specific IgM-secreting B cells in skin and gill leukocytes obtained from *Y. ruckeri*-infected and mock-infected fish was determined through ELISpot. To determine total IgM-secreting cells, 5×10^3 skin or gill leukocytes isolated as described above and resuspended in L-15 medium with P/S and 5% FCS, were transferred to ELISpot plates previously coated with anti-IgM. For the coating, ELISpot plates (Merck) were activated with 70% ethanol for 5 min, then washed with sterile Milli-Q water, and incubated overnight at 4 °C with an anti-trout IgM mAb (2 μg/ml). Thereafter, wells were washed 5 times with PBS (Sigma Aldrich) and incubated with 2% bovine serum albumin (BSA, Sigma Aldrich) in PBS for 2 h at RT, to block non-specific binding. Leukocytes were then transferred to the ELISPOT plates and incubated for 24 h at 20 °C. Thereafter, leukocytes were removed, and wells washed 5 times with PBS and blocked with PBS with 2% BSA for 1 h at RT. Biotinylated anti-trout IgM conjugated to biotin using the Biotin Conjugation Kit Lightning-Link (Innova Biosciences) was then added to the wells (1 μg/ml) and incubated for 1 h at RT, followed by several washing steps with PBS. Finally, the plates were developed by using streptavidin-HRP (Thermo Fisher Scientific) at 100 ng/ml for 1 h at RT, washed again with PBS, and incubated with 3-amino 9-ethylcarbazole (Sigma Aldrich) for 30 min at RT in the dark. Substrate reaction was stopped by washing the plates with distillated water. Once the membranes had dried, they were digitally scanned, and the number of spots determined using an AID iSpot Reader System (Autoimmun Diagnostika GMBH, Germany).

To determine the number of cells secreting *Y. ruckeri*-specific IgM, the protocol followed was the same but 5×10^4 leukocytes were transferred to each well, and the plates were coated with 100 μl of *Y. ruckeri* (2.8×10^8 cfu/ml) that had been previously heat-inactivated (60 °C for 1 h).

2.7. Statistics

Data were analyzed using GraphPad software (GraphPad Prism v8.0.1, La Jolla California, USA). Data from flow cytometry and ELISpot were presented as mean + SEM, while those from real time PCR analysis as mean + SD. 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 $p \leq 0.001$. Prior to analysis, data were checked for normality using the Shapiro-Wilk test and q-q plots. A two-tailed Student's *t*-test was used for normally distributed data, whereas non-normally distributed data was compared with a non-parametric Wilcoxon matched-pairs signed-rank test.

3. Results

3.1. Effect of Y. ruckeri on the transcription of genes related to B cell function in the kidney

We analyzed the levels of transcription of a series of genes related to B cell function in fish exposed to inactivated or live *Y. ruckeri* cells and compared them to those of control fish exposed to culture media alone. We included *il1b* as an indicator of global inflammation, the secreted and membrane forms of all Ig isotypes, genes related to B cell differentiation (*prdm1a-1*, *prdm1a-2*, *prdm1c-1* and *prdm1c-2*, *irf4* and *pax5*) as well as cytokines involved in B cell activation (*baff*, *april* and *balm*) and

their receptors (*baff-r*, *taci* and *bcma*). As an initial step, we also estimated the bacterial load by amplification of the *Y. ruckeri* 16S RNA in all tissue samples obtained from fish exposed to the live bacteria after 4 or 7 days post-infection. The results obtained indicated that the bacteria was detected in all four tissues sampled at days 4 and 7 post-infection (Fig. S1). At day 4 post-infection, the bacterial load was significantly higher in systemic tissues than in mucosal surfaces (Fig. S1). Although these differences were maintained at day 7 post-infection, differences were no longer significant due to the large variability observed between fish (Fig. S1). No bacterial 16S RNA was ever detected in control uninfected fish.

In the kidney, already after 2 days post-infection, the live bacteria was able to significantly up-regulate *il1b*, *prdm1a-1*, *prdm1c-1*, *prdm1c-2* and *baff* mRNA levels (Fig. 1A). Interestingly, the inactivated bacteria provoked at this point a significant down-regulation of the levels of *april* and *balm* mRNA in the kidney (Fig. 1A). At day 4 post-inoculation, the number of genes regulated in the kidney was much higher. At this moment, the live bacteria significantly increased the transcription of *il1b*, all Ig isotypes (membrane and secreted), *prdm1a-1*, *prdm1c-1*, *prdm1c-2*, *april*, *balm*, *baff-r* and *bcma* (Fig. 1B). The inactivated bacteria, on the other hand, only increased the transcription of secIgM, memIgD, *irf4*, *balm* and *bcma* at this point (Fig. 1B). At day 7 post-challenge, only *baff-r* mRNA levels were significantly increased in the kidney of fish exposed to the live bacteria when compared to controls (Fig. 1C).

3.2. Effect of *Y. ruckeri* on the transcription of genes related to B cell function in the spleen

Many genes were transcriptionally up-regulated at day 2 post-exposure in the spleen in response to live *Y. ruckeri*. These included

