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A BCWD-resistant line of rainbow trout exhibits higher abundance of IgT⁺ B cells and heavy chain tau transcripts compared to a susceptible line following challenge with *Flavobacterium psychrophilum*

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Abstract

Bacterial Cold Water Disease (BCWD) is a common, chronic disease in rainbow trout, and is caused by the gram-negative bacterium *Flavobacterium psychrophilum* (*Fp*). Through selective breeding, the National Center for Cool and Cold Water Aquaculture has generated a genetic line that is highly resistant to *Fp* challenge, designated ARS-Fp-R (or R-line), as well as a susceptible “control” line, ARS-Fp-S (S-line). In previous studies, resistance to *Fp* had been shown to correlate with naïve animal spleen size, and further, naïve R-line trout had been shown to have a lower abundance of IgM⁺ and IgM⁺⁺ cells compared to S-line fish. Here we wished to first determine whether the abundance of IgT⁺ and/or IgT⁺⁺ cells differed between the two lines in naïve fish, and if so, how these patterns differed after *in vivo* challenge with *Fp*. *Fp* challenge was by intramuscular injection of live *Fp* and tissue collections were on days 5, 6, and/or 28 post-challenge, in two independent challenge experiments. Flow cytometric and gene expression analyses revealed that naïve R-line fish had a higher abundance of IgT⁺ B cells in their anterior kidney, spleen, and blood, compared to S line fish. Further, that after *Fp* challenge, this difference was maintained between the two lines. Lastly, abundance of IgT⁺ B cells and expression of *secHCtau* correlated with lower *Fp* pathogen loads in challenged fish. In the anterior kidney, IgM⁺ B cell abundance correlated with increased *Fp* loads. Together, these results suggest that IgT⁺ B lineage cells may have a protective function in the immune response to *Fp*.

INTRODUCTION

Bacterial Cold Water Disease (BCWD) is a common, chronic disease in rainbow trout, and is caused by the gram-negative bacterium *Flavobacterium psychrophilum* (*Fp*). Through

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selective breeding, the National Center for Cool and Cold Water Aquaculture (NCCCWA) has generated a genetic line that is highly resistant to Fp challenge, designated ARS-Fp-R (or R-line), and in addition, a reference susceptible line, ARS-Fp-S (S-line) is available for phenotypic comparisons (Silverstein et al., 2009), (Leeds et al., 2010). (Wiens et al., 2013a) The molecular mechanisms that drive increased resistance to Fp remain unknown, but studies so far have revealed several immune components that differ between the lines. R-line fish have lower pathogen loads after challenge with *Fp* by intraperitoneal injection (Hadidi et al., 2008) and exhibit higher survival by 5–10 days post-infection (Wiens et al., 2013a) suggesting enhanced capacity for pathogen control relative to the S-line. Resistance to Fp has also been correlated with naive animal spleen size (Hadidi et al., 2008), with Fp-resistant trout having larger spleens (Wiens et al., 2013b)(Wiens et al., 2013c), although its functional significance remains unclear, as splenectomized animals do not show changes in resistance to Fp (Wiens et al., 2015). We have also reported on differences in humoral immunity between the lines, with naïve R-line trout having lower abundance of IgM⁺ and IgM⁺⁺ cells (ISCs) compared to S-line fish (Zwollo et al., 2015).

Rainbow trout rely heavily on the IgM isotype, which is the most prevalent Ig in plasma, but they also produce IgT/Z (Hansen et al., 2005a), and IgD (Wilson et al., 1997). IgM is the most abundant isotype in teleosts, is essential for both systemic immunity and for mucosal responses, and is the best-studied class of Igs (Salinas et al., 2011). IgT/Z is functionally equivalent to immunoglobulin alpha (IgA) in mammals and functions as part of the mucosal defense against pathogens. While IgM and IgD can be co-expressed on a given B cell, this has not been observed for IgM and IgT, and it has been proposed that IgM and IgT represent two different B lineages (Zhang et al., 2010).

A total of three igt genes has been described in the rainbow trout, including igt1, igt2 and igt3, encoding IgT1, IgT2, and IgT3 (Zhang et al., 2017). Each of these genes has a different expression pattern. igt1 was found to be expressed both in systemic and mucosal tissues, with highest expression in blood and spleen, followed by anterior kidney and gut; igt2 was expressed mostly in systemic tissues, with highest expression levels in thymus and blood, but virtually absent from anterior kidney, gut and skin; igt3 levels were undetectable by PCR except in spleen, but IgT3 Ig could be detected in serum (Zhang et al., 2017). *In vivo* challenge with LPS, but not poly(I:C) resulted in upregulation of igt1 and igt2 in gut, but effects on the spleen were minor. A monoclonal antibody has been generated (Zhang et al., 2010) that recognizes all three isotypes of IgT (Zhang et al. 2017). A few gene-expression studies have reported on the roles of IgT in response to bacterial pathogens in recent years. In one study, a protective role for IgT against Fp was suggested because secreted heavy chain tau1 (HCtau1) levels increased in the gills following immersion or anal intubation with Fp (Makesh et al., 2015).

No studies to date have yet determined whether cellular abundance of IgT⁺ B lineage cells in rainbow trout plays roles in immune protection to Fp challenge. To begin to address the immunological basis of Fp-resistance, here we determined whether S-line and R-line fish differed in their abundance of IgT⁺ and IgM⁺ B cells and IgT⁺⁺ and IgM⁺⁺ secreting cells, both before and after Fp challenge, and further, whether cellular abundance or gene

expression patterns for secreted HC tau1 and HCmu correlated with Fp-loads in infected fish.

MATERIALS AND METHODS

Animals and facilities

All trout were bred at the National Center for Cool and Cold Water Aquaculture (NCCCWA) and reared following NCCCWA Standard Operating Procedures for the Care and Use of Research Animals (Rainbow trout). All fish utilized in this study were naive with respect to *Fp* challenge. Broodstock and progeny were maintained in a specific pathogen-free facility, monitored by twice yearly inspections testing for bacterial and viral pathogens as previously described, and were negative for *F. psychrophilum* (Wiens et al., 2013b). Fish used in these experiments were reared at the NCCCWA until the post-hatch age of 4–6 months, after which they were maintained at The College of William and Mary Trout Facility in 100-gallon tanks with a recirculating system employing biologically-filtered water at 12° C. IACUC committees at each institution approved this study (NCCCWA Protocol #076 and William and Mary protocol 2012-06-14-8016pxzwol) and steps were taken to ameliorate suffering in all work involving experimental trout. Trout used here were spawned either in year class 2013 (YC13) or 2015 (YC15). YC13 fish were challenged in 2014 (Fp2014 challenge experiment) and YC15 were challenged in 2016 (Fp2016 experiment).

