B cell signatures of BCWD-resistant and susceptible lines of rainbow trout: a shift towards more EBF-expressing progenitors and fewer mature B cells in resistant animals.

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INTRODUCTION

Breeding for improved disease resistance in farm animals has become a major focus in applied immunogenetics. Most disease resistance breeding programs target one or several pathogens having high economic impact (Bishop et al. 2011). Little is known about how breeding for a specific disease resistance phenotype results in correlated changes in immune response parameters and how such changes relate to improved survival following pathogen challenge. Rainbow trout (*Oncorhynchus mykiss*) are a unique resource to address these questions as this species has been domesticated for many years, large numbers of trout are available per cross (1000's), pedigreed lines are available and rainbow trout are a widely used in biomedical research (Thorgaard et al., 2002). Bacterial Cold Water Disease (BCWD) is a common, chronic disease in rainbow trout, and is caused by the gram-negative bacterium *Flavobacterium psychrophilum* (Fp). Since 2005, the National Center for Cool and Cold Water Aquaculture (NCCCWA) has implemented a rainbow trout selective breeding program to increase survival following BCWD challenge (Hadidi et al. 2008, Silverstein et al. 2009, Leeds et al. 2010). A genetic line that is highly resistant to Fp challenge has been established through family-based selective breeding, designated ARS-Fp-R (or R-line), and in addition, a reference susceptible line, ARS-Fp-S (S-line) is available for phenotypic comparisons. The S-line has a similar founder genetic contribution as the R-line (Hadidi et al. 2008), was evaluated at the same age and size, and was selected for one generation for increased susceptibility (Wiens et al. 2013a). When challenged with Fp by intraperitoneal injection, the R-line exhibits splenic lower loads (Hadidi et al. 2008) and higher survival by 5-10 days post-infection (Wiens et al. 2013a), suggesting enhanced capacity for pathogen control relative to the S-line. Whether these differences are the result of base-line changes in immunological parameters (i.e. cell numbers) or are manifested following pathogen exposure, or a combination of both possibilities, is unknown.

BCWD typically affects fry and juvenile stages (Nematollahi et al. 2003, Barnes and Brown 2011), and as such, the disease can manifest itself before the acquired immune system has been fully developed. Many genes and immune proteins are regulated following infection that include serum amyloid A, antimicrobial peptides, MHC I and II, complement components and a number of chemokines and cytokines (Villarroel et al. 2008, Evenhuis and Cleveland 2012, Orioux et al. 2012, Langevin et al. 2012, Henriksen et al. 2014). Resistance to Fp has been correlated with naive animal spleen size, with Fp-resistant trout having larger spleens, although the functional significance remains unclear (Hadidi et al. 2008, Wiens et al 2013b). Adaptive immunity (IgM antibody) is generated in convalescent fish, and serum adoptive transfer provides partial protection against experimental challenge (LaFrentz et al. 2002). Based on these observations, mechanisms that provide resistance against Fp infection and/or disease progression could include innate and acquired immune systems and potentially manifested either prior to, or induced following, pathogen exposure.

The developmental pathways of the teleost immune system are not well defined compared to highly studied mammalian systems. However, some important progress has been made recently, as the main developmental and terminally differentiating B cell populations have now been defined in rainbow trout (reviewed in Zwollo, 2011). Trout B lymphoid cell stages are similar to those of the mammalian system, including the earliest stages, Common Lymphoid Progenitor (CLP)/pro-B and pre-B cell stages, followed by immature B, mature B, plasmablast, and finally, the plasma cell stage. Using flow cytometric analysis, tissue-specific B cell patterns or “B cell signatures” have been defined for the major immune organs in trout, based on combinatorial expression of transcription factors, which provide accurate developmental markers that are highly conserved between species (Zwollo et al, 2011, Barr et al 2011, MacMurray et al 2013). Early developing B lymphoid cells can be characterized by the expression of the transcription factor Early B cell Factor (EBF) and the recombinase RAG1 (Zwollo et al. 2008, Zwollo, 2011). EBF is first expressed in CLPs, and its expression is highest

in large pre-B cells (Lukin et al., 2008, McMurray 2013; Figure 1). Late developing B cells (including small pre-B and (im)mature B cells) have low levels of EBF and lack RAG1, but strongly express the transcription factor Pax5.PD (the Pax5 isoform containing a paired domain) and the immunoglobulin heavy chain mu (HCmu), while terminally differentiated plasma cells lack Pax5 and membrane IgM, but secrete high levels of IgM (HCmu⁺⁺; Zwollo et al., 2010, Barr et al., 2011, MacMurray et al, 2013; Figure 1). Plasmablasts continue to co-express Pax5 and HCmu, and can be detected using proliferation marker EdU (Barr et al., 2011).

In teleosts, including the rainbow trout, the main site for hematopoiesis is the anterior kidney or K1 (Zwollo et al., 2005), and as the main primary immune site, it has the highest concentration of developing B cells (Zapata and Cooper, 1990, Zwollo et al. 2010). It is also believed to be the site for storage of long-lived plasma cells (LLPCs; Bromage et al., 2004, Ma et al, 2013). In contrast, posterior kidney (named K5) possesses both renal and immune tissue, the latter containing mature and activated B cells (Zapata and Cooper, 1990, Zwollo et al., 2010). The trout spleen retains some hematopoietic function in adult animals, as it expresses low levels of RAG1 (Hansen et al., 1997, Zwollo et al 2010), and EBF (McMurray 2013); however, it primarily functions as a secondary immune organ where mature B cells are activated by antigen. As in mammalian species, trout spleen has abundant levels of mature B cells (Zapata and Cooper, 1990, Kaattari and Irwin, 1985, Bromage et al., 2004, Zwollo et al., 2005, 2008). The blood of the rainbow trout is a poorly understood and complex immune site. It contains many (im) mature, IgM⁺ B cells as well as progenitor cells, but very few plasmablasts/plasma cells (Zwollo et al, 2008, McMurray, 2013, Ma, 2013).