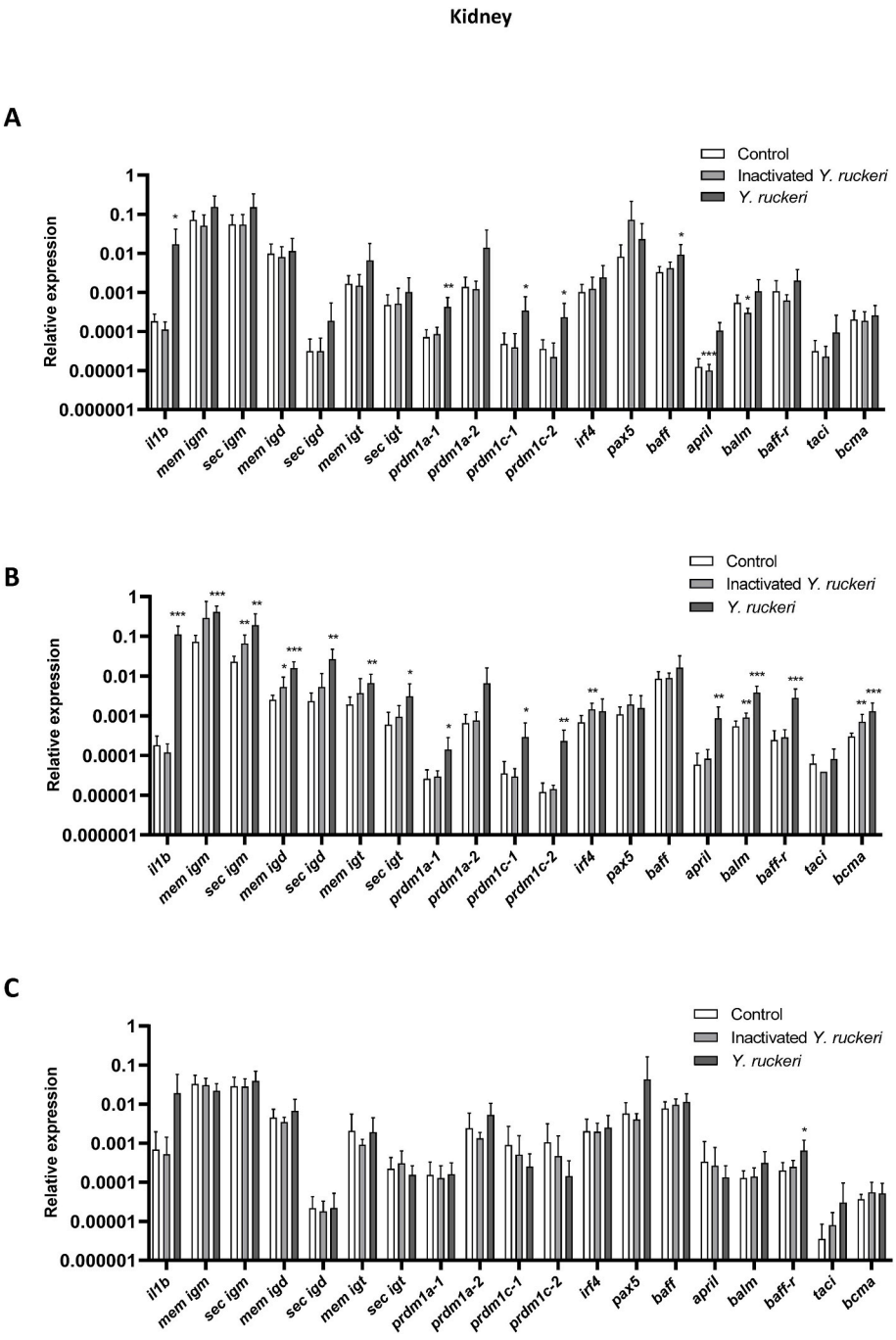

Fig. 1. Effect of *Y. ruckeri* on the transcription of genes related to B cell function in the kidney. Rainbow trout were exposed to inactivated or live *Y. ruckeri* cells (strain Yr 026-01/15) by bath as described in the Materials and Methods section. Fish exposed to culture media alone were also included as controls. At days 2 (A), 4 (B) and 7 (C) post-exposure, rainbow trout were euthanized and the kidney collected to analyze the levels of transcription of different genes related to B cell function by real time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF1-α + SD ($n = 10$). Asterisks denote significantly different transcription levels than those of control fish (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$).

il1b, *prdm1a-1*, *prdm1a-2*, *irf4*, *baff*, *april*, *balm*, *baff-r* and *taci* (Fig. 2A). Additionally, these animals showed decreased mRNA levels of memIgM in the spleen when compared to control fish (Fig. 2A). At this point, only *pax5* was transcriptionally down-regulated in the spleen by the inactivated bacteria (Fig. 2A). At day 4, some of the genes regulated at day 2 by the live bacteria were still transcribed in the spleen at levels significantly higher than those of control fish. These included *il1b*, *prdm1a-1*, *prdm1a-2*, *irf4* and *april*. Additional genes that were up-regulated at this point in infected fish when compared to control fish were *prdm1c-1* and *prdm1c-2* (Fig. 2B). Interestingly, at this point, a number of genes were transcriptionally down-modulated in the spleen of infected fish when compared to controls. These were memIgM, memIgD, secIgD, memIgT, *pax5*, *baff*, *balm*, *taci* and *bcma* (Fig. 2B). As occurred before, only slight transcriptional changes were induced in the spleen by the inactivated bacteria. Thus the spleen of fish exposed to inactivated *Y. ruckeri* had *il1b*, *prdm1a-1, prdm1a-2* and *baff-r* mRNA levels significantly lower levels than those of control fish (Fig. 2B). As happened in the kidney, the number of genes transcriptionally regulated in the spleen by exposure to the bacteria was lower after 7 days. At this point, the mRNA levels of *prdm1c-1, prdm1c-2* and *april* were significantly higher in the spleen of infected fish, when compared to controls, whereas the memIgD, secIgD, memIgT, *irf4* and *balm* mRNA levels in the spleen of infected fish were significantly lower than those of controls (Fig. 2C). At this time point, the inactivated bacteria provoked a down-modulation of memIgD and memIgT, as well as the up-regulation of *prdm1c*-1 mRNA levels (Fig. 2C).