In vivo Fp challenge

For the Fp2014 challenge, fish weight averaged 426 grams, and for the Fp2016 experiment, the average weight was 113 grams. For the Fp2016 challenge, on day 0, fish were anesthetized with 100 mg/L MS-222 (Tricaine-S, Western Chemical, Inc., Ferndale, WA) and intramuscularly injected below the cranial aspect of the dorsal fin with either 0.1 ml sterile PBS or 0.1 mL of freshly grown Fp CSF259-93 cultured in TYES, and resuspended in sterile PBS prior to challenge to give a challenge dose of 4.4e7cfu/fish, as determined by plate count. One Fp-challenged S-line fish died before collection on day 5 in the Fp2016 experiment, and 4 Fp-challenged S-line fish died in the Fp2014 experiment before day 6. On each collection day, fish were euthanized with 200 mg/L MS-222 and blood and peritoneal cavity fluid collected in heparinized tubes. Spleen indexes [spleen wt (mg)/body weight (g)] were determined at the time of tissue collection as previously described (Hadidi et al., 2008). Spleen (SPL) and anterior kidney (AK; equivalent to K1, as defined in (Zwollo et al, 2005)) were collected from each fish and placed in 5 mls ice cold RPMI. Cells were also collected from peritoneal cavity fluid (PCF) in RMPI containing heparin. Red blood cells (rbcs) were removed using Histopaque-1077 (Sigma-Aldrich). Live cells were counted using Trypan blue with a hemocytometer. For peripheral blood lymphocytes (PBLs), 25% of each sample was spun separately, cell pellets were resuspended into 1 ml RNazol, and cells stored at –20C until use, and 75% was fixed for use in flow cytometry. The SPL, PCF, and AK cells were fixed for flow cytometric analysis (see below). For the Fp2014 challenge, a similar procedure was followed, but collecting AK, SPL, and posterior kidney (PK; equivalent to K5, as defined in (Zwollo et al, 2005)) cells instead, and only processed for gene expression (RT-qPCR) purposes, for days 6 and 28.

Antibodies

The monoclonal mouse anti-trout immunoglobulin heavy chain mu (HCmu, or I-14; (DeLuca, 1983) was a gift from Dr. Greg Warr. The monoclonal mouse anti-trout immunoglobulin tau antibody (41.8; (Zhang et al., 2010) recognizes IgT1, IgT2, and IgT3 (Zhang et al., 2017) and was a gift from Dr. Oriol Sunyer. The rabbit polyclonal Pax5.PD antibody (previously called ED-1; (Zwollo et al., 2008)) recognizes the paired domain of vertebrate Pax5 and detects trout Pax5 in pre-B through plasmablast stages (McMurray et al, 2013). The rabbit-anti-human RAG1 IgG (H300; recognizing amino-acids 744–1043 of the human RAG protein) was purchased from Santa Cruz Biotech. Isotype control antibodies included rabbit IgG or mouse IgG (eBiosciences) conjugated to Alexa Fluor 555 or Alexa Fluor 647. Antibody aliquots were stored in 1% BSA at -20°C .

Fixation, permeabilization, and flow cytometry

Cells were fixed in 1% ice-cold paraformaldehyde (10% stock, EM-grade; Electron Microscopy Sciences) and permeabilized in 1 mL ice-cold 80% methanol, as described previously (Zwollo et al., 2010). After overnight incubation at -20°C , cells were either resuspended in permeabilizing solution (BD perm wash in PBS, BD Biosciences) and stained as described previously, or refixed and stored in FBS containing 10% DMSO at -80°C (Zwollo et al., 2010, MacMurray 2013). Approximately 30,000 events were acquired per sample using a BD FACSArya (BD Biosciences). Duplicate samples for each staining combo were run for each experiment. Contour graphs were generated using WinMDI 2–8 (J. Trotter 1993–1998) software. Contour graphs are shown as log algorithms with intervals of 50%.

RNA Extraction and cDNA synthesis

Total RNA was isolated using RNeasy Lysis Buffer (Molecular Research Center, Inc). Frozen tissue samples stored in RNeasy Lysis Buffer were thawed and approximately 50mg of tissue was placed in 2 ml screw-cap RNeasy Lysis Buffer tubes containing 1mL of RNeasy Lysis Buffer. AK and PK samples were homogenized using RNeasy Lysis Buffer without beads, while bead-containing Lysing Matrix F (MP Biomedicals) tubes were used for SPL tissue. Tissues were homogenized using an Omni BeadRuptor 24 (Omni International), with one 20-second cycle at speed 5. RNA was purified according to manufacturer's instructions. PBL samples in RNeasy Lysis Buffer were processed similarly. RNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc.) and RNA stored at -80°C for future use. cDNA was synthesized using iScriptTM Reverse Transcriptase Supermix for RT-qPCR, using random primers (Bio-Rad Laboratories, Inc.).

DNA Isolation

DNA isolation was performed using DNeasy (Molecular Research Center, Inc.) in RNeasy Lysis Buffer tubes containing 1mL of DNeasy using an Omni BeadRuptor24 with one 20-second cycle at speed 5 and DNA was purified according to manufacturer's protocols. DNA was stored at -80°C for future use.

Real-Time PCR

Quantitative real-time PCR to determine expression of secHCmu has been described previously using primers that amplify both igm1 and igm2 constant regions genes (Schouten et al., 2013). Expression levels of the secreted form of isotype tau1 (named “secHCtau” in this study) were determined using a custom TaqMan® Gene Expression Assay (Applied Biosystems), with a FAM reporter, NFQ quencher, and a ROX reference. Primers were: Forward primer sequence: 5′ CGGGTAACTCATGTGAAGACAAGT-3′, reverse primer: 5′-AGTCAATAAGAAGACACAACGACACA-3′, and reporter sequence 5′-CACACAGGTTAAAATC-3′. Trout α -tubulin (Tubulin 60) was used as the endogenous control as previously described (Schouten et al., 2013). The Taqman assay for Fp loads has been described elsewhere (Marancik and Wiens, 2013). All qPCR assays were performed using a StepOne Real-Time PCR instrument (Applied Biosystems Inc).