As part of series of studies designed to define the immunological phenotypes of the R-line and S-line rainbow trout both prior to, and following, pathogen exposure, herein, we used combinations of B lymphoid and monocytic markers to determine if innate and humoral immune differences exist between naive R-line and S-line trout. Since few trout-specific markers to detect innate immune cells have been described, especially those for use in flow cytometric

analysis, we developed a trout-specific antibody against the pro-inflammatory cytokine IL-1 β , which is secreted abundantly by monocytic cells, granulocytes, and activated B1 cells (Verburg-van Kemenade et al., 1995, Weyts et al, 1997, Zhong et al., 2007). We initially tested the hypothesis that resistance to Fp correlates with increased abundance of naive animal IgM⁺ B cells, plasmablasts and immunoglobulin-secreting cells (ISCs). However, our data suggest the opposite, as R-line trout had lower abundance of both IgM⁺ mature B cells and ISCs, but higher abundance of EBF⁺ progenitor populations, in both primary and secondary immune tissues. Based on their staining patterns, early developing cell populations had characteristics of both B lymphoid and myeloid progenitors, and as such, may play important roles in directing immune development more towards myeloid lineage cells in Fp-resistant trout, relative to Fp-susceptible trout in naive animals.

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. To summarize briefly, the NCCCWA breeding program utilizes phenotypic records generated at our disease challenge facility to estimate family breeding values, and subsequently using these values to select pathogen-naive full-siblings maintained in a physically separate, facility as broodstock (Leeds et al. 2010). 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. 2013a). All NCCCWA broodstock eggs are iodophore disinfected after fertilization and reared using filtered and UV-treated well water. 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 Virginia Institute of Marine Science/The College of William and Mary Trout Facility in 100-gallon tanks with a recirculating system employing biologically-filtered water at 12° C wherein no *Fp* challenge occur. IACUC committee 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. The original founder composition and response to selection have been previously described (Silverstein et al. 2009, Leeds et al. 2010). Trout used here were spawned in year class (YC) 2011 and 2012 (YC2011 and YC2012). Resistant trout, ARS-Fp-R (R-line) were pooled from 37 families in 2011 and 43 families in 2012, while the ARS-Fp-S (S-line) were pooled from 16 families in 2011 and 11 families in 2012. Trout were cultured at ambient water temperature (12–13°C) and fed standard trout diet rations (Ziegler-Bros). Spleen indexes (spleen wt (mg)/body weight (g)) were determined at the time of tissue collection as previously described (Hadidi et al., 2008). For YC2011, the average spleen index at the time of the experiment was 1.56 (+/- 0.19) for R trout (n=25), and 1.46 (+/- 0.14) for S trout (n=25) respectively, with an average age at time of collection of 12-22 months for both groups. For YC2012, the average spleen index was 1.65 (+/- 0.18) for R trout (n=30), and 1.02 (+/- 0.16) for S trout (n=30), with an average age of 12-15 months for each line at time of collection.

Isolation of leukocytes.

Fresh trout kidney cells were obtained using methods previously described (Zwollo et al., 2005). Upon isolation through Histopaque 1077 cushions (Sigma Aldrich) the lymphocyte layer was removed and cells were either washed in cold HBSS for culturing, in PBS (1.9 mM $\text{NaH}_2\text{P}_04 \cdot \text{H}_2\text{O}$, 8.1 mM $\text{Na}_2\text{HP}_04 \cdot 7\text{H}_2\text{O}$, 137 mM NaCl, and 2.6 mM KCl, pH 7.4) containing 0.02% sodium azide in preparation for fixation (see Fixation), or used immediately to prepare cytoplasmic extracts for western blot analysis (below).

Cell culture and mitogens.

Freshly isolated trout peripheral blood lymphocytes (PBL), spleen (SPL), and anterior kidney (K1) cells were cultured at 1.0×10^7 cells/mL in trout culture medium (TCM) consisting of RPMI 1640 with 10 mM L-glutamine, 10% fetal bovine serum (FBS), 50 µg/mL gentamicin, 50 µM 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine (final concentration 10 µg/mL; Sigma-Aldrich). Cells were maintained at 18° C in the presence of blood gas and fed every other day with one tenth of the culture volume of a 10x tissue culture cocktail containing 500 µg/mL gentamycin, 10x essential aas, 10x non-essential aas, 70 mM l-glutamine, 70 mg/mL dextrose, 10x nucleosides, and 33% FBS. PBL and SPL cultures were activated using LPS, either from *Escherichia coli* 055:B5 (Sigma), pasteurized for 30 min at 70° C in distilled water, or from *F. psychrophilum*, at a final concentration of 50 µg/mL. LPS from *F. psychrophilum* strain CSF259-93 was extracted by the phenol/water method of Westphal and Jann (Westphal and Jann, 1965) at the Complex Carbohydrate Research Center, University of Georgia. Chemical composition of the extracted Fp LPS confirmed the presence of rhamnose, fucosamine and a dideoxy diamino sugar, consistent the reported structure of L-Rhamnose, N-Acetyl-L-Fucosamine and an acetylated derivative of N-Acetyl-D-Quinavosamine (MacLean et al., 2001). For cell proliferation analysis after LPS-activation, SPL or PBL cells were incubated in the presence of 3 µM 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) for 17 hours immediately before collection as described previously (Barr et al, 2011). For proliferation analysis of anterior kidney immune cells in the absence of mitogens, freshly isolated cells were incubated in the presence of membrane dye PKH26 using a PKH26 red fluorescent cell linker kit for membrane labeling according to manufacturer's instructions (Sigma-Aldrich), and cells collected after 2, 24, or 48 hours, fixed, and analyzed by flow cytometric analysis. Control samples were same cells mixed with PKH26 and collected at 0 hrs.

Antibodies.