3.3. Effect of *Y. ruckeri* on the transcription of genes related to B cell function in the skin

To determine how bacterial exposure modulated B cells in mucosal surfaces, we studied the transcription of all these genes in the skin of infected and mock-infected fish. At day 2 post-exposure, the skin of fish infected with *Y. ruckeri* transcribed *il1b* at levels significantly higher than that of control fish (Fig. 3A), while it transcribed memIgD, memIgT

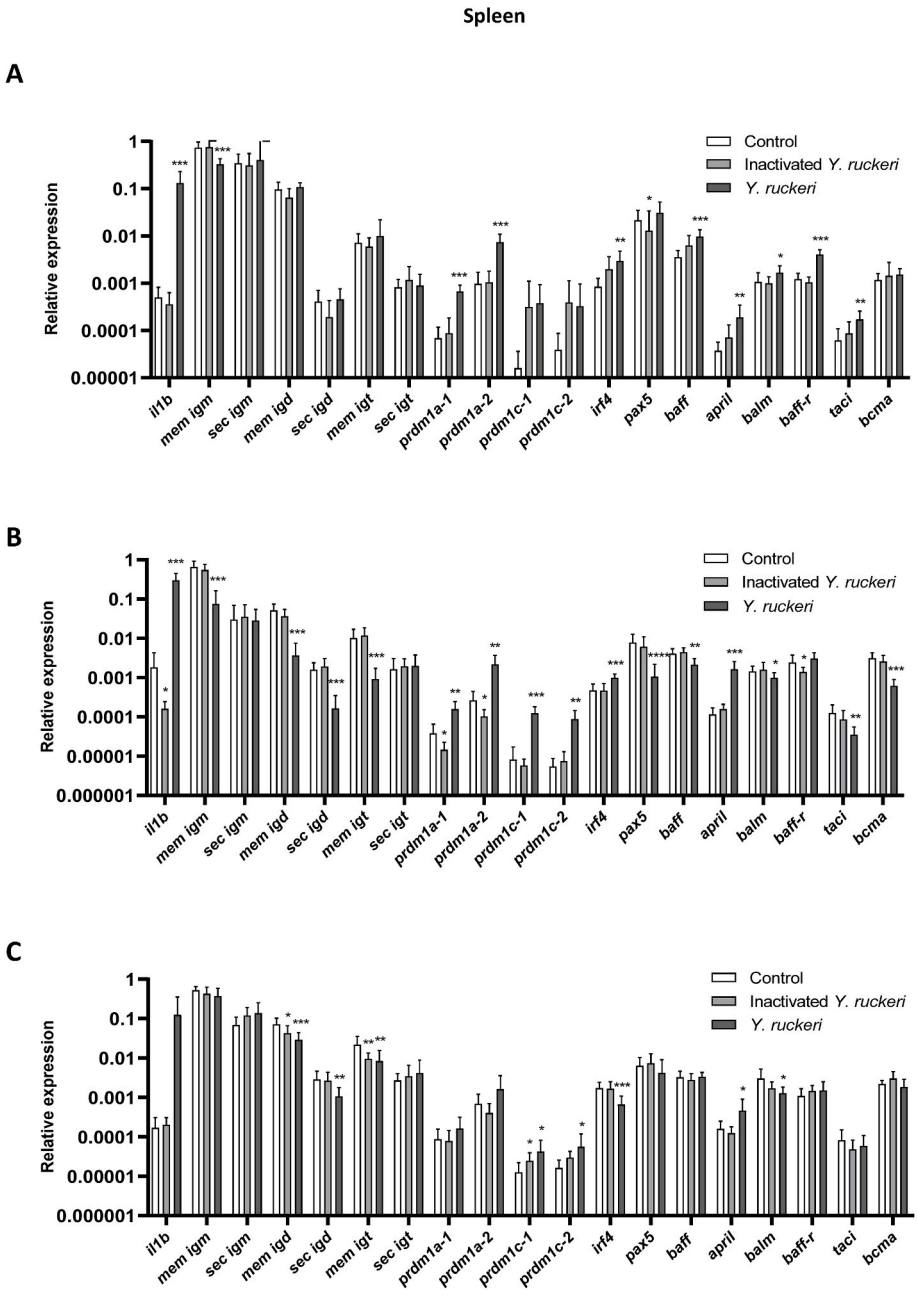

Fig. 2. Effect of *Y. ruckeri* on the transcription of genes related to B cell function in the spleen. Rainbow trout were exposed to inactivated or live *Y. ruckeri* cells (strain Yr 026-01/15) by bath as described in the Materials and Methods section. Fish exposed to culture media alone were also included as controls. At days 2 (A), 4 (B) and 7 (C) post-exposure, rainbow trout were euthanized and the spleen collected to analyze the levels of transcription of different genes related to B cell function by real time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF1-α + SD ($n = 10$). Asterisks denote significantly different transcription levels than those of control fish (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$ and ****$p \leq 0.001$).