A standard containing a desired number of amplicons for the Fp assay was ordered via gBlocks® Gene Fragments (Integrated DNA Technologies, Inc.). 500ng of standard DNA was reconstituted in 500 μ L of TE buffer (10mM Tris pH 8, 1mM EDTA pH 8; 1ng/ μ L). Copy number was determined using the conversion of 1.096×10^{-21} g/bp. Dilutions were created in molecular grade water to create concentrations between 100,000 and 1 copy/ μ L.

Statistical analysis

Data from the analysis of secHCmu and secHCtau were initially taken in the form of average CT scores from triplicate samples. Relative fold change (RFC) for secHCmu was determined by subtracting the delta [CT(secHCmu_{ref})-CT(tubulin_{ref})] of a control fish from the delta[CT(secHCmu_{sample})-CT(tubulin_{sample})] of each sample to obtain ddCT, and 2^{-ddCT} calculated for each sample. Similarly, for the RFC of secHCtau, delta [CT(secHCmu_{ref})-CT(tubulin_{ref})] of the same control fish was subtracted from the delta[CT(secHCtau_{sample})-CT(tubulin_{sample})] of each sample to obtain ddCT, and 2^{-ddCT} calculated.

QPCR measurement of Fp load was calculated using a standard curve and expressed as Fp genome equivalents per 100 ng DNA. The statistical programming environment R was used to calculate p values, create strip charts, and create scatterplots (R Core Team, 2015). Fp load data were Log₁₀ transformed using the package “dplyr” (Wickham and Francois, 2016) for comparison with percentages of IgM⁺ and IgT⁺ B cells. Data comparing challenged and unchallenged fish were checked for normal distribution using the Shapiro-Wilk normality test. Once a normal distribution was established, ANOVA tests were run to check for significance between groups. After that, a Tukey-Kramer multiple comparisons of means test was performed using the package “multcomp” (Hothorn et al., 2008). Nonparametric data was Log₁₀ transformed and tested using a Shapiro-Wilk normality test. If the data still did not follow a normal distribution, a Kruskal-Wallis test was performed using the package “pgirmess” (Giraudaux, 2016). A nonparametric version of the multiple comparisons of means test, referred to as a Nemenyi test, was performed through the package “PMCMR” (Pohlert, 2016). The Nemenyi test used a Tukey distribution approximation for consistency with the parametric test.

RESULTS

The two main research questions we wished to address were as follows: Do naive R-line fish differ from S-line fish in their abundance of IgT⁺ B cells and/or IgT⁺⁺ Immunoglobulin Secreting Cells (ISCs), and further, do those patterns differ after *in vivo* challenge with Fp? Our approach involved intramuscular injection of live Fp, followed by euthanizing the animals and tissue collections on day 5 (Fp2016), day 6 (Fp2014), or day 28 (Fp2014) post-challenge. Mock challenges involved injecting fish with PBS instead of Fp. Flow cytometric and/or gene expression analyses were then performed on PBLs, spleen, AK and peritoneal cavity fluid (PCF) for the Fp2016 experiment, or AK, PK, and SPL for the Fp2014 experiment.

The Fp challenge experiment: leukocyte numbers

The average number of leukocytes (after Histopaque purification) was determined for each of the 4 groups as part of the Fp2016 experiment. The four groups included Mock R-line fish (MR), Mock S-line fish (MS), Fp-challenged R-line fish (FpR), and Fp-challenged S-line fish (FpS), as shown in Supplemental Table I.

For Fp2016, no significant differences in number of white blood cells (wbcs) were observed between the two groups prior to Fp challenge. However, on day 5 post Fp-challenge, there were several significant differences, summarized in Supplemental Table I. The average number of AK leukocytes was significantly higher in FpR fish compared to FpS fish (a 3.8× increase; 64.9 E6 versus 17.2 E6 respectively). PCF leukocyte numbers were also significantly higher in FpR compared to FpS fish (3.09 E6 versus 0.73 E6 respectively). Further, FpS fish had significantly fewer PBLs than any other groups. As expected, based on earlier observations (Hadidi et al., 2008) the spleen index (SI) of both fish lines increased significantly after Fp-challenge, however, a significant difference between MR and MS was not observed (Suppl Table I).

Characterization of IgT⁺ cells using Pax5 and RAG1

PBLs, AK, SPL, and PCF cells (from Fp2016) were subjected to fixation and surface/ intracellular staining with trout-specific IgT and IgM antibodies and analyzed by flow cytometry, as reported previously (Zwollo et al, 2008) (Zhang et al, 2008). Figure 1 shows examples of the contour graphs for PBLs, SPL and PCF cells with gated populations of interest shown, including IgM⁺ B cells (HCmu⁺/HCTau⁻, gate 2), IgT⁺ B cells (HCmu⁻/HCTau⁺, gate 3), IgM⁺⁺ ISCs (HCmu⁺⁺/HCTau⁻, gate 1), and IgT⁺⁺ ISCs (HCTau⁺⁺/HCmu⁻, gate 4). To confirm that these gated populations represented B lineage cells, AK cells were stained with B cell marker Pax5.PD in combination with the HCTau and HCmu antibodies in 3-color flow cytometry (Figure 2A–2E). All HCmu⁺/HCTau⁻ cells (Gate 2, Figures 2A, 2B) expressed Pax5.PD, while the majority of HCmu⁻/HCTau⁺ cells stained positive for Pax5.PD (white arrows, Gate 3, Figures 2A–2C).

A third population of AK cells co-stained for both HCmu and HCTau (Gate 5), and surprisingly, had considerably stronger staining for Pax5.PD compared to IgT⁺ or IgM⁺ B cells. This cell type also had a relatively high FSC, and was assigned phenotype

Pax5.PD⁺⁺/IgM⁺/IgT⁺/FSC^{high} (Gate 5, black arrows in Figure 2). In AK, the abundance of this population *increased* significantly after Fp challenge ($p=0.002$ in the R-line, and $p=0.007$ for the S-line; Table I). However, there were no significant differences in the AK between S- and R-line fish (Table I).

Pax5.PD⁺⁺/IgM⁺/IgT⁺/FSC^{high} cells were also detected in PBLs, SPL, and PCF. As in AK, no strain differences were detected in between S and R line fish for SPL and PCF (Table I). In SPL, this population was significantly *reduced* after Fp-challenge ($p=0.01$ for R-line fish, and $p=0.02$ for S line fish). PBLs were the only tissue with a significant difference in cell abundance between R and S line fish, with R-line fish having more than twice as many Pax5.PD⁺⁺/IgM⁺/IgT⁺/FSC^{high} compared to S-line fish, in Mock challenged fish. The nature of these cells remains unknown, but it should be noted that the only known immune cells in trout that express such high levels of Pax5.PD are activated B cells (Barr et al., 2011) (MacMurray et al., 2013). We speculate that these cells express FcRs, which would explain how each cell can stain for both IgM and IgT, but this question will have to be resolved in future studies.