The polyclonal anti-paired domain (Pax5.PD) antibody (previously called ED-1; Zwollo et al., 1998) recognizes the paired domain of vertebrate Pax5 and detects trout Pax5 isoforms in pre-B through plasmablast stages (McMurray et al, 2013). The monoclonal mouse anti-trout immunoglobulin heavy chain mu (HCmu, or I-14; DeLuca et al, 1983) was a generous gift from Dr. Greg Warr. The polyclonal rabbit-anti-human EBF (H300; detecting aas 1–300 of EBF1, and reactive with mouse/human EBF2, 3, and 4) was purchased from Santa Cruz Biotech. The polyclonal rabbit-anti-trout IL-1 β antibody (tIL-1 β) was generated by Lampire Biological Laboratories, by injecting a peptide (RRNTGVDISTLESASFRN), covering aas 208-225 of trout IL-1 β 1 (Genbank Acc# CAA11684.1) into rabbits. The peptide included a C-terminal cysteine for conjugation to KLH for primary immunization, and BSA for boosting. Rabbits were boosted twice and a final production bleed obtained on day 50. Prior to injection, a pre-bleed serum sample was collected and antibody purified with protein-A for use as negative control. The tIL-1 β 1 antibody is predicted to detect trout IL-1 β 1 with high affinity, and trout IL-1 β 2 with low affinity (5/18 aas mismatch), while not detecting tIL-1 β 3. A computer-generated 3D model of trout IL-1 β protein, using SWISS-Model, is shown in Figure 2A. For flow cytometric analyses, protein-A purified antibodies were conjugated (Alexa Fluor 555 and/or Alexa Fluor 647) using protein-labeling kits according to manufacturer's instructions (Molecular Probes/Invitrogen Inc.). Antibody aliquots were stored in 1% BSA at –20° C. Isotype control antibodies included rabbit IgG or mouse IgG (eBiosciences) conjugated to Alexa Fluor 555 or Alexa Fluor 647.

Western blot analysis

Cytoplasmic extracts from trout spleen, K1, and liver were isolated as reported previously (Zwollo et al. 1998) and 2.5 μ g of protein lysate was mixed with 40 μ L of a 1x sample buffer containing 2.5% 2-ME and proteins separated by size using denaturing 12% SDS-PAGE

gels. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). Membranes were incubated in blocking solution of 5% dry milk in PBS containing 0.5% Tween (PBST) for 1 h, followed by a 2 h incubation in blocking solution in the presence of primary antibody in a final concentration of 0.25 µg/mL final dilution for anti-tIL-1β antibodies. A rabbit-anti-human α-tubulin IgG (H300; Santa Cruz Inc., which also cross-reacts with rainbow trout, zebrafish and *Xenopus* α-tubulin, was used at 0.25 µg/mL as a control for protein quality and loading (Zwollo et al, 2010). After primary staining with each antibody, four 5 min washes in PBST were then followed by a 1 h incubation with the secondary antibody goat anti-rabbit IgG-HRP conjugate (0.1 µg/mL; Zymed Laboratories) in blocking solution and membranes were washed four more times for 5 minutes each in PBST. Filters were developed using a chemiluminescence kit (ECL-Plus; GE Healthcare Life Sciences).

Fixation, permeabilization, and flow cytometry.

Cell pellets 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 resuspended in permeabilizing solution (BD perm wash in PBS, BD Biosciences) and stained as described previously (Zwollo et al., 2010, MacMurray 2013). For three-color flow cytometry, EBF antibody was conjugated directly with Alexa-647 (Invitrogen Inc), and the anti-IL-1β antibodies with Alexa555 or Alexa-647. An indirect staining approach was used for the third antibody by staining cells with a biotinylated HCmu (Warrs) in conjunction with streptavidin APC-750 (Sigma). For cell proliferation analysis, the nucleoside analog EdU and a Click-It kit (Invitrogen) was used according to manufacturer's instructions, as described previously (Barr et al., 2011). Approximately 30,000 events were acquired per sample using a BD FACSAarray (BD Biosciences). Duplicate samples were analyzed for each experiment. A minimum of ten fish was

used for each experiment with duplicate samples for each (indicated in the Figure legends). Contour graphs were generated using WinMDI 2-8 (J. Trotter 1993–1998) software. Contour graphs are shown as log algorithms with intervals of 50%. Statistics were obtained from WinMDI 2-8 software. Arithmetic means of population frequencies, standard errors, and P-values (2-tail student t test) were calculated for each experiment using Microsoft Excel software.

Selection of populations using magnetic beads.

PBLs were purified as above, and membrane IgM⁺ cells positively selected using a biotinylated anti-HCmu antibody (final conc 1 µg/1 x 10E7 cells/ml), according to the manufacturers' instructions (StemCell Research) with enrichment being between 88-93%, as described previously (Zwollo et al., 2008). Next, both selected (membrane HCmu⁺) and non-selected (membrane HCmu⁻) cells were resuspended in RNazol-RT (Molecular research Center, Inc.) at < 1 x 10E7 cells/ml, and stored frozen until RNA-purification. The experiments were repeated twice.

RNA, cDNA, endpoint and real-time PCR

mRNA was isolated using RNazol-RT and cDNA made using an iScript random labeling cDNA kit (BioRad). 1.0 µL of cDNA was initially used in a 25 µL volume end-point PCR using the Platinum PCR SuperMix (Invitrogen Inc.). To amplify IL-1β cDNA, two sets of primers were used, either targeting *IL1β1* or *IL1β2*, using PCR primers as used by others (Husain et al, 2012). Each PCR run was carried out on an ABI-Veriti 96-well Fast Thermocycler (ABI) and consisted of 2 min. at 95° C, 40 cycles of denaturing at 95° C for 45 sec, annealing at 65° C for 15 sec, and extension at 72° C for 15 sec, and ending with 7 min. at 72° C. Products were analyzed on a 2% agarose gel. Quantitative real-time PCR was performed using a PerfeCTa SYBR Green SuperMix, ROX PCR kit (Quanta Biosciences). Each sample was measured in quadruplicate,

and repeated twice. Trout α -tubulin was used as a reference as reported previously (Zwollo et al., 2008). The reaction was performed on an Applied Biosystems StepOne Real-Time PCR instrument. Expression levels were calculated as fold-change relative to the reference samples. The fold change, or amount of target, was calculated according to the Fold Change = $2^{-\Delta\Delta CT}$ equation (Livak and Schmittgen, 2001).

RESULTS

Characterization of the tIL-1 β antibody.