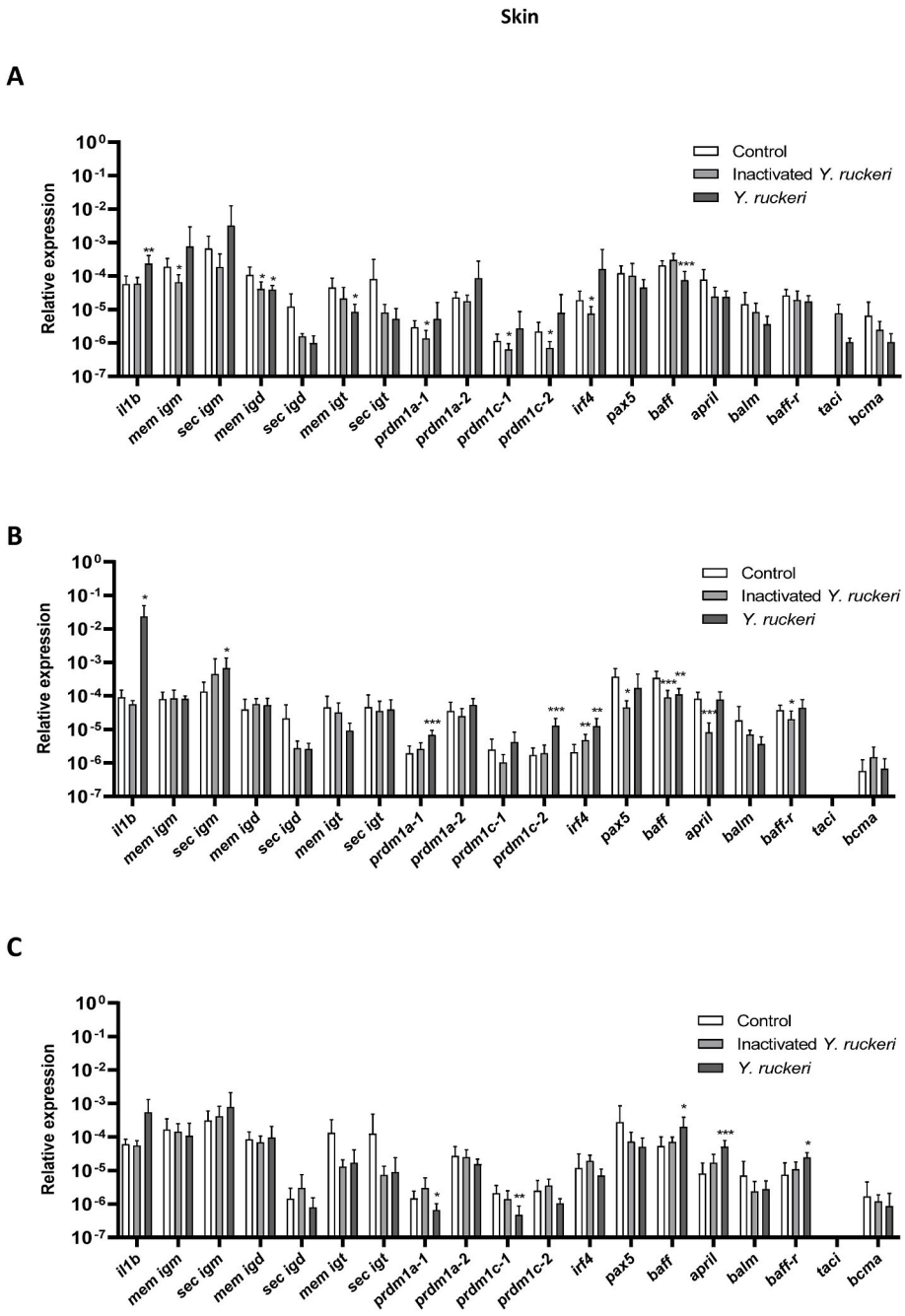

Fig. 3. Effect of *Y. ruckeri* on the transcription of genes related to B cell function in the skin. Rainbow trout were exposed to inactivated or live *Y. ruckeri* cells (strain Yr 026-01/15) by bath as described in the Materials and Methods section. Fish exposed to culture media alone were also included as controls. At days 2 (A), 4 (B) and 7 (C) post-exposure, rainbow trout were euthanized and skin collected to analyze the levels of transcription of different genes related to B cell function by real time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF1-α + SD ($n = 10$). Asterisks denote significantly different transcription levels than those of control fish (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.005$).

and *baff* at levels significantly lower than those of controls (Fig. 3A). At this point, the inactivated bacteria provoked the down-modulation of memIgM, memIgD, *prdm1a-1*, *prdm1c-1*, *prdm1c-2* and *irf4* mRNA levels (Fig. 3A). After 4 days, fish exposed to live *Y. ruckeri* up-regulated *il1b*, secIgM, *prdm1a-1*, *prdm1c-2*, *irf4* RNA levels and down-modulated *baff* mRNA levels in the skin, when compared to controls (Fig. 3B). At this moment, fish exposed to the inactivated bacteria up-regulated *irf4* and down-modulated *pax5*, *baff*, *april* and *baff-r* in the skin, when compared to controls (Fig. 3B). At day 7 post-infection, live *Y. ruckeri* provoked an up-regulation of *baff*, *april* and *baff-r* transcription in the skin, along with a down-modulation of *prdm1a-1* and *prdm1c-1* transcription (Fig. 3C). No significant effects were provoked in the skin by the inactivated bacteria at this point (Fig. 3C).

3.4. Effect of Y. ruckeri on the transcription of genes related to B cell function in the gills

Finally, we also studied the levels of transcription of genes related to B cell function in the gills. At day 2 post-infection, *Y. ruckeri* provoked an up-regulation of *il1b*, *prdm1a-1*, *prdm1a-2*, *prdm1c-1*, *prdm1c-2* and *baff-r* mRNA levels in the gills when compared to control fish (Fig. 4A). The inactivated bacteria, however, only up-regulated *il1b* and *prdm1c-2* transcription in the gills at this time point (Fig. 4A). At day 4, *il1b*, *prdm1c-1* and *prdm1c*-2 mRNA levels were still up-regulated in the gills of infected fish, along with secIgT (Fig. 4B). At this point, the levels of transcription of *balm* and *baff-r* in the skin of infected fish were significantly lower than controls (Fig. 4B). No genes were modulated in the gills by the inactivated bacteria at day 4 nor at day 7 post-exposure (Fig. 4B and C). At day 7, the live bacteria provoked the significant up-regulation of *il1b*, secIgT, *prdm1a-2*, *prdm1c-2*, *baff* and *balm* mRNA