To determine whether IgT⁺ B cells, like IgM⁺ B cells, can develop in the AK, we co-stained AK cells with the IgT antibody and an antibody against recombination marker RAG1 in 2-color flow cytometry (Figure 2F). Results show that a significant portion of HCtau⁺ cells expressed RAG1, supporting the assumption that IgT⁺ B cells, like IgM⁺ B cells (Zwollo et al., 2010), develop in the AK. As shown previously for HCmu⁺ developing B cells, FCS^{low} cells were all RAG1⁻, while RAG1⁺ cells had a higher FSC (Zwollo et al., 2010).

We previously characterized the phenotype of Late developing IgM⁺ B cells in the AK, and such cells are FCS^{low} and RAG1⁻, and include late pre-B, immature, and mature B cells (Zwollo et al., 2010). Similarly, in this study we focused on late developing IgT⁺ B cells (FCS^{low} HCtau⁺/Pax5⁺) (Figures 2D, 2E). RAG1⁺/IgT⁺ (early developing B) cells will be investigated as part of a different study.

Abundance of IgT⁺ and IgM⁺ B cells

Strikingly, R-line fish had a significantly *higher abundance* of IgT⁺ B cells in their AK, PBL and SPL tissues, compared to S-line fish, in Mock-challenged animals (Table I). Further significant differences between the S and R lines were seen after Fp-challenge, including a significant reduction in abundance of IgT⁺ B cells in the AK and SPL of FpS fish compared to FpR fish (Table I). Generally, after Fp challenge, the abundance of IgT⁺ cells was reduced compared to Mock-challenged fish. In SPL, these changes were *line-independent*, eg, both R- and S-line fish showed a significant reduction in abundance (from 9.7% to 1.92% for R-line fish, and from 3.25% to 0.46% for S-line fish; Table I). Reduced abundance of IgT⁺ cells after challenge was also seen in the AK, although only the R-line showed a significant drop (from 12.1% to 2.38%; Table I).

In a previous study, we had shown that S-line fish had a significantly higher abundance of IgM⁺ B cells in their AK compared to R line fish (Zwollo et al., 2015). Here, we confirm that pattern in PBL, albeit that no such significant differences were detected in AK or SPL (Table I). It should be noted that the sample size for Mock AK and SPL samples were much

lower in this study compared to the previous study (Table I). After Fp challenge, both lines had significant *increases* in abundance of IgM⁺ B cells in AK, with S-line fish responding more strongly compared to the R-line fish; FpS line fish had significantly higher abundance of IgM⁺ B cells compared to FpR fish (42.2% versus 20.7% respectively; Table I).

No significant differences between the S- and R-lines were seen for IgM⁺ cell abundance in the SPL, either in Mock or Fp-challenged groups. Lastly, FpR fish had a significantly higher abundance of IgM⁺ B cells in their PCF compared to FpS fish ($p=0.001$). (No PCF cells were collected for Mock challenged fish). Insufficient material was available to analyze PBL patterns in Fp challenged fish, as FpS fish had very low numbers of PBLs, as mentioned above (Suppl Table I).

In summary, R-line fish had a significantly *higher* abundance of IgT⁺ B cells compared to S-line fish, a pattern that persisted upon Fp-challenge. The average abundance of IgT⁺ cells was greatly *reduced* upon challenge in both fish lines in all three tissues. In contrast, the abundance of IgM⁺ B cells was similar in both lines before challenge, but was greatly *increased* after challenge in the AK, with S-line fish responding more strongly than R-line fish.

Abundance of IgM and IgT secreting cells

Next, we determined the abundance of Ig-secreting cells for both isotypes (ISCs; for gating see Figure 1), using flow cytometry. Only one significant difference was observed in Mock-challenged fish, and for IgM⁺⁺ ISCs only, in the spleen: MS fish had more than twice the abundance of IgM⁺⁺ ISCs than R line fish (0.89% and 0.43% respectively; Table I), in agreement with our earlier study (Zwollo et al., 2015). After Fp-challenge, FpS fish had a significantly higher abundance of IgM⁺⁺ ISCs in their AK compared to FpR fish, similar to the IgM⁺ B cell pattern in Mock-challenged fish (Table I). The opposite pattern was observed for PCF cells, with a higher abundance of IgM⁺⁺ ISCs in FpR fish compared to FpS fish. The latter also correlates with the PCF pattern for IgM⁺ B cells in the Mock-challenged groups (Table I).

Both MR and MS fish had a very low abundance of IgT⁺⁺ ISCs in the three tissues tested, with no significant differences between the lines (Table I). After Fp-challenge, the abundance of IgT⁺⁺ cells in the AK was twice as high as that for S-line fish (0.04% versus 0.02%), a pattern similar to what was observed for IgT⁺ B cells (Table I).

In summary, the Ig patterns for ISCs followed those for B cells in most tissues and for both classes of Ig, although it was difficult to establish this by flow cytometry, considering that the abundance of ISCs was very low (0.02–1.48%; Table I).

Gene expression of secreted immunoglobulin tau and mu

To independently measure IgM⁺⁺ and IgT⁺⁺ ISCs abundance in the four groups, we utilized an alternative approach: we measured gene expression for the secreted Ig heavy chains of each isotype, secHCtau and secHCmu, using RT-qPCR. Available RNA samples included PBL (collected as part of the Fp2016 experiment), and AK, PK, and SPL (Fp2014 experiment). Relative Fold Change (RFC) was determined for each sample (see Methods).

We first compared the Mock-challenged fish from each line, MR and MS, collected on day 6 post challenge. No significant differences were seen between R- and S-line fish in AK, SPL, or PBL tissues, for either secHCmu or secHCtau1 (Supplemental Table II). The only significant difference between the two lines was seen in the PK, with MR fish expressing significantly higher levels of secHCmu than MS fish at day 6 (5.92 versus 1.85 RFC; Suppl. Table II).

