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Innate Immune Cell Signatures in a BCWD-Resistant Line Of Rainbow Trout Before and After *In Vivo* Challenge With *Flavobacterium psychrophilum*

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Abstract

Phenotypes of myeloid-lineage cells remain poorly understood in the rainbow trout, and were the focus of this study, including effects of *in vivo* challenge to *Flavobacterium psychrophilum* (Fp), the cause of Bacterial Cold Water Disease (BCWD). A genetic line was used that is highly resistant to BCWD (R-line) as well as a susceptible control line (S-line). Using flow cytometry, we describe two Pax5-negative, myeloid-lineage populations: Population 1 consisted of small cells with high SSC and strong staining for Q4E, MPO, Pu1, EBF, and IL-1 β , which we named “neutrophil-like” cell. Population 2 had high Q4E, but weaker MPO, Pu1, EBF, and IL-1 β staining. Five days after Fp-challenge, both genetic lines had a reduced abundance of neutrophil-like cells in anterior kidney, PBL, and spleen. Pop. 2 abundance was reduced in anterior kidney, and increased in spleen. S-line fish responded more strongly to Fp-challenge compared to R-line fish. Challenged fish with a higher abundance of neutrophil-like cells had significantly lower Fp-loads after challenge, suggesting that these cells aid in the resistance to BCWD.

INTRODUCTION

Myeloid-lineage immune cells, including neutrophils, monocytes and macrophages, play essential roles in the immune response through antimicrobial killing, antibody- or complement-mediated phagocytosis, MHC Class II presentation, and release of cytokines and chemokines. In humans, neutrophils and monocytes develop in the bone marrow and are released into the blood after they reach maturation and/or during inflammatory responses. While human myeloid-lineage cells have been studied extensively, in teleosts such cells remain poorly defined. In teleosts, myeloid-lineage cells are formed in the anterior kidney (AK), the main hematopoietic tissue in fish and the functional equivalent of bone marrow. Myeloid-lineage cells that have been reported in teleost (Zapata and Cooper, 1990) (Balla et al., 2010) (Overland et al., 2010) display varying degrees of phagocytic capacity, and can be further characterized based on their size (Forward Scatter; FSC) and complexity (Side Scatter; SSC); unstimulated neutrophils have the phenotype FSC^{low}/SSC^{high}, while activated

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macrophage and neutrophils are FSC^{high}/SSC^{high} cells. Further, macrophages and neutrophils have been defined based on their respiratory burst activity (Tumbol et al., 2009) (Sharp and Secombes, 1993).

Only few antibodies are currently available to characterize myeloid-lineage cells in rainbow trout. A trout-specific monoclonal antibody named Q4E recognizes neutrophil-like cells and a subset of myeloid cells (Kuroda et al., 2000). Expression of the enzyme myeloperoxidase (MPO) has been used as a marker to distinguish between neutrophils and monocytes in teleost. Zebrafish have the MPO-homologue myeloid-specific peroxidase (mpx), as proposed by Lieschke et al (2001). In zebrafish and goldfish, neutrophils express high levels of MPO/MPX, monocytes only stain weakly for this enzyme, while zebrafish basophil/eosinophil populations lack MPO/MPX staining (Katzenback and Belosevic, 2009) (Bennett et al., 2001). Sudan black has been used to stain neutrophil granules in zebrafish and gold fish (LeGuyader et al, 2008),(Bennett et al., 2001) (Katzenback and Belosevic, 2009). Macrophage Colony Stimulating Factor-Receptor 1 (M-CSFR) has been used in goldfish to distinguish between neutrophils and monocytes as it is expressed on monocytes and macrophages, but not neutrophils (Katzenback and Belosevic, 2009). M-CSFR1 was cloned and sequenced in the rainbow trout, and is expressed in AK, spleen (SPL), and peripheral blood leukocytes (PBL) (Honda et al., 2005). Three additional M-CSFR-like sequences have since been identified in the rainbow trout (Berthelot et al., 2014). Lastly, antibodies against trout cytokine IL-1 β can aid in the identification of myeloid-lineage cells in combination with other markers (Zwollo et al., 2015).