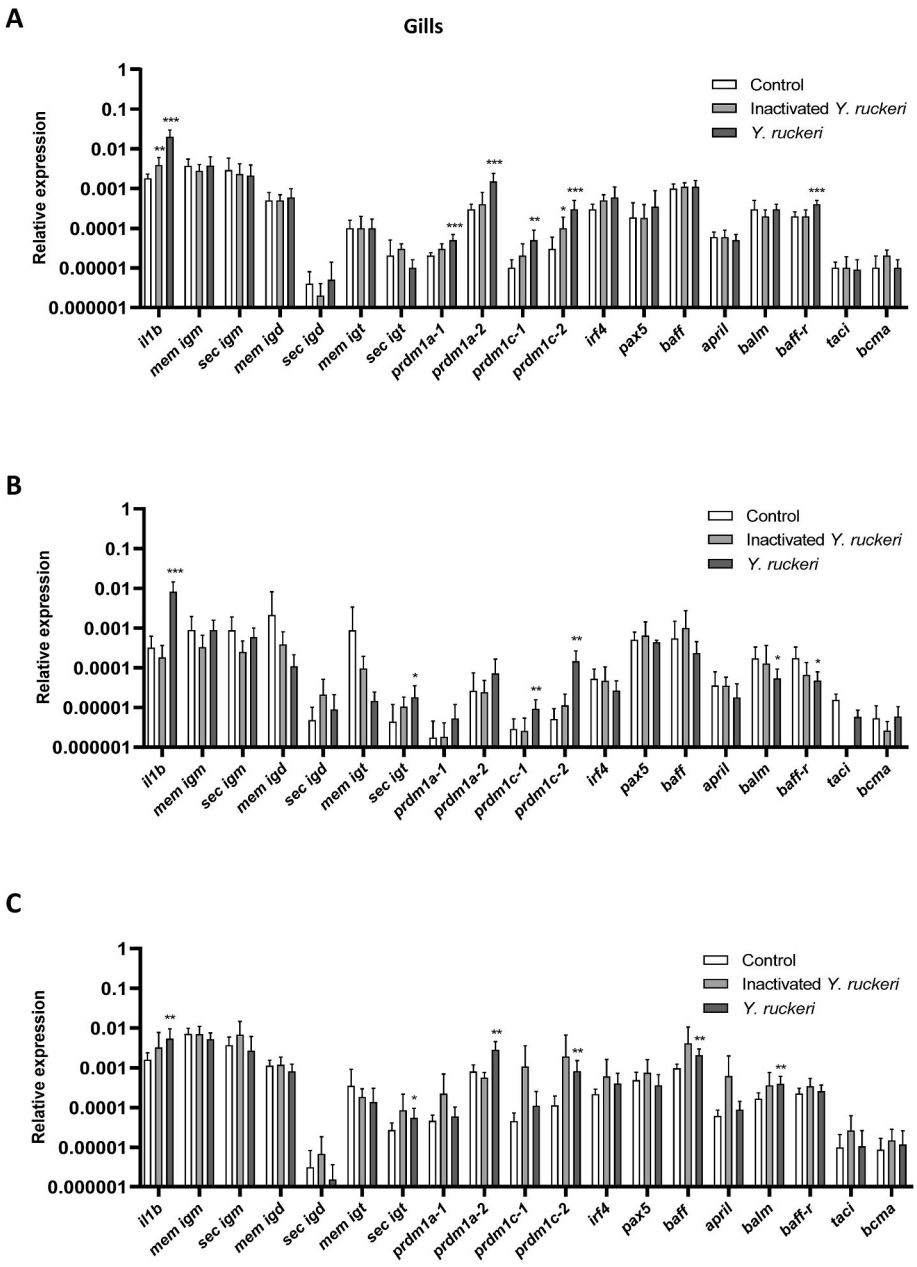

Fig. 4. Effect of *Y. ruckeri* on the transcription of genes related to B cell function in the gills. Rainbow trout were exposed to inactivated or live *Y. ruckeri* cells (strain Yr 026-01/15) by bath as described in the Materials and Methods section. Fish exposed to culture media alone were also included as controls. At days 2 (A), 4 (B) and 7 (C) post-exposure, rainbow trout were euthanized and gills collected to analyze the levels of transcription of different genes related to B cell function by real time PCR. Data are shown as the mean relative gene expression normalized to the transcription levels of the housekeeping gene EF1-α + SD ($n = 10$). Asterisks denote significantly different transcription levels than those of control fish (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.005$).

levels in the gills (Fig. 4C).

3.5. Effect of Y. ruckeri on IgM $^{+}$ B cells in skin and gills

After having analyzed how *Y. ruckeri* affected the transcription of genes related to B cell function in skin and gills, we studied by flow cytometry how the bacterial infection modulated the number of B cells in these mucosal tissues. For this, fish were infected with the live bacteria or mock-infected. At days 3 and 5 post-infection, fish from both groups were sacrificed, and skin and gill leukocytes isolated to determine the percentage of $IgM^{+}IgD^{-}$ and $IgD^{+}IgM^{-}$ B cells by flow cytometry. The percentage of $IgM^{+}IgD^{-}$ B cells among skin leukocytes was not significantly modified by the infection (Fig. 5A, Fig. S2). In gills, however, there was a significant decrease in the percentage of $IgM^{+}IgD^{-}$ B cells provoked by *Y. ruckeri* at day 3 post-infection (Fig. 5A, Fig. S2). Interestingly, the size of $IgM^{+}IgD^{-}$ B cells in the skin significantly increased in response to the bacterial infection at both days sampled (Fig. 5B, Fig. S2). In the gills, this increase in size was only significant at day 3 post-infection (Fig. 5B, Fig. S2). $IgD^{+}IgM^{-}$ B cells were also identified in skin and gills leukocytes (Fig. S2), as previously described [13]. However, in this case, the percentage of this B cell subset and the size of these cells did not vary throughout the infection in skin nor gill (Figs. S2 and S3).