After Fp-challenge, the only significant difference between the two lines was detectable in PBLs on day 5 post-challenge (Figure 3, Suppl. Table II). For both secHCtau and secHCmu, expression levels of FpS fish were significantly lower compared to the other 3 groups, suggesting a drop in IgM⁺⁺ and IgT⁺⁺ ISCs in the blood of S-line fish after challenge.

Several *line-independent* changes were also observed after Fp-challenge: first, a highly significant drop in secHCtau expression in the SPL of R-line fish by day 6 (FpR-D6 fish had 14-fold *lower* secHCtau expression than MR-D6 fish (a RFC value of 4.1 in MR and 0.29 in FpR-D6). There was a similar (3.8-fold) drop in expression of secHCtau in the SPL of S-line fish (a RFC value of 1.54 in MS and 0.40 in FpS-D6), but the change was not significant (p=0.09).

Second, a significant, 7-fold *increase* in expression of secHCmu in AK of R-line fish when comparing Mock-challenged (MR-D6) to day 28 post-challenge fish (FpR-D28): a RFC value of 30.2 in MR and 213.3 in FpR-D28. Lastly, an interesting difference was noted for PK: when comparing Mock-challenged (Day 6) to Fp-D28 challenged fish, there was a significant *increase* in secHCmu expression in the PK of S line fish, but a significant *decrease* in the PK of R-line fish. One caveat in the D28 comparisons was the lack of Mock-Day 28 control (we only had Mock-Day 6 control fish), but one would assume that Mock Day 6 and Mock Day 28 fish would be fairly similar.

Because RFC values for both secHCtau and secHCmu varied widely between fish samples, we next calculated the expression ratio of the two targets for each fish (Table II). Highly significant differences were evident between S- and R-lines in Mock-challenged fish, with R-line fish having relatively higher secHCtau:secHCmu ratios than S-line fish, in AK, SPL, and PBL (Table II). After Fp-challenge, the only difference between the two lines was found on day 28, with a higher secHCtau:secHCmu ratio in the AK for R-line fish compared to S-line fish. *Line-independent* changes were only seen in the SPL, and for both S- and R-line fish, with a significantly reduced ratio of secHCtau:secHCmu in day 6 post-challenged fish (Table II), in agreement with the reduced expression of secHCtau (shown in Suppl. Table II).

Correlations between Fp-loads and Ig expression

As a measure of infection level, splenic Fp-loads (the number of Fp genome copies/100 ng extracted trout DNA) were determined for each fish on day 5 (Fp2016) or day 6 (Fp2014). For both experiments, Fp loads were much higher in S-line fish compared to R-line fish on both days. However, by day 28 post-challenge, Fp loads were much lower than for Day 6, and not significantly different between R- and S-line fish (Suppl. Table III).

Next, we wished to determine whether significant correlations existed between splenic Fp loads and abundance of IgM⁺ or IgT⁺ B cells in the various immune tissues. Based on the observation that MR line fish had a significantly higher abundance of IgT⁺ B cells compared to MS fish (in AK, SPL, and PBLs), we hypothesized that Fp loads would negatively correlate with IgT⁺ B cell abundance, IgT⁺⁺ ISC abundance, and/or secHCtau gene expression. We calculated p values for correlations in the combined FpR and FpS fish (for a higher N value), but also for the individual FpR and FpS groups. Significant correlations between Fp load and cell populations or gene expression are shown in Table III.

In AK, a highly significant *positive correlation* was seen between abundance of IgM⁺ B cells and Fp load, both in the combined (FpR and FpS) group ($R = 0.76$; Table III), as well as for FpR fish ($R=0.74$) and FpS fish ($R=0.79$). An opposite pattern was seen for IgT⁺ B cells: a higher abundance of IgT⁺ B cells in the AK was significantly correlated with lower Fp loads ($R=-0.55$; Table III), but only in the combined group. Lastly, no significant correlations were seen between Fp-loads and IgM⁺⁺ or IgT⁺⁺ ISCs and Fp loads in the AK, although FpR fish showed a close to significant positive correlation between Fp load and percentage IgM⁺⁺ ($R=0.54$, $p=0.059$; Table III). However, a strong negative correlation was detected between secHCtau and Fp loads in the combined group ($R=-0.43$), and a close to significant correlation in FpS group ($R=0.48$, $p=0.059$), but not the FpR group (Table III). Together, these data suggest that in the AK, fish with lower Fp loads may have more abundant IgT⁺ B cells, more IgT⁺⁺ ISCs, *and* fewer IgM⁺ B cells. Whether a higher ratio of IgT⁺/IgM⁺ B cells in the AK make a fish more resistant to Fp, or whether higher Fp-loads result in a stronger shift towards more IgM⁺ cells and fewer IgT⁺ cells, remains unresolved.

Interestingly, for SPL, strong negative correlations were detected between Fp loads and both IgT⁺ and IgM⁺ B cell abundance, but only in the combined group ($R= -0.68$ and -0.84 respectively; Table III). Further, no correlations with Fp load were significant for IgT⁺⁺ or IgM⁺⁺ ISCs in the spleen. This may be partly the consequence of the low N values for SPL samples available for flow cytometry (Table III), but this is less likely, as no significant correlations were seen by qPCR either (Table III).

PCF showed the most significant correlations with Fp loads of all four tissues tested (Table III): strong negative correlations were seen with abundance of IgM⁺ B cells, both in the combined group and in FpR fish ($R= -0.76$ and $R= -0.41$ respectively; Table III). Further, negative correlations were seen with IgT⁺ B cell abundance, both in the combined group and in the FpR group ($R= -0.41$ and -0.64 respectively; Table III). Strikingly, PCF was also the only site that showed significant negative correlations with abundance of IgM⁺⁺ and IgT⁺⁺ ISCs, both in the combined group and for FpR fish (Table III).

Although no flow cytometric data could be obtained on PBLs from challenged fish, significant negative correlations were found using qPCR: Fp loads correlated negatively with secHCtau expression, both in the combined group, and in FpR fish ($R=-0.65$ and -0.85 respectively; Table III). A significant negative correlation was also found between secHCmu expression and Fp loads in PBLs, but only in the combined group, a likely consequence of low N values for the PBL samples. No other significant correlations were found between ISCs and Fp-load in the PK.

DISCUSSION

This study aimed to describe the kinetics of IgT and IgM immune responses to Fp infection in two selectively bred lines of rainbow trout, a BCWD-resistant line (R-line), and a BCWD-susceptible line (S-line). In a previous study, we reported that naïve R-line fish had a lower abundance of IgM⁺ B cells and IgM⁺ISCs compared to naïve S-line fish (Zwollo et al., 2015). The results from the current study confirm these findings, and provide evidence that R-line fish have a higher abundance of IgT⁺ B cells compared to S line fish, both before and after challenge, and that IgT abundance and secHCtau expression correlate with lower pathogen loads in challenged fish.