Lineage-specific expression by transcription factors provides useful developmental markers in poorly defined species, including the rainbow trout (Zwollo, 2011). One marker of potential use in delineation of teleost myeloid-lineage cells is ETS-domain transcription factor Pu1, essential for early cell-fate decisions in mammalian B lymphoid, myeloid, and granulocyte development (Dahl and Simon, 2003). High levels of Pu1 enforce myeloid/granulocytic development, while low levels promote B-cell development and neutrophil maturation (Mercer et al., 2011) (Friedman, 2007). Further, in humans a transient Pu1 “boost” is believed to be necessary to complete neutrophil maturation (Mercer et al., 2011) (Friedman, 2007). Pu1 has been cloned and characterized in the rainbow trout, and is expressed at high levels in macrophage primary cultures derived from the AK. Further, Pu1⁺ (hematopoietic precursor) cells are abundant in AK (Ribas et al., 2008). In zebrafish, Pu1 is expressed in myeloid progenitors and in immature neutrophils (Bennett et al., 2001). Larval zebrafish neutrophils are unusual, as they retain their Pu1 expression, even after they are differentiated and in the periphery (Le Guyader et al., 2008).

Here we first determined the abundance of myeloid-lineage cells in rainbow trout, using a combination of six different immune markers. Next, we tested cellular changes after an *in vivo* immune response to *Flavobacterium psychrophilum* (Fp), a Gram-negative bacterium that causes Bacterial Cold Water Disease (BCWD). We took advantage of the availability of a genetic line that is highly resistant to Fp challenge, designated ARS-Fp-R (or R-line), and used the available reference susceptible line, ARS-Fp-S (S-line) for comparison (Silverstein et al., 2009) (Leeds et al., 2010) (Wiens et al., 2013a). Both genetic lines were generated through a selective breeding program at the National Center for Cool and Cold Water

Aquaculture (NCCCWA). A number of innate and acquired immune components reportedly differ between the two lines, with R-line fish having an enhanced capacity for pathogen control relative to the S-line and R-line fish having significantly larger spleens (Marancik et al., 2014b)(Wiens et al., 2013b)(Hadidi et al., 2008), although the latter does not appear to have a protective role (Wiens et al., 2015). Additionally, R-line fish have a higher abundance of Early B-cell Factor-expressing (EBF) cells compared to S-line trout (Zwollo et al., 2015), although the identity of EBF⁺ cells is more complicated than previously assumed (this study). Further, R-line fish have a higher abundance of IgT⁺ B lineage cells (Zwollo et al., 2017), a lower abundance of IgM⁺ B lineage cells (Zwollo et al., 2015), and higher expression of secreted heavy chain tau transcripts, compared to S-line fish (Zwollo et al., 2017), suggesting that R-line fish have stronger mucosal responses than S-line fish.

Because resistance to BCWD has been observed during the very early stages of the life cycle (when fish weight 0.2 grams; Hadidi et al., 2008), it is likely that innate immune differences exist between the two lines. However, no studies have yet assessed whether myeloid-lineage cells play a role in resistance to BCWD. Here we begin addressing that question. We describe two populations of Q4E⁺ myeloid-lineage cells which differed in expression of MPO, Pu1, EBF, and Il1 β . We show that the abundance of the two populations differed between the genetic lines. Further, after *in vivo* challenge with Fp, S-line fish responded more intensely than R line fish although both lines had similar patterns of changes. Possible consequences of specific myeloid-signatures for resistance to BCWD are discussed.

MATERIALS AND METHODS

Animals and facilities.

All trout were bred at the National Center for Cool and Cold Water Aquaculture (NCCCWA, Leetown, West-Virginia) 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., 2013a). Fish were either spawned in 2013 (Year Class 13, YC13) or YC15. Fish 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 well 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.

In vivo Fp challenge.

The Fp challenge on YC15 fish has been described previously (Zwollo et al., 2017). Immune tissues were collected on day 5 post-challenge. The SPL, PBL and AK cells were fixed and permeabilized for flow cytometric analysis.

RNA, cDNA, Real-Time PCR and sequence determination.