3.6. Effect of Y. ruckeri on IgM-secreting cells in skin and gills

Finally, we also determined how the bacterial infection affected the local differentiation of B cells to IgM-secreting cells (plasmablasts/plasma cells). For this, we quantified the number of cells secreting total and specific IgM among skin and gill leukocytes obtained at different times post-infection. Surprisingly, the number of cells secreting total IgM decreased in response to infection with *Y. ruckeri* both in skin and gills, with numbers being significantly lower than those of control fish in skin at day 5 post-infection (Fig. 6A, Fig. S4A). Nonetheless, the number of cells secreting *Y. ruckeri*-specific IgM significantly increased at day 5 post-infection in both skin and gills (Fig. 6B, Fig. S4B).

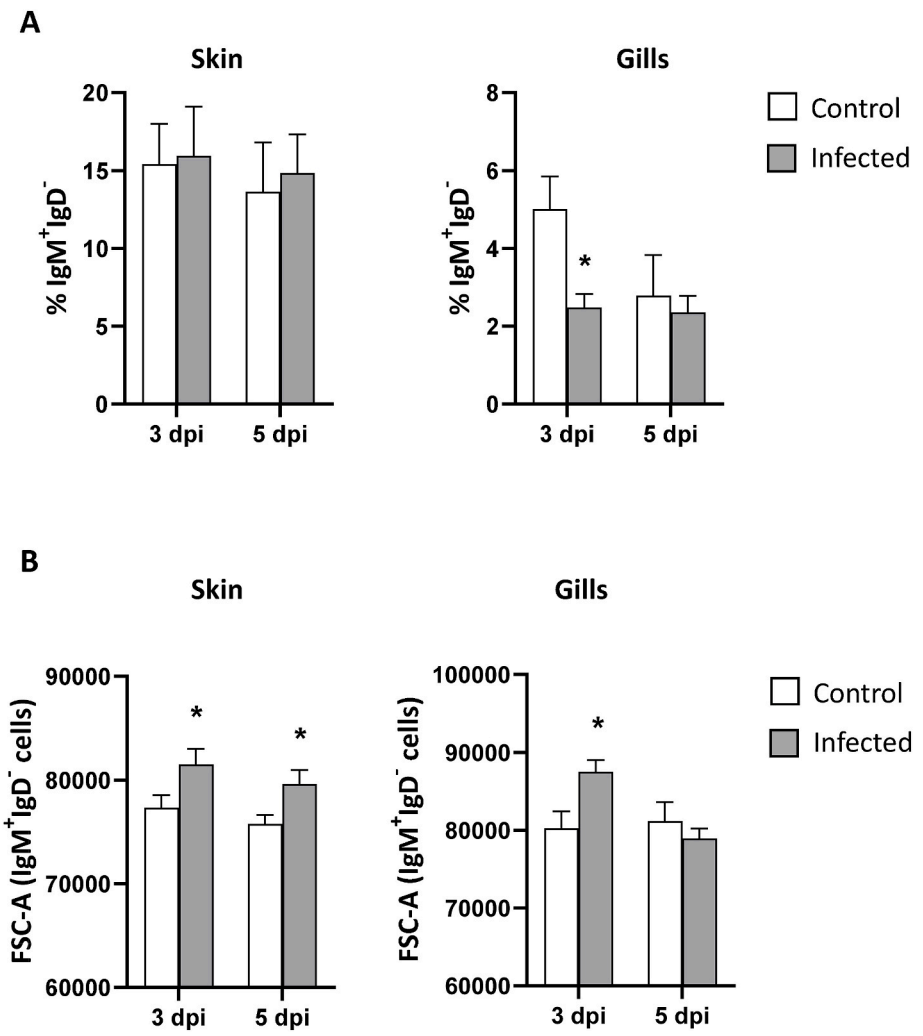

Fig. 5. Effect of *Y. ruckeri* infection on $IgM^{+}IgD^{-}$ B cell abundance and size in rainbow trout skin and gills. Rainbow trout were infected with *Y. ruckeri* (strain Yr 026-01/15) by bath as described in the Materials and Methods section. Fish exposed to culture media alone were included as controls. At days 3 and 5 post-infection, rainbow trout were euthanized and skin and gills collected to isolate total leukocytes. The percentage of $IgM^{+}IgD^{-}$ B cells in total leukocyte cultures was estimated by flow cytometry (A). Data are shown as the mean percentage of singlet, live (DAPI negative) $IgM^{+}IgD^{-}$ B cells (mean + SEM; $n = 7$). The size of these cells was also estimated and is shown as mean fluorescence intensity (MFI) of FSC (mean + SEM; n = 7) (B). Asterisks denote significant differences between infected and control fish ($*p \leq 0.05$).

4. Discussion

Because mucosal surfaces are continuously exposed to a high load of microorganisms present in the aquatic medium, they constitute the first immunological barrier to prevent pathogen entry inside the host. The first line of defense is orchestrated by innate immune elements focused at eliminating the pathogens at the entry site. Nonetheless, adaptive immune elements present in these mucosal tissues, also contribute to this initial clearance and shape further local adaptive responses with consequent systemic effects [5]. However, as fish lack organized lymphoid structures such as those present in mammalian mucosae, the key question remains as to how fish B cells mount mucosal adaptive responses in the absence of these structures in which B and T cells closely interact.