The tIL-1 β antibody was initially tested using western blot analysis, in spleen (SPL), anterior kidney (K1), and liver cells from an S-line trout. Western blot analysis in Figure 2B shows that an approximately 16-20 kD band was present in spleen, and, more strongly, in K1, but only weakly in the liver sample, similar to the predicted size of denatured IL-1 β 1 from carp (16-20 kD), trout (24 kD) and human (18 kD) mature, processed IL-1 β 1 (Verburg-van Kemenade et al., 1995, Mizutani et al, 1991, Zou et al., 1999, Hong et al., 2004). A α -tubulin antibody was used as a control for amount and quality of extract (Figure 2B). Next, the tIL-1 β antibody was tested in two-color flow cytometry in combination with the anti-HCmu antibody, using fixed cells (Figure 2C). Protein A-purified pre-bleed antiserum (PB; obtained from the same rabbit prior to immunization) showed no reactivity with immune populations from PBLs (Figure 2C). Two cell populations reacted with the tIL-1 β antibody: a minor population of HCmu⁻ cells expressing high levels of IL-1 β , and a major population that co-stained for IL-1 β HCmu, but with much lower staining intensity for IL-1 β . (Figure 2C).

The co-staining of HCmu⁺ B cells with tIL-1 β antibody suggested that B cells expressed (low levels of) IL-1 β protein. To confirm this, *IL-1 β* RNA expression was determined in PBLs that were positively selected with the anti-IgM antibody, using magnetic beads. RT-qPCRs were

performed on mRNA from both surface IgM⁺-selected, and non-selected cells, and *IL-1β* levels determined using *IL1β1* and *IL1β2* as target genes. Results in Figure 2D show that the levels of *IL1β1* RNA transcripts were detectable, and not significantly different, between surface IgM⁺ (selected) and non-selected cells, supportive of both expressing *IL1β1* mRNA. Interestingly, the precursor transcript *IL1β2* was expressed at significantly higher levels in IgM⁺ selected cells ($p<0.01$) compared to non-selected cells (Figure 2D). Together, these experiments support the specificity of the tIL-1β antibody to its target cytokine IL-1β in flow cytometry.

HCmu signatures in R- and S-line trout.

The abundance of immunoglobulin HCmu-expressing B cells was compared between R-line and S-line trout. Flow cytometry was performed on freshly isolated, non-immune, fixed and permeabilized cells using the anti-HCmu antibodies. All flow cytometric analyses in this study were performed using fixed and permeabilized cells, unless noted otherwise. First, the abundance of HCmu⁺ B cells was determined. For YC2011 trout, three tissues were tested: SPL, PBLs, and K1. Although the average percentage of HCmu⁺ cells was generally lower in tissues from R-line trout of that YC, no significant differences were detected between the S and R-lines in any of the three tissues (Figure 3A, Table I). For YC2012 trout, all four tissues were tested. R-line trout had significantly lower percentages of HCmu⁺ B cells than S-line trout, and this pattern was significant for SPL, PBL, and K1, but not K5 (Figure 3A, Table I).

Next, because R trout have larger spleens than S trout, we wished to determine the total number of HCmu⁺ spleen cells per trout, using the total number of isolated WBCs per trout spleen (after Histopaque purification). No significant differences were observed in total HCmu⁺ numbers between R and S line trout (Table II), suggesting that trout from each line have similar total numbers of splenic IgM⁺ B cells. Total numbers were not calculated for K1, K5, and PBLs, as too much variation is introduced during dissections and cell isolation for these tissues.

Next, the abundance of HCmu⁺⁺-expressing, immunoglobulin secreting cells (ISCs) was tested in both lines, and for both year classes. HCmu⁺⁺ ISCs include activated B cells, plasmablasts, and plasma cells (Barr et al, 2011). Results (Figure 3B, Table I) indicate that overall, R-line trout had lower percentages of HCmu⁺⁺ ISCs than S trout, with significantly lower percentages in PBL (for YC2012 only) and K1 (both YCs 2011 and 2012; Figure 3B and Table I). When the total number of HCmu⁺⁺ splenic ISCs per trout was calculated, no significant differences between R and S line trout were detected (Table II).

B cell signatures for B lymphocytes that do not express the isotype IgM.

Previous work by our lab and others has shown that although the majority of small (low FSC), (im)mature B cells express the IgM class, populations of IgM-lacking B cells (including IgT⁺ B cells) are also present in each tissue (Zwollo 2008, Zhang et al, 2010). To determine if differences in abundance of such B cells existed between R and S-line trout, two-color flow cytometry was performed using the combination of markers Pax5.PD and HCMu. The Pax5.PD marker was used here because it detects all (im)mature B cells independent of isotype or class (McMurray et al, 2013).

Results showed that, as expected, the majority of Pax5.PD⁺ cells in from SPL and PBL were IgM⁺ (HCmu⁺) B cells, as summarized in Table III. In agreement with the data shown in Figure 3A, PBLs of S trout had significantly higher percentages of Pax5.PD⁺/HCmu⁺ cells than R trout (50.5% and 42.3% respectively; p=0.001), while the total abundance of Pax5.PD⁺ cells as well as the abundance of Pax5.PD⁺/HCmu⁻ cells (eg, B cells that do not express IgM) did not differ between the lines (p=0.18 and 0.08 respectively), suggesting that the reduced abundance of (im)mature B cells in R trout is limited to the isotype IgM. For spleen, no significant differences were detected between R and S trout in abundance of either IgM⁺ or IgM⁻ (Pax5.PD⁺/HCmu⁺ or Pax5.PD⁺/HCmu⁻) cells (Table III). Both anterior and posterior kidney (K1 and K5) tissues had higher Pax5.PD⁺/HCmu⁻ to Pax5.PD⁺/HCmu⁺ ratios (eg, relatively more

IgM⁻ B cells) compared to PBL and SPL (Table III). No significant differences were detected in K1 tissue between R and S lines. However, K5 tissue of R trout had significantly lower percentages of IgM⁺ B cells (Pax5⁺/HCmu⁺) compared to S trout (Table III), similar to the trend seen in Figure 3A.

B cell signatures for developing B cells.

To investigate possible differences in the abundance of early developing B cells between R and S lines, cells were stained using the combination of tIL-1 β , EBF, and HCmu antibodies, and analyzed by flow cytometry. The IL-1 β marker was used for detection of myeloid lineage cells, and EBF to detect developing B cells (see Figure 1). Only YC2012 trout were used for this and the remaining experiments in this study.