IgT⁺ B cells in the anterior kidney

The anterior kidney of R-line fish had a significantly higher abundance of IgT⁺ B cells compared to S-line fish, both in the Mock-challenged and Fp challenged fish. This finding is relevant when considering the possible causes of resistance to Fp. The anterior kidney comprises the main hematopoietic organ in rainbow trout, and is the site where both IgM⁺ B cells and IgT⁺ B cells develop, based on the co-staining with RAG⁺, as shown here (IgT⁺ B cells), and elsewhere (for IgM⁺ B cells; (Zwollo et al., 2010).

The abundance of IgT⁺ B cells was significantly reduced in R-line fish on day five post challenge, but not in S-line fish. This result is in agreement with a finding by another group that HCtau expression is reduced after Fp challenge in rainbow trout fry (using Fp strain 950106-1/1)(Henriksen et al., 2013). A drop in expression may reflect increased outmigration of IgT⁺ B cells, a shift towards making more IgM⁺ B cells, or an increase in influx of other cells into the AK. While R-line fish had a significantly higher abundance of IgT⁺ B cells in their AK, S-line fish had a higher abundance of IgM⁺ B cells compared to R-line fish. However, R-line fish also differed from S-line fish in that they had a strong increase in the total number of wbc's in their AK after challenge. Although the abundance of IgT⁺ populations after challenge was reduced more than 5-fold in R line fish, the *total number* of IgT⁺ B cells did not change. In S-line fish, where Fp challenge did not change the total number of AK wbc's, there was a net drop of IgT⁺ B cells (2.2-fold). Hence, R-line fish may generate more IgT⁺ B cells than S-line fish, both before and after Fp challenge.

Tau versus mu: the structure of the Ig HC locus in rainbow trout

What is the molecular mechanism that generates IgT⁺ versus IgM⁺ B cells in the anterior kidney, and how are R-line fish different in this regard from S-line fish? The answer likely lies within the large and complex structure of the rainbow trout Ig HC locus: HCtau gene segments are encoded within the HCmu/delta loci, in a nested gene structure (Hansen et al., 2005) (Zhang et al., 2017). D τ and J τ segments are located between (isotype-shared) VH segments (at the 5' end of the Ig HC gene), and the C τ 1 segment is located immediately downstream from the D τ 1 and J τ 1 segments. C τ 3, immediately downstream from 5' VH segments, has its own D τ and J τ segments. Separate D μ /δ and J μ /δ segments are located downstream from C τ 1, with C μ and Cδ constant segments residing 3' of the D μ /δ and J μ /δ segments. During gene rearrangement, a progenitor B cell can either combine upstream VH segments with D τ J τ segments, or alternatively, combine these upstream VH segments with

downstream DJ μ / δ segments. The former will transcribe into VDJC τ (encoding the HCtau1/3), the latter into VDJC μ or VDJC δ (encoding HCmu and HCdelta respectively).

This choice between HCmu or HCtau lineage B cells is likely driven by epigenetic factors (Hansen et al., 2005). In this regard, the rainbow trout Ig locus is similar to the nested organization of the mammalian T-cell Receptor genes Trca and Tcr δ , an organization that precludes co-existence of both $\alpha\beta$ and $\delta\gamma$ TCRs on the same T cell. Two enhancers have been described for the Tcr genes (E δ and E α), the activity of which determines which locus is activated first (Hernández-Munain, 2015). Such enhancers have not been uncovered in the rainbow trout, but the possibility of differences in cis-acting elements within the Ig HC gene between R- and S-line fish is attractive. Alternatively, or perhaps additionally, R-line fish may have cytokine signatures that promote gene rearrangement and/or expression of HCtau over HCmu. At this time, nothing is known about such mechanism during rearrangement in the rainbow trout.

The importance of IgT secreting cells in relation to *Fp* resistance

R-line fish had significantly higher secHCtau to secHCmu ratios when compared to S line fish. This difference was especially striking in Mock-challenged fish. Three of the four tissues (AK, SPL, and PBL) showed this pattern, suggesting that prior to challenge, R-line fish don't just generate more IgT⁺ B cells in the AK, but also produce higher levels of the IgT antibody and/or IgT⁺⁺ cells in secondary immune sites. If IgT would in fact be more protective against *Fp* infection than IgM, then having more IgT lineage cells would be expected to correlate with lower *Fp* loads after challenge. Our data support this notion: the abundance of IgT⁺ B cells in all 3 tissues was strongly correlated with lower pathogen loads in challenged fish, and secHCtau expression in the AK and in blood (PBLs) also correlated negatively with *Fp* loads in challenged fish.

Our finding that R-line fish had a higher abundance of IgT⁺ B cells is supported by previous results of a whole-body transcriptome study, which reported on immune genes that differed between R-line and S-line fry on day 5 post challenge (using *Fp* strain CSF-259-93) (Marancik et al., 2014). One *Fp*-responsive gene was the membrane form of Ig HCtau (memHCtau). Before challenge, R-line fish expressed higher levels of memHCtau than S line fish (0.88 versus 0.56 RFC), and expression increased almost 5-fold increase on day 5, while *Fp*S fish had less than a 3-fold increase. Hence, both lines had increased memHCtau expression after *Fp* challenge, but R-line fish started out with higher levels of memHCtau and increased its expression at a higher rate, compared to S-line fish.

Another study in support of IgT in protection against *Fp* infection reported that levels of secreted IgT were significantly increased in gills of fish, using immersion or anal intubation with *Fp* strain 259-93-B.17. A protective role for IgT was also reported in a recent study in gilthead sea bream. Both serum IgM and mucosal IgT levels were increased after bath challenge with *Photobacterium damsela* (Piazzon et al, 2016). In the same paper, the authors also showed that when gilthead sea bream were put on a plant-based diet followed by challenge with the intestinal parasite *Enteromyxum leei*, the IgT responses necessary to neutralize the parasite were suppressed, while both IgM responses and pathogen loads increased. The authors proposed that fish on the plant-based diet attempted to compensate

for the lack of IgT response by increasing their IgM response, but this correlated with worse disease outcome.