Although the presence of the three fish Ig isotypes has been demonstrated in mucosal secretions from both gills and skin [2,16,24], studies characterizing specific B cell response in these mucosal tissues are very scarce. In the skin, different Igs seem to be differently implicated in the response to pathogens. For example, when rainbow trout is infected with the ciliate parasite *Ichthyophtirius multifiliis* (Ich), IgT responses and IgT^{+} B cells were shown to be prevailing in the skin [15]. In that work, although the systemic responses to the parasite were mainly IgM, the skin IgM responses were negligible when compared to those of IgT [15]. However, a subsequent study performed with this same parasite but sampling at earlier time points, reported significant transcriptional up regulations of IgT, IgD and IgM in the skin, although again IgT responses were higher [25]. Another study performed in Atlantic salmon exposed to sea lice (*Lepeophtheirus salmonis*) also reported the up-regulation of both IgM and IgT transcription in the skin in response to the parasite [26]. The involvement of IgD in skin local responses to pathogens was clearly demonstrated in rainbow trout affected by red mark syndrome (RMS), a skin disease thought to be caused by a *Midichloria*-like bacteria. Rainbow trout affected by RMS infection increased IgD^{+} B-cells 61 and 82 days post-cohabitation, while IgM^{+} B-cells increased 82 and 97 days post-cohabitation. In this study, skin IgT^{+} B-cells were scarce and remained unchanged during the whole trial [27]. Therefore, it seems that depending on the pathogen and the time post-exposure, different Igs can be involved in skin immune responses, yet the exact role that each of these Ig plays remains to be elucidated.

In gills, the response to Ich infection was found to be similar to that of skin. Hence, locally, the response was dominated by IgT and IgT^{+} cells, whereas at systemic level, the response was mainly IgM [16]. A similar prevailing IgT response was obtained in response to infection with *Flavobacterium columnare* [28]. In contrast, specific IgM production was demonstrated in the gill mucus of grass puffer (*Takifugu niphobles*) in response to the monogenean ectoparasite *Heterobothrium okamotoi* [29]. Additionally, many other studies have reported the up-regulation of the three different Igs at a transcriptional level in response to a range of pathogens [30–32]. Although these transcriptional studies demonstrate the implication of different B cell subsets in the immune response elicited, they do not demonstrate a specific response to these pathogens.

In the current work, we have focused exclusively on cells of the IgM/

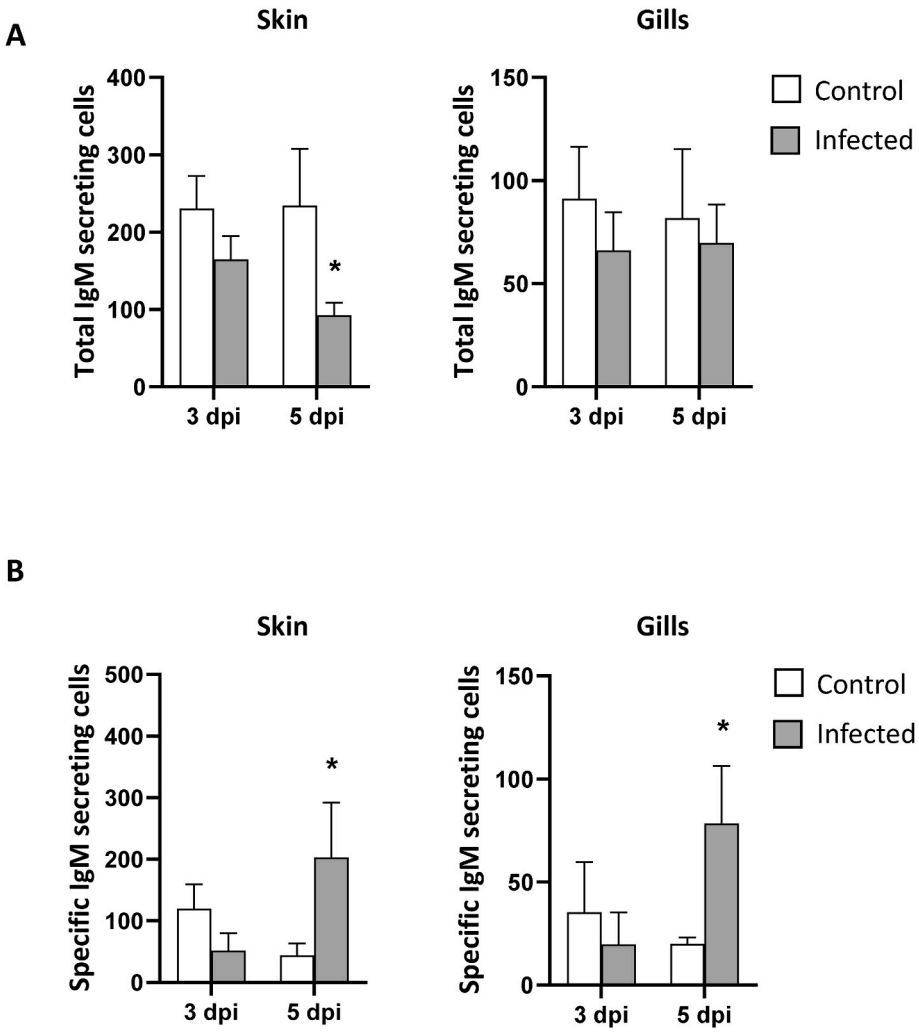

Fig. 6. Effect of *Y. ruckeri* infection on the number of cells secreting total or *Y. ruckeri*-specific IgM in rainbow trout skin and gills. Rainbow trout were infected with *Y. ruckeri* (strain Yr 026-01/15) by bath as described in the Materials and Methods section. Fish exposed to culture media alone were included as controls. At days 3 and 5 post-infection, rainbow trout were euthanized and skin and gills collected to isolate total leukocytes. The number of cells secreting total (A) or *Y. ruckeri*-specific (B) IgM was then determined by ELISpot. Results are shown as the mean number of IgM-secreting cells per 5×10^3 cells + SEM in the case of total IgM and the mean number of IgM-secreting cells per 5×10^4 cells + SEM in the case of *Y. ruckeri*-specific IgM (n = 7). Asterisks denote significant differences between infected and control fish ($^*p \leq 0.05$).