Strikingly, when the abundance of all EBF-expressing cells was measured [either EBF⁺ or EBF⁺⁺, indicated as “EBF+(+)”], PBL, K1, and K5, but not SPL, showed significantly higher percentages of such cells in the R-line trout compared to S-line trout, with almost twice the abundance in R-line trout (Figure 4A). As expected, abundance of EBF⁺⁽⁺⁾ was highest in K1 (a hematopoietic site) and lowest in spleen (a secondary immune site). Significant differences between R- and S-line trout were also detected for cells co-expressing IL-1 β and EBF. Cells that expressed high levels of IL-1 β and stained moderately for EBF, but lacked HCmu (IL-1 β ⁺⁺/EBF⁺/mu⁻) were significantly more abundant in R-line trout than S-line trout, (P<0.05 for K1 and K5; Figure 4B). Because this population most likely represents early B/myeloid progenitors (Figure 1), it was not surprising that cells at this developmental stage are most abundant in K1, and virtually absent from the spleen. Lastly, a somewhat less abundant population of cells that stained moderately with IL-1 β , strongly with EBF and weakly with HCmu (IL-1 β ⁺/EBF⁺⁺/HCmu^{int}) was also significantly more abundant in R trout, but only in PBL (P<0.01) and K5 (P<0.01) (Figure 4C). IL-1 β ⁺/EBF⁺⁺/HCmu^{int} cells are predicted to be at the pre-B cell stage, based on

their high EBF expression (MacMurray et al., 2013), and low HCmu expression (Figure 1).

Tissue distribution of this population is fairly evenly across tissues.

Next, the total numbers of both progenitor and pre-cells in the spleen were calculated; no significant differences were detected between R and S line trout, although on the average, the spleens of R line trout had almost twice the number of early pre-B cells ($IL-1\beta^+/EBF^{++}/HCmu^{int}$) compared to S line trout (0.93×10^5 versus 0.47×10^5 cells for R and S line respectively; Table II).

Proliferation of progenitor cells in the anterior kidney of R-line and S-line trout.

To further explore the nature of B cell progenitors, K1 cells were labeled with the tracking marker PKH26 to compare proliferation patterns between S and R lines. PKH26 was used in this experiment because it allows identification of freshly isolated, slowly dividing progenitor cells while not requiring Edu-incubation and can detect multiple rounds of cell proliferation. PKH26-labeled K1 cells were collected after 2, 24, or 48 hours of culturing, fixed, and then analyzed by flow cytometry using either EBF or HCmu as the second marker. Lower intensity of PKH26 per cell reflects more cell division, hence, PKH26⁺ stained cells have undergone one more round of cell division compared to PKH26⁺⁺ stained cells (illustrated in Figure 5A). Overall, K1 cells from R line trout underwent significantly less proliferation at 24 and 48 hours (but not at 2 hours) compared to K1 cells from S fish (Figure 5A, 5B). Using EBF in combination with PKH26 (Figure 5C), and gating on EBF⁺ cells only, it was found that EBF⁺ progenitors from R line-derived K1 cells had proliferated less than progenitors from S line trout. This was reflected in a significantly lower ratio of EBF⁺/PKH26⁺ to EBF⁺/PKH26⁺⁺ cells in R-line trout, at both 24 and 48 hours, but not 2 hours (+/+ ratio, see Figure 5C). The proliferation of HCmu^{int} pre-B cells from K1 was tested similarly, by gating on HCmu^{int} cells only. Results indicated that pre-B cells from R line trout underwent significantly fewer cell-cycles than S line trout (the ratio of HCmu^{int}/PKH26⁺ to HCmu^{int}/PKH26⁺⁺ was lower for R line trout; Figure 5D) both after 24 and 48 hours, but not 2 hours. In summary, freshly isolated K1 progenitor and pre-

B cells from S line trout proliferated more rapidly in culture compared to those isolated from R line trout.

***In vitro* LPS-induction of plasmablast populations in R- and S-line cultures.**

To test for potential differences in sensitivity to LPS-induction in spleen and PBL, cells were cultured *in vitro* in the presence of FpLPS and the percentage plasmablasts determined on days 2, 4, and 7 post-induction. In addition, induction with FpLPS was compared to that using Ecoli LPS; the latter has been a routine mitogen for trout B cell proliferation. Cells were collected on days 2, 4, and 7 after LPS induction, fixed, and IgM⁺ plasmablasts (HCmu⁺/Edu⁺) detected in 2-color flow cytometry using the Edu-Click-iT kit and the anti-HCmu antibody.

Results for LPS induction in the spleen are shown in Figure 6A. FpLPS was significantly more potent than EcoLPS in both trout lines, resulting in more than 3-fold higher percentages of IgM⁺ plasmablasts on days 4 and 7 after induction. Importantly, no significant differences were observed between the two trout lines. Induction of PBLs also resulted in FpLPS inducing significantly more IgM⁺ plasmablasts than EcoLPS (on day 7 only), but no differences were detected between the two trout lines for either type of LPS (Figure 6B). Lastly, when anti-Pax5 antibody Pax5.PD was used instead of HCmu in the above experiments to include IgM⁻ plasmablasts (eg, IgT), no differences between the trout lines were detected (data not shown). Together, this suggests that both trout lines are equally capable of generating plasmablasts in response to *in vitro* LPS challenges.

Other differences in B cell signatures after *in vitro* FpLPS induction.

Next, possible maturation/differentiation effects of *in vitro* LPS-induction were determined in PBL and SPL using 3-color flow cytometry. Populations of interest are illustrated in the form of contour graphs in Figure 7A, for each of the three days collected. To rule out loss of myeloid lineage cells due to plastic adherence, wells were checked under the microscope

after each collection, and no adherent cells remained in the wells. In PBL cultures, the percentage of EBF-expressing cells dropped rapidly after FpLPS induction, and at approximately the same rate for both R- and S-line trout, with R-line trout having significantly more EBF⁺ cells than S-line trout on days 0 and 4 (Figure 7B). Further, the progenitor population (IL-1 β ⁺⁺/EBF⁺/mu⁻) dropped approximately 10-fold, from more than 5% on day 0 to less than 0.5% by day 7 post induction, and this pattern was the same for both S- and R-line trout (Figures 7A and 7C). The opposite pattern was seen for an EBF-expressing population staining with lower levels of IL-1 β (IL-1 β ⁺/EBF⁺/mu⁻): this population increased over time at a similar rate in both trout lines, but with R trout having gained significantly more cell abundance on day 4. (Figures 7A and 7D). In summary, *in vitro* FpLPS induction of PBL cultures reduced the abundance of EBF-expressing progenitors with high IL-1 β expression, and increased the abundance of EBF-expressing cells with lower IL-1 β expression, suggesting that at least in culture, LPS-sensitive progenitors can be induced to mature into B lymphoid cells.