IgM and Fp loads

One striking result from this study was that IgM⁺ and IgM⁺⁺ cell abundance correlated positively with Fp load, but only in the AK. As discussed above, this may be related to a difference in the generation of IgM⁺ versus IgT⁺ B cell production in the AK of S-line fish. Alternatively, it may be a consequence of a more activated immune system in fish with higher pathogen loads, which leads to increased production of IgM⁺ B cells in highly infected fish (which may or may not be protective).

In secondary immune organs like SPL and PCF, FpS fish showed significant negative correlations between abundance of IgM⁺ B cells and Fp loads, and the same pattern for the expression of secHCmu transcripts and Fp loads in PBLs. The only negative correlation between IgM⁺⁺ ISCs and with Fp loads was seen in the PCF, suggesting a protective response, or alternatively, a stronger humoral immune response in fish with higher Fp loads.

The available literature lends some support in favor of protective roles for IgM in the response to Fp infection. In one Fp challenge study in rainbow trout, using intraperitoneal injection (but not immersion) with killed bacteria, a serum IgM response was induced at week 6, while mucus IgM peaked at week 9 (LaFrentz et al., 2009). In a different study, when rainbow trout were immunized intraperitoneally with attenuated Fp strain CSF259-93-B, the levels of IgM increased in serum, gill, and skin mucus by day 28 post-challenge (Makesh et al., 2015).

Our data also showed a 7-fold increase in expression of secHCmu by day 28, in the AK of R-line fish (relative to Mock-challenged R). Together, this indicates that new, IgM⁺⁺ plasmablasts enter the AK during the period between day 6 and day 28, where they may differentiate into Fp-specific IgM⁺⁺ LLPCs. However, another study found a lack of correlation between plasma IgM titers and visual health status in Fp-infected rainbow trout, suggesting that even if new IgM is produced, it is not necessarily protective (Orieux et al., 2013). Lastly, a recent study in rainbow trout supports a protective role for IgM⁺⁺ ISCs in the PC, as peritoneal injections with *E.coli* or VHSV resulted in a rapid (days 2 and 3) and strong influx of such cells to this site, as measured in the PCF using flow cytometry (IgT was not measured in this study) (Castro et al., 2017).

Clearly, future studies are needed to increase our understanding of the roles of both IgT and IgM in protection against Fp, including studies that focus on the abundance of IgT⁺ B lineage cells in gills and skin, and patterns of Fp-specific IgT antibodies in serum, mucus, and muscle of infected fish.

In summary, our studies show that Ig patterns in a BCWD-resistant line of rainbow trout have shifted towards higher abundance of IgT⁺ B cells and lower abundance of IgM⁺ cells, and this correlates with lower Fp loads after challenge. However, IgM⁺ B cells may also play a protective role during Fp exposure at secondary lymphoid sites like SPL and PCF. Future

studies should focus on genetic differences between the lines, including potential regulatory elements associated with the IgHC gene that may drive the BCWD-resistant phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- * A genetic line of rainbow trout resistant to *Flavobacterium psychophilum* has a higher abundance of IgT⁺ B cells compared to a susceptible control line both before and after bacterial challenge.
- * Higher abundance of IgT⁺ B cells and expression of secHCtau correlated with lower pathogen loads in challenged fish.
- * The data suggest that IgT⁺ B lineage cells may have a protective function in the immune response to *Fp*.

Figure 1.

Flow cytometric analysis for the *in vivo* Fp-challenge experiment, using two-color, HCtau and HCmu staining. Shown here are PBL, spleen (SPL), and peritoneal cavity fluid (PCF). Illustration of patterns in the form of contour graphs, on day 5 post-challenge, using MR samples for PBL and SPL tissue, and an FpR sample for PCF. Gate 1, IgM⁺ ISC; gate 2, IgM⁺/IgT⁻ B cells; gate 3: IgM⁻/IgT⁺ B cells; gate 4: IgT⁺ ISC, gate 5: IgM⁺/IgT⁺ cells (see Figure 2).

Figure 2.

Characterization of IgT^+ cells in the AK using flow cytometry. The anterior kidney sample used here is pooled from four FpS fish. **A–E:** 3-color flow cytometry. Grey arrow: control antibody/non-staining population. Black arrow: $\text{IgM}^+/\text{IgT}^+$ cells. White arrow: IgT^+ B cells. **A.** Example of a contour graph with HCTau and HCTmu staining shown. Gates numbered as in Figure 1: gate 1, IgM^{++} ISCs; gate 2, $\text{IgM}^+/\text{IgT}^-$ B cells; gate 3: $\text{IgM}^-/\text{IgT}^+$ B cells; gate 4: IgT^{++} ISC, gate 5: $\text{IgM}^+/\text{IgT}^+$ cells. **B.** Pax5.PD expression of selected subpopulations from Figure 2A. Grey filled line: all cells; black-white dotted line: $\text{IgM}^+/\text{IgT}^-$ cells (gate 2); black solid line: $\text{IgM}^-/\text{IgT}^+$ cells (gate 3); black-striped: $\text{IgM}^+/\text{IgT}^+$ cells (gate 5). **C.** Similar to B., but without $\text{IgM}^+/\text{IgT}^-$ cells shown to enlarge the $\text{IgT}^+/\text{IgM}^+$ population. Grey line represents negative control stain. Black arrow indicates a small population of $\text{IgM}^+/\text{IgT}^+$ cells (gate 5) with Pax5.PD $^{++}$ staining. Grey arrow: control antibody. **D.** Contour of FSC and HCTau staining, showing an IgT^+ population of Late developing B cells (low FSC), and an unknown, IgT^+ population with high FSC. **E.** Histogram with Pax5.PD staining of the two gated populations from Figure 2D: Late developing B cell (grey filled, with white arrow), and an unknown population with Pax5.PD $^{++}$ staining (black line). Grey arrow: control antibody. **F.** HCTau and RAG1 staining using 2-color flow cytometry.

Figure 3. Strip plot showing changes in gene expression for secHCmu and secHCtau transcripts in PBLs. The mean fold change values of gene expression ($\pm$ SE) are shown on the Y-axis. Each of the four groups is shown on the X-axis. Day 5 post Fp-challenge (Fp2016). **A.** secHCtau. **B.** secHCmu. *, <0.05 , ** <0.01 .

Table 1

Comparisons of cell abundance (in %) between the four groups, using flow cytometric analysis, means with SE and p values.