D lineage. Among them, in both rainbow trout skin and gills, IgM^+IgD^+ populations were reported to be scarce, while IgM^+IgD^- and IgD^+IgM^- populations constituted the main IgM/D-bearing populations [13]. Interestingly, that study also revealed that these populations had already started a differentiation process towards plasmablasts/plasma cells when compared to naïve blood IgM^+IgD^+ populations [13]. In the current study, we have demonstrated that in the first stages of an infection with *Y. ruckeri*, IgD^+IgM^- B cells from skin or gills do not seem affected in percentage nor size. In contrast, IgM^+IgD^- B cell populations significantly increase in size in response to the bacteria, suggesting a further differentiation, as B cells are known to increase in size along the differentiation process to plasmablasts/plasma cells [8,9]. Additionally, some of the genes previously associated with a B cell differentiation process, both in mammals [19,33] and fish [20,34] such *prdm1 or irf4* are also further up-regulated in both mucosal surfaces in response to *Y. ruckeri.* These included two rainbow trout homologues of the mammalian *prdm1* gene (*prdm1a-1* and *prdm1c-2*) and *irf4* in the skin. In the gills, all four homologues of the mammalian *prdm1* gene (*prdm1a-1*, *prdm1a-2*, *prdm1c-1* and *prdm1c-2*) were significantly up-regulated in response to the infection. Surprisingly, in some cases, the up-regulation of *prdm1a* genes did not go along with increased levels of mRNA of secreted IgM or down-regulated *pax5* mRNA levels, suggesting that other cell types could also account for *prdm1a* or *pax5* transcription. Nonetheless, the overall transcriptional profile and the increase in size of IgM^+IgD^- B cells suggests that these cells that were already in a more differentiated state than naïve blood B cells, are able to differentiate further in skin and gills at the early stages of a bacterial infection. Interestingly, the total number of IgM-secreting cells was not significantly increased in these tissues, and it was exclusively the number of cells secreting *Y. ruckeri*-specific IgMs that was strongly augmented in infected fish. These specific responses were detected as early as 5 days post-infection, demonstrating that fish only take a few days to locally organize a specific IgM response aimed at blocking the pathogen at the entry site. Of course, the kinetics of IgM response in fish is greatly affected by temperature, as previously reported [35], hence, this specific IgM response to *Y. ruckeri* might have taken longer if we had performed the infection at lower temperatures.

Although B cell responses were also studied by flow cytometry and ELISpot at skin and gills, the transcriptional study conducted was also undertaken in spleen and head kidney. In both of these systemic immune organs, transcriptional changes in genes related to B cell function were also evident, including Ig genes, genes associated with B cell differentiation and also cytokines from the TNF superfamily and their receptors, which have been shown to play a role in B cell activation in rainbow trout [36–38]. All these changes suggest that B cell differentiation also takes place in distal systemic tissues, in addition to the local differentiation of B cells in skin and gills. Interestingly, the involvement of cytokines of the TNF superfamily and their receptors seemed to be lower in mucosal than in systemic compartments, since the number of these genes transcriptionally regulated in skin and gills was much lower than that of spleen and kidney. These results seem to indicate that these cytokines and their receptors are not as implicated in the differentiation of

mucosal B cells as they are in that of systemic B cells, yet this is something that should be further investigated. Nonetheless, it also has to be taken into consideration, that at days 4 and 7 post-infection, the bacterial load detected in systemic tissues is higher levels than that of mucosal tissues. On another note, the up-regulation of BAFF mRNA levels detected in the spleen in response to *Y. ruckeri* contrasts the down-regulation of BAFF mRNA levels detected in response to this same pathogen in rainbow trout after an intraperitoneal injection [39], suggesting that the route of infection is key in determining the response to a certain pathogen.

In conclusion, the transcriptional study of a wide range of genes related with B cell function strongly suggested that *Y. ruckeri* infection provokes systemic and local changes in B cells. In skin and gills, although $IgD^{+}IgM^{-}$ B cells were not affected by the infection at the time points sampled in number or size, early IgM responses to the bacteria were evident. Although the number of $IgM^{+}IgD^{-}$ cells and the number of cells secreting total IgM remained unaltered, these cells significantly increased their size and the number of cells secreting *Y. ruckeri*-specific IgM augmented. Altogether, these results reveal the capacity of fish mucosal IgM^{+} B cells to mount local specific responses at very early time points.

CRediT authorship contribution statement

J.G. Herranz-Jusdado: Data curation, Formal analysis, Methodology, Writing – original draft. **E. Morel:** Data curation, Formal analysis, Methodology, Writing – original draft. **M.C. Ordás:** Methodology, Writing – review & editing. **D. Martín:** Methodology, Writing – review & editing. **F. Docando:** Methodology. **L. González:** Methodology. **E. Sanjuán:** Methodology. **P. Díaz-Rosales:** Conceptualization, Formal analysis, Writing – review & editing. **M. Saura:** Conceptalization. **B. Fouz:** Conceptualization, Writing – review & editing. **C. Tafalla:** Conceptualization, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.108989.

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