The percentage of (im)mature B cells (HCmu⁺) did not significantly change after *in vitro* LPS induction in PBLs, using either S or R line cells. Further, although the average HCmu⁺ percentage remained higher in PBL cultures from S-line trout compared to R-line trout throughout the LPS-induction period, the differences were not significant (Figure 7E). The percentage of HCmu⁺⁺ ISC^s peaked on day 2 and then dropped down, in agreement with earlier studies using EcoLPS (Barr et al., 2011), but this pattern was identical for cells from both trout lines (Figure 7F).

Lastly, a transient cell population was detected in PBL cultures from both R- and S-line trout, with the phenotype IL-1 β ⁺⁺/EBF⁻/mu^{low}. This LPS-induced population expanded on day 2 and had dropped back down by day 7 (Figures 7A and 7G). This was not a B-progenitor/pre-B cell population, as it lacked EBF. It also expressed high levels of IL-1 β , suggesting it had myeloid characteristics. The nature of this population is addressed in the Discussion.

Spleen cells from R- and S-line trout were also tested in response to *in vitro* FpLPS induction. In S-line trout, the EBF⁺⁽⁺⁾ population increased significantly between Day 0 and Day 7 (P<0.05); this was not observed in R-line trout (Figure 8A). By Day 2, S trout already had a higher abundance of EBF⁺⁽⁺⁾ cells than R trout, although the difference was small. This pattern was similar to that for a population of maturing B (IL-1 β ⁺/EBF⁺/mu^{int}) cells, with a significant increase in abundance between Day 0 and Day 7 in S spleen cultures, and significantly higher abundance of such cells in S spleen compared to R trout by Day 7 (Figure 8B). The LPS-induced increase in abundance of EBF⁺ cells suggests that LPS-sensitive EBF⁺ pro/myeloid/pre-B cells are able to mature in culture, and that spleen cells from R trout mature slower towards HCmu⁺ cells than those from S trout. For IgM⁺ mature B cells and ISCs, no significant differences were detected upon *in vitro* FpLPS induction between the two lines (Figure 8C and 8D).

DISCUSSION

This study focuses on the immunophenotypes of naïve Fp-resistant vs. Fp-susceptible trout, with the anticipation of applying this knowledge within future global efforts to resolve the mechanisms of innate BCWD resistance. In this study, we compared the tissue distribution of B lineage and myeloid lineage cells between two lines of rainbow trout. Surprisingly, we found differences in the abundance of developing and mature B lymphoid cells between naïve animals from the two lines. This is discussed below.

R-line trout have a lower abundance of IgM⁺ (im)mature B cells and ISCs.

R line trout had significantly lower percentages of HCmu⁺ (im)mature B cells in their tissues than S-line trout, and this pattern was consistent for spleen, blood and anterior kidney (K1). Following along the same pattern, abundance of ISCs was also significantly lower in PBLs and K1 (but not spleen) of R-line trout. The lower abundance of ISCs in the K1 of R-line trout is

intriguing, as this site, being the functional equivalent of mammalian bone marrow, provides a home for long-lived plasma cells (LLPCs; Bromage et al, 2004). Hence, R-line trout have potentially fewer LLPCs.

Several hypotheses could explain this pattern. First, because R-line and S-line trout were selected based on survival after Fp-challenge at about 2.4 g average body weight (Silverstein et al. 2009, Leeds et al. 2010, Wiens et al. 2013a), they are selected based on the strength of their innate, rather than their acquired (humoral) immune system, which may not yet be fully developed at this stage. A stronger innate immune response might reduce the necessity of a strong humoral response, as bacterial pathogen loads are kept lower. Alternatively, it is still plausible that lower abundance of LLPCs in R trout correlates with a higher affinity of anti-Fp antibodies. Both hypotheses will be tested in future studies.

So how are the two lines similar? Although R trout had lower abundance of IgM⁺ B cells and ISCs, both trout lines had similar *total* numbers of IgM⁺ B cells and ISCs in their spleens, likely because the spleens of R trout are larger compared to S trout (Hadidi et al., 2008, Wiens et al 2013b). Further, from our *in vitro* LPS activation experiments we concluded that there were no clear differences in the terminal B cell differentiation pathways between the lines, as reflected by the rate of plasmablast formation and abundance of plasmablasts in either PBL or SPL.

Plasticity of progenitor lineages; making choices that increase Fp resistance?

Earlier studies had already identified the EBF⁺/HCmu⁻ population as progenitor stage cells (McMurray 2013); these cells also co-expressed the marker Pax5.E6 (which is highly expressed in progenitor cells), but not Pax5.PD, a B cell marker that is not strongly expressed until the late-pre-B cell stage. Here we expanded this phenotype and showed that the progenitors also stained strongly for IL-1β, a cytokine secreted by myeloid cells. The IL-

1 β ⁺⁺/EBF⁺/mu⁻ progenitor population also stained strongly with a trout-specific anti-TNF α antibody (Wiens, Zwollo and Ray, unpublished data), further supporting myeloid lineage characteristics, as TNF α is secreted by trout monocytes/macrophages (Qin et al 2001, Hong et al., 2013). However, myeloid lineage cells lack B cell markers Pax5.PD and EBF, at least in mammalian species (Lukin, 2008). Because the IL-1 β ⁺⁺/EBF⁺/mu⁻ progenitor population described here expressed both early B cell markers (EBF, Pax5.E6) and myeloid markers (IL-1 β , TNF α), we propose that it represents a bi-potential B lymphoid/myeloid progenitor (BMP), which has previously been described in mammalian systems (Montecino-Rodriguez, 2001, Blin-Wakkach et al, 2006), and is able to differentiate into B lymphoid cells, monocytes, or neutrophils. The putative trout BMPs were most abundant in the anterior kidney, as would be expected, as this is the main hematopoietic site in trout. However, the relatively high abundance of EBF⁺ cells found in PBLs suggests that blood is an important site where these cells are stored, induced, and/or perhaps in transit to spleen and K5. A model depicting the multi-lineage potential of BMPs, including their ability to trans-differentiate between IgM⁺ B and myeloid lineages, is shown in Figure 1.