RNA purification and cDNA synthesis was reported previously (Zwollo et al., 2015). Expression levels of rainbow trout M-CSFR were based on sequence GSONMT00023347001; (Honda et al, 2005). Expression in AK, spleen (SPL), peripheral blood leukocytes (PBL) and posterior kidney (PK) from YC13 or YC15 fish were determined using a custom TaqMan® Gene Expression Assay (Applied Biosystems), with a FAM reporter, NFQ quencher, and a ROX reference. The Taqman assay was designed using the design tools from the company (forward primer MCSFR.F: 5'-TGACCCTCAGTCCCTCAACT-3'; reverse primer MCSFR.R: 5'-CCAGCAAAGTTAAAGGTGACACA-3'; probe: 5'-AGGAGTTCACAGTGGAGTGTGTGCAC-3'; Amplicon length; 60 nt). These primers do not have the required homology in the amplicon region to anneal to three additional rainbow trout MCSFR genes (GSONMT00016920001, GSONMT00017434001, and GSONMT00036252001, using the NCBI Blast software (Berthelot et al., 2014). qPCR reactions were performed using a StepOne™ Real-Time PCR System 48 Well Instrument (Applied Biosystems). A melt curve analysis was performed to ensure purity of PCR product. Each sample was run in triplicate. Negative controls included “no cDNA”, using dH₂O, and were included in each experiment. Relative fold change (RFC) was determined by calculating the 2^{-ddCT} for each sample using α -tubulin as the control target as reported previously (Schouten et al., 2013). The Taqman assay for Fp loads has been described elsewhere (Marancik and Wiens, 2013) (Zwollo et al., 2017).

Percoll gradients and purification of neutrophils.

Rainbow trout and human blood cells were enriched for myeloid-lineage cells through separation over a Percoll gradient (GE-Healthcare Inc), as described previously (Zwollo et al., 2005). Three layers were used, 50%, 60%, and 70%, and after centrifugation, cells from the 50% and 60% layers were removed and pooled. Cells were then fixed and permeabilized using standard methods (see below). Human neutrophil purification has been described elsewhere (Voyich et al., 2005).

Antibodies.

The rabbit-anti-human EBF IgG (H300; detecting aas 1–300 of EBF1, and reactive with mouse/human EBF2, 3, and 4) were purchased from Santa Cruz Biotech. The polyclonal rabbit-anti-trout IL-1 β antibody has been characterized previously (Zwollo et al., 2015). The polyclonal rabbit-anti-human Pu1 IgG (H-135, recognizing aas 1–135 of human Spi1) was purchased from

Santa Cruz Inc. The Q4E monoclonal antibody was a gift from Drs. Kuroda (and shipped by Dr. Bernd Kollner (Friedrich-Loeffler Institute, Federal Research Institute, Germany), and recognizes rainbow trout granulocytes, monocytes and macrophages, but not resting lymphocytes or thrombocytes (Kuroda et al., 2000). The polyclonal rabbit antibody MPO IgG (H300, detecting aa 446–745 at the C-terminus of the heavy chain of human MPO) was purchased from Santa Cruz Inc. This antibody cross-reacts with myeloperoxidase-like (XP_021472524) and eosinophil-peroxidase-like proteins that have been identified in the rainbow trout (GSONMT00079314001), the two proteins are 99% similar to each other, and

have approximately 69% homology, with the heavy chain of human myeloperoxidase within the aa 446–745 region (ZP_021472524.1). The rabbit polyclonal Pax5.PD antibody recognizes the paired domain (PD) of Pax5 in rainbow trout B-lineage cells (Zwollo et al., 2008). Isotype control antibodies included rabbit IgG or mouse IgG (eBiosciences). All antibodies were conjugated to Alexa Fluor 555 or Alexa Fluor 647, and stored in 1% BSA at –20° C as described previously (Barr et al., 2011).

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 –80C (Zwollo et al., 2010). Approximately 30,000 events were acquired per sample using a BD FACSArray (BD Biosciences). Duplicate samples 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%.

Statistical analysis.

Statistical analysis used the environment R to calculate p values and has been described previously (Zwollo et al., 2017).

RESULTS

Our first goal was to determine whether R-line fish differed from S-line fish in expression of monocyte marker M-CSFR, using RT-qPCR. Next, we wished to define the phenotype of myeloid-lineage cells using six antibodies (Q4E, MPO, IL-1 β , Pu1, Pax5.PD, and EBF) in a flow cytometric approach. Our third goal was to use this information to determine whether the abundance of myeloid-lineage cells changed after an *in vivo* challenge with Fp and whether these changes differed between R-line and S-line fish.

Expression patterns for M-CSFR.