B cells		Differences between lines:				Line-independent changes after challenge:			
		Mock challenge		Fp challenge:		R line:		S line:	
		MR	MS	p	FpR	FpS	p	MR	FpS
IgM+	AK	9.0 ± 1.3	11.6 ± 1.6	0.30	20.7 ± 2.3	42.2 ± 4.8	0.001	9.0 ± 1.3	11.6 ± 1.6
	PBL	49.9 ± 3.3	59.0 ± 2.0	0.05	ND	ND	ND	ND	ND
	SPL	42.5 ± 4.22	38.3 ± 4.1	0.49	31.2 ± 4.05	29.4 ± 2.44	0.71	42.5 ± 4.22	38.3 ± 4.1
	PCF	ND	ND	ND	17.7 ± 2.92	4.85 ± 1.23	0.001	ND	ND
	IgT+	MR	MS	p	FpR	FpS	p	MR	FpS
	AK	12.1 ± 2.3	1.86 ± 0.71	0.004	2.38 ± 0.41	0.82 ± 0.17	0.002	12.1 ± 2.3	2.38 ± 0.41
	PBL	15.7 ± 2.38	4.4 ± 0.71	0.001	ND	ND	ND	ND	ND
	SPL	9.7 ± 2.22	3.3 ± 0.52	0.02	1.92 ± 0.54	0.46 ± 0.28	0.04	9.7 ± 2.22	1.92 ± 0.54
ISCs	PCF	ND	ND	ND	1.68 ± 0.43	0.77 ± 0.18	0.08	ND	ND
	IgM++	MR	MS	p	FpR	FpS	p	MR	FpS
	AK	0.90 ± 0.09	1.48 ± 0.34	0.17	1.62 ± 0.24	2.55 ± 0.31	0.03	0.90 ± 0.09	1.62 ± 0.24
	PBL	0.11 ± 0.03	0.11 ± 0.02	0.88	ND	ND	ND	ND	ND
	SPL	0.43 ± 0.10	0.89 ± 0.14	0.05	0.54 ± 0.10	0.47 ± 0.09	0.62	0.43 ± 0.10	0.54 ± 0.10
	PCF	ND	ND	ND	2.16 ± 0.50	0.42 ± 0.08	0.004	ND	ND
	IgT++	MR	MS	p	FpR	FpS	p	MR	FpS
	AK	0.07 ± 0.02	0.06 ± 0.04	0.87	0.04 ± 0.01	0.02 ± 0.01	0.03	0.07 ± 0.02	0.04 ± 0.01
?	Pax5++	MR	MS	p	FpR	FpS	p	MR	FpS
	AK	1.1 ± 0.33	1.0 ± 0.25	0.88	5.4 ± 0.96	3.8 ± 0.68	0.21	1.1 ± 0.33	5.4 ± 0.96
	PBL	1.42 ± 0.30	0.51 ± 0.53	0.02	ND	ND	ND	ND	ND
	SPL	1.45 ± 0.39	0.94 ± 0.26	0.34	0.24 ± 0.05	0.20 ± 0.10	0.77	1.45 ± 0.39	0.24 ± 0.05
	PCF	ND	ND	ND	6.67 ± 2.1	1.59 ± 0.33	0.07	ND	ND

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N values: for AK: MR and MS, N=5, FpR and FpS, N=14. For SPL: N=5 for MR, MS, FpR, and FpS. For PBL and PCF: N=14 for all groups.
Pax5++ cells were IgM+/IgT+/FSCHigh; “?”; question mark indicates that the cell type is unknown.

Table II

Ratios of relative fold change (RFC) of secHCtau/secHCmu for Fp2014 (top) and Fp2016 (bottom) experiments, with p values.

Fp2014	Differences between R and S line fish:										Line-independent changes after challenge:					
	Mock		Day 6 challenge				Day 28 challenge				R line, day 6:			S-line, day 6:		
			MR	MS	p	FpR	FpS	p	FpR	FpS						
AK	0.014	0.002	0.0005	0.007	0.001	0.07	0.0011	0.0004	0.03	0.014	0.007	0.09	0.002	0.001	0.19	
	0.016	0.005	0.0003	0.001	0.002	0.30	ND	ND	ND	0.016	0.001	0.001	0.005	0.002	0.04	
PK	0.004	0.009	0.27	0.033	0.024	0.52	0.32	0.80	0.27	0.004	0.033	0.07	0.009	0.024	0.16	
Fp2016	Mock		Fp challenge				R line:			S-line:						
			MR	MS	p	FpR							FpS	p		
	0.070	0.010	0.02	0.03	0.03	0.99	0.07	0.03	0.2	0.01	0.03	0.12				

N values for SPL: MR-D6 and MS-D6, N=9. FpR and FpS-D6; N=15. AK: MR, N=9, MS, N=7, FpR and FpS; N=18. PK: MR and MS; 10, FpR and FpS, 18.

Table III
Correlations between B cell markers and Fp-loads; Pearson's R values are shown for significant correlations

B cells (Flow)	All Fp-challenged fish				FpR fish				FpS fish			
	% IgM+	N	% IgT+	N	% IgM+	N	% IgT+	N	% IgM+	N	% IgT+	N
Day 5												
AK	0.76***	25	-0.55***	25	0.74***	13		13	0.79***	12		12
SPL	-0.84**	10	-0.68*	11		6		5		6		6
PCF	-0.76***	28	-0.41*	28	-0.53*	15	-0.64**	14		13		13
ISCs (Flow)												
	% IgM++	N	% IgT++	N	% IgM++		% IgT++	N	% IgM++	N	% IgT++	N
Day 5												
AK		26		20	0.54 ^a	13		12		13		12
SPL		11		11		5		6		6		5
PCF	-0.79***	27	-0.43*	28	-0.63*	14	-0.59*	14		13		13
ISCs (qPCR)												
	secHCmu	N	secHCtau	N	secHCmu	i	secHCtau	N	secHCmu	N	secHCtau	N
Day 6												
AK		33	-0.43**	36		19		20		16	-0.48 ^a	16
SPL		32		32	-0.53a	13		14		15		16
PK		32		32		15		13		16		16
Day 5												
PBL	-0.81***	14	-0.65***	15		8	-0.85*	7		6		8

* p 0.05;

** 0.01,

*** 0.001.

^a correlations close to significance (p=0.059). Flow, flow cytometric analysis.

Top rows: Flow for B cells, middle rows: flow for ISCs, bottom rows: ISC data by qPCR.

"All Fp-challenged fish: combined R- and S-line fish. Middle and right columns: data separated by R- or S-line.