Cell plasticity of immune cells in mammalian species includes differentiation pathways from EBF⁺ B progenitors towards either more mature B lymphoid cells, or towards monocyte/neutrophil lineage cells (EBF⁻/CSF-1R⁺/C/EBP α ⁺), but also includes trans-differentiation of B lymphoid cells to the monocyte/neutrophil lineage (reviewed in ref Nakajima et al, 2012). Because of the phagocytic nature of trout B cells (Li et al, 2006), our model also provides a potential mechanism by which cells could reversibly switch from phagocytic to non-phagocytic B lineage cells.

A transient cell population with the phenotype IL-1 β ⁺⁺/EBF⁻/mu^{low} was induced after *in vitro* FpLPS-induction of PBLs. This LPS-sensitive population peaked on day 2 and then dropped back to pre-induction levels, with both S and R line trout responding similarly. In

support of possible trans-differentiation this transient population was not a B-lineage cell, as it lacked EBF, did not stain with B cell marker Pax5.PD, and expressed high levels of IL-1 β . The puzzling characteristic of this cell type was its expression of low levels of HCmu. We speculate that this cell type is switching from B lineage towards myeloid lineage cells, and still possesses some HCmu protein, as observed in murine BMPs (Yu et al, 2003, Popi et al., 2008; Figure 1). To convincingly show that this transient bi-potential population can indeed generate monocytes (Figure 1), future studies will need to include additional myeloid-specific markers.

Differences in early B cell development between the trout lines.

Abundance of EBF-expressing cells was consistently higher in immune tissues from the R-line trout compared to S-line trout, including IL-1 β^{++} /EBF $^{+}$ /mu $^{-}$ progenitors and IL-1 β^{+} /EBF $^{++}$ /mu int precursors. Interestingly, from this stage on, S-derived pre-B cells appear to favor differentiation towards the IgM $^{+}$ immature B cell stage, reflected by higher abundance of IgM $^{+}$ B cells in S trout immune tissues compared to R trout. Alternatively, the higher percentage of BMPs and pre-B cells found in R trout tissues could reflect a slower progression towards EBF-driven B-lineage cells, increasing their abundance. This is supported by the cell cycle experiments in freshly isolated K1 cells, which showed that in culture, both EBF-expressing progenitors and HCmu int pre-B cells from R line trout divide more slowly compared to cells from S-line trout.

Effects of *in vitro* FpLPS-induction on EBF $^{+(+)}$ progenitors were studied in both PBL and spleen. When PBL cultures were induced with FpLPS, this *decreased* the abundance of both BMPs and pre-B cells, suggesting that they either die, or mature after LPS-exposure, with both trout lines having a similar pattern. Interestingly, we observed an opposite pattern in spleen-induced cultures, namely an *increase* in percentage of EBF $^{+(+)}$ and EBF $^{+}$ /HCmu int (B lymphoid lineage) cells upon FpLPS induction, but only in S-line trout (Figure 1; MacMurray et al, 2013).

The higher percentage of such cells in 7-day spleen cultures from S-line trout, supports the idea that B cell precursors can expand more rapidly in the spleens of S-line fish. The molecular pathways driving such lineage decisions or differentiation pathways will need future investigation.

Do BMPs play a role in IL-1 β secretion in response to infection? Sunyer et al. (2013) proposed a model where mammalian phagocytic B1 cells isolated from the peritoneal cavity, when activated by pathogenic bacteria to phagocytose, will secrete proinflammatory cytokines including IL-1 β and TNF α . The authors further hypothesized that phagocytic B cells in trout may have similar characteristics, so, it is feasible that the BMPs identified here provide an important source of IL-1 β and may promote cell recruitment to sites of Fp infection. Because R-trout have higher abundance BMPs in their blood, K1 and K5 compared to S line fish, R-line trout may have stronger capacity for innate responses than S-line trout through myeloid lineage differentiation.

Conclusions.

Fp-resistant trout had higher percentages of BMPs cells in PBL, K1 and K5 tissues as compared to S trout, potentially lower differentiation rates towards IgM⁺ B lymphoid cells, and lower percentages of IgM⁺ mature B cells. Tissues from Fp-resistant trout also had a significantly lower abundance of IgM⁺⁺ ISCs, most significantly in PBL and K1. However, mature B cells from both trout lines responded similarly to LPS-induction in culture. Future studies that focus on the dynamics of early developing B/myeloid populations appear to be the most promising route to identify and select for BCWD-resistant trout.

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FIGURE LEGENDS

Figure 1. Predicted phenotype of cells based on immune markers used. EBF, early B cell factor; HCmu, immunoglobulin heavy chain mu, Pax5.PD, Pax5-paired domain-specific. Dotted arrows with question marks indicate hypothetical pathways. Only B cell maturation pathways for the isotype IgM are depicted here.

Figure 2. Antibody design and testing of tIL-1 β antibody using western blot analysis. **A.** SWISS models and position of the immunogen (peptide) for trout tIL-1 β . **B.** Western blot analysis using the tIL-1 β antibody, and α -tubulin as control antibody. 2.5 μ g of S100 cytoplasmic fraction per lane SPL, spleen; K1, anterior kidney; Li, liver. Protein size (in kD) shown on the right. **C.** Flow cytometric analysis of fixed PBLs, using pre-bleed antibody (PB) labeled with Alexa555 (PB-555) and Alexa647 (PB-647), (left contour graph), or using tIL-1 β and HCmu antibodies (right panel). **D.** qPCR to detect *IL1 β 1* (left graph) and *IL1 β 2* (right graph) on HCmu-selected (Mu⁺) or non-selected (Mu⁻) PBLs, selected using magnetic beads. On the Y-axis: fold-change relative to Mu⁻. Mean $\pm$ SD. ** P<0.01.

Figure 3. Differences in HCmu⁺ (im)mature B and HCmu⁺⁺ ISC populations between S and R trout, using flow cytometry. N= 22 for YC11, and N=14 for YC12. S-line trout shown in grey bars, R-line trout in black bars. For K5 samples, no data were collected for YC2011. **A.** Percentage of HCmu⁺ cells, shown as mean $\pm$ SD. **B.** Percentage of HCmu⁺⁺ immunoglobulin-secreting cells, shown as mean $\pm$ SE. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 4. Differences between the progenitor populations of S-line and R-line trout, using flow cytometry. Grey bars: S line trout, black bars: R line trout. YC12, N=10 for each line.