Four immune tissues from naïve animals were tested for potential differences in M-CSFR gene expression between R-line and S-line fish, namely AK, PBL, SPL, and PK, using a Taqman assay. Both YC13 and YC15 samples were available for analysis of AK tissue, while for PK, only YC13 samples were used. SPL and PBL samples were from YC15 fish. Results showed that M-CSFR expression was significantly higher in AK and PK from R-line fish compared to S-line fish, for YC13 (AKYC13 and PKYC13, Figure 1). However, no differences between the genetic lines were observed for YC15 samples (AKYC15, SPLYC15 and PBLYC15; Figure 1). Limitations of this approach include 1. that potential differences are diluted through the average expression level of M-CSFR per tissue, 2. the sample size for these experiments was low (between 8 and 13, and See Figure 1 legend for values for each tissue, year, and for each line), and 3. that we only measured expression of one of the (4) M-CSFR genes present in the rainbow trout genome.

Identification of myeloid/granulocytic cell populations using flow cytometry.

As an alternative approach towards uncovering potential differences in myeloid-lineage populations, we used flow cytometry. The six antibodies used here are described in the Methods section and listed in Table I. The rainbow trout-specific Q4E antibody reportedly detects the majority of neutrophils, and a subset of monocytes and macrophages (Kuroda et al., 2000). When cells were stained using Q4E in combination with Pu1, IL-1 β , or MPO antibodies, four Q4E-reactive populations were detected, which differed in intensity of MPO staining: Q4E⁺/MPO⁺/IL-1 β ⁺/Pu1⁺, Q4E⁺/MPO^{int}/IL-1 β ^{int}/Pu1^{int}, Q4E⁺/MPO⁻/IL-1 β ^{low}/Pu1⁺, and Q4E^{int}/MPO⁻/IL-1 β ^{int}/Pu1^{int}, as shown in Table I and Figure 2. Further, 2-color staining using Pu1 and MPO, and using IL-1 β and MPO, generally showed co-staining, although there were differences in staining intensity of Pu1 and IL-1 β in AK compared to PBL (Supplemental Fig 1A and 1B, Table I). Isotype control antibodies included rabbit IgG (Molecular probes) or mouse IgG (eBiosciences) conjugated to Alexa 555 or Alexa 647 were used to set gates to distinguish between positive and negative populations, as described previously (Barr et al, 2011).

To aid in the identification of neutrophil-like cells in rainbow trout, wbcs from both trout and human blood were enriched for myeloid-lineage cells using Percoll gradients (50–60%), cells fixed and permeabilized, and stained using MPO and Pu1 antibodies. As a positive control, human neutrophils were purified using the method of Voyich (Voyich et al, 2005). Repeated attempts to purify trout neutrophils using Voyich's method failed, suggesting they differ from human neutrophils. Attempts to purify neutrophils from blood using the method developed for goldfish also failed in our hands (Katzenback and Belosevic, 2009).

As expected, purified human PNCs stained strongly for MPO, but not for Pu1 (Figure 3). In contrast, MPO⁺ trout PBLs co-stained with Pu1, and no obvious MPO⁺/Pu1⁻ population could be detected (Supplemental Figure 1A). The majority of MPO⁺ (neutrophil-like) cells from either human or trout origin were relatively small cells (Figure 3) with high SSC (Supplemental Figure 1C), a characteristic of neutrophil identity, and in agreement with other studies (Tumbol et al., 2009). Staining AKs and PBLs with both IL-1 β and MPO resulted in a clear MPO⁺/IL-1 β ⁺ population in both tissues, as well as a population with intermediate IL-1 β staining and no MPO staining, in PBLs (Supplemental Figure 1B). We have reported earlier that B cells also stain somewhat for IL-1 β ⁺ (Zwollo et al, 2015), so (a subset of) MPO⁻/IL-1 β ^{int} cells likely represent B cells.

Next, cells were stained with Q4E and B lineage-specific antibodies Pax5.PD or EBF. Patterns differed between tissues: in AK, one major population of Q4E⁺/Pax5.PD^{low-int} cells was observed, as well as a population of Q4E⁻/Pax5.PD⁺ (late developing B) cells (Figure 2A). The Q4E⁺/Pax5.PD^{low-int} population varied in intensity of Pax5.PD staining between fish (between low and intermediate staining; Table I). Further, Q4E⁺/Pax5.PD^{low-int} cells stained strongly for Pu1 (Table I). The variation of Pax5 staining intensity in Pu1⁺ cells is interesting because (B cell-specific transcription factor) Pax5 is considered an antagonist of Pu1, and in order to induce myeloid differentiation, Pu1 has to overcome inhibitory action of Pax5 (Mercer et al., 2011), a potentially dynamic process within the AK. Hence Pop. 4 may represent developing B/myeloid progenitors. PBL and SPL showed three Q4E⁺ populations that differed in intensity of Pax5.PD staining: Q4E⁺/Pax5.PD⁻, Q4E⁺/Pax5.PD⁺, and Q4E⁺/