Percentage of cells is shown as mean $\pm$ SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **A.** All EBF-expressing cells (EBF⁺). **B.** IL-1 β ⁺/EBF⁺/HCmu⁻ cells, **C.** IL-1 β ⁺/EBF⁺/Hcmu^{int} cells.

Figure 5. Comparison of cell proliferation between R-line and S-line trout in freshly isolated K1 cultures, using membrane-tracking dye PKH26. Flow cytometric analysis of PKH26-labeled anterior kidney cells after 2, 24, and 48 hours of culture. Solid line, R-line fish, dotted line, S-line fish. N=10 for each line. **A.** Representative histogram with PKH26 intensity gated on all lymphocytes, at 24 hours. In filled gray: 0 hr PKH26 incubation control, S-line fish. **B.** Proliferation rate of all lymphocytes, gated on all lymphocytes, as determined by the ratio of % PKH26⁺ to % PKH26⁺⁺ cells. Average of ratio with SE. **C.** Proliferation rate of progenitors (EBF⁺), gated on EBF⁺ cells only, as determined by the ratio of % EBF⁺/PKH26⁺ to EBF⁺/PKH26⁺⁺ cells. N=10. Average of ratio with SE. **D.** Proliferation rate of HCmu^{int} (pre-B) cells, gated on HCMu^{int} only, as determined by the ratio of % HCMu^{int}/PKH26⁺ to HCMu^{int}/PKH26⁺⁺ cells. N=10. Average of ratio with SE. * $P < 0.05$, ** $p < 0.01$.

Figure 6. B cell proliferation patterns in *in vitro* cultures after after LPS induction with either Ecoli LPS or Fp LPS (50 μ g/ml), and comparison of R-line and S-line trout. Numbers on the Y-axis are the mean percentage proliferating (HCmu⁺/Edu⁺ cells) or plasmablasts, as determined using flow cytometry. YC12, age 11-21 months. N=13 fish for each group. **A.** Spleen, **B.** PBLs. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 7. Patterns of EBF, IL-1 β and HCmu expression in FpLPS-activated PBL cultures **A.** Contour graph illustrating changes in flow cytometric patterns after FpLPS activation. **6B-G.** The specific subpopulations are indicated on top of each graph. Solid line: R-line trout; dotted line:

S-line trout. Numbers on the Y-axis are the mean percentage of cells +/- SE, comparison of R versus S trout (YC2012). N=15-20 fish per group. * P < 0.05, ** P < 0.01, *** P < 0.001.

Figure 8. Patterns of EBF, IL-1 β and HCmu expression in FpLPS-activated spleen cultures. **A-D.** The specific subpopulations are indicated on top of each graph. Solid line: R-line trout; dotted line: S-line trout. Numbers on the Y-axis are the mean percentage of cells +/- SE, comparison of R versus S trout (YC2012). N=12-15 fish per group. *S: P < 0.05 comparing day 0 and day 7, significant for S fish only.

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STAGE:	2012	PBL			Spleen			K1			K5		
		S line	R line	P value	S line	R line	P value	S line	R line	P value	S line	R line	P value
B/myeloid progenitor		6.2	8.2	0.100	0.2	0.2	0.810	7.9	16.3	0.040	2.3	5.2	0.014
Large pre-B (mu int)		2.7	5.0	0.005	0.6	0.9	0.006	1.1	1.0	0.390	2.0	7.6	0.004
(im)mat B (mu +) and late pre-B		44.8	34.0	0.0003	53.2	44.1	0.030	21.0	10.5	0.002	23.5	21.7	0.06
ISC (mu++)		0.09	0.05	0.011	0.54	0.46	0.47	1.01	0.66	0.04	0.68	0.49	0.08

STAGE:	2011	PBL			Spleen			K1		
		S line	R line	P value	S line	R line	P value	S line	R line	P value
(im)mat B (mu +) and late pre-B		41.4	37.8	0.36	55	49.5	0.2	14.5	14.6	0.97
ISC (mu++)		0.36	0.26	0.14	0.72	0.63	0.58	1.24	0.73	0.01

Table I. Percentage of cells for each developmental stage with P values, and comparing R-line and S-line trout.

Cell stage	Phenotype	total # S	total # R	P value
both BMP and pre-B	EBF+(+)	1.93 * E5	1.97 * E5	0.95
B/myeloid progenitor	IL-1 β +/EBF+/mu-	0.14 * E5	0.13 * E5	0.82
Large pre-B	IL-1 β +/EBF+/mu int	0.47 * E5	0.93 * E5	0.23
(Im)mature B	HCmu+	0.44 * E7	0.41 * E7	0.86
ISC (mu++)	HCmu++	0.46 * E5	0.44 * E5	0.93

Table II: Spleen total cell numbers for each stage.

All B cells				IgM+ B cells				IgM– B cells			
<i>Total Pax5-PD</i>				<i>Pax5.PD+/mu+</i>				<i>Pax5.PD+/mu–</i>			
	R	S	<i>p</i>	R	S	<i>p</i>		R	S	<i>p</i>	
SPL	46.7 (3.3)	54.0 (4.1)	0.22	35.1 (2.5)	44.0 (3.9)	0.06		12.5 (1.7)	10.1 (1.3)	0.26	
PBL	64.2 (1.7)	67.9 (2.0)	0.18	42.3 (1.7)	50.5 (1.3)	0.001		21.9 (1.9)	17.3 (1.6)	0.08	
K1	26.9 (1.4)	31.4 (3.1)	0.22	13.6 (1.4)	16.0 (1.5)	0.26		13.3 (1.7)	15.4 (2.0)	0.42	
K5	50.9 (3.9)	55.0 (3.4)	0.25	22.4 (1.6)	26.3 (2.0)	0.03		28.5 (2.5)	28.8 (2.6)	0.94	

Table III: Percentages of HCmu+ and HCmu– B cells using Pax5.PD, with P values.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5

A. SPL

B. PBL

Figure 6

Figure 7A.

Figure 7B-G:

Figure 8