Pax5.PD⁺⁺ (Figure 2B and Table I). Next, using Q4E in combination with EBF antibody, a small population of Q4E⁻/EBF⁺ cells, most likely early developing B cells, was detected in AK (Figure 2A). Unexpectedly, a major population of EBF⁺ cells co-stained with Q4E (Q4E⁺/EBF⁺; Figure 2). This result is addressed in the Discussion.

The combined use of the six antibodies resulted in assignment of 5 different cell populations, as listed in Table I. Population 1 (Pop. 1) reacted with Q4E and co-stained strongly with MPO, IL-1 β , Pu1, and EBF, but not with Pax5, and based on its high MPO-reactivity and FSC^{low}/SSC^{high} phenotype, was referred to as a “neutrophil-like cell”. Population 2 (Pop. 2) cells also reacted with Q4E, but generally had weaker staining for MPO, IL-1 β , Pu1, and EBF, and did not stain for Pax5. Population 3 cells (Pop 3) were, based on earlier reports (MacMurray et al., 2013) late developing B cells (including late pre-B, immature, and mature B cells; Q4E⁻/MPO⁻/EBF^{low}/Pax5.PD⁺). Further, MPO^{low}/IL-1 β ^{int} cells may represent either B cells (Pop 3, no MPO staining) or Pop. 2 cells (int MPO staining). A fourth population (Pop 4) co-stained for Q4E and Pax5, but had lower (and varying) Pax5 staining in the AK. These cells may be myeloid/B progenitors. A fifth population (Pop 5), mostly seen in PBL and SPL, stained moderately for Q4E, and strongly for Pax5.PD (++) , and could represent activated or phagocytic B cells. These cells were identical to a cell type described previously, which stained positive for both IgM and IgT (Zwollo et al, 2017), and which is currently being investigated as part of a different study.

Patterns of myeloid-lineage cells in mock-challenged fish.

Using combinations of the six antibodies, three tissues were analyzed using flow cytometry, AK, PBL, and SPL, and results are summarized in Tables II–IV. Significant differences were found between R-line and S-line fish in AK and SPL, but not in PBLs. In the AK, the only difference was a significantly higher abundance of Pop. 2 cells (Q4E⁺/EBF^{low}) in Rline fish compared to S-line fish (Table II). In SPL, the abundance of Pop. 1 cells (IL-1 β ⁺/MPO⁺ cells) was almost twice as high for S-line fish compared to R line fish (Table IV).

Changes in immune cell signatures after Fp-challenge.

In S-line fish, Fp challenge dramatically reduced the abundance of both Pop. 1 and 2 cells in the AK while R-line fish only had a significant reduction in Pop. 2 cells (Table II, right panel). Abundance of B cells (Pop. 3) in the AK increased in both lines (Table II), while the opposite pattern was observed for Pop. 4. Together, for AK, Fp-challenge resulted in a shift from mostly innate (Q4E⁺) cells to mostly B-lymphoid cells (Q4E⁻/Pax5.PD⁺), and this response was more strongly seen in S-line fish.

In PBLs, R-line fish showed no significant changes in abundance of either Pop. 1 or Pop. 2 cells after challenge (Table III). In contrast, S-line fish reacted strongly to challenge as reflected by a dramatic reduction in Pop. 1 cells (Table III). Changes in Pop. 3 and Pop 4 abundance could not be determined due to insufficient cells for flow analysis.

In SPL, as in AK, Fp-challenge was much more severe in S-line fish compared to R-line fish. A reduced abundance of Pop. 1 cells was seen in both lines, while abundance of Pop. 2 cells increased significantly, but only in S-line fish. Abundance of B cells (Pop. 3) was

significantly reduced in both lines after challenge (Table IV). Lastly, Pop. 4 abundance was increased after challenge, but only in S-line fish (Table IV).

Patterns of tissue abundance.

The average abundance of the various immune populations differed between the three immune tissues. Pop. 1 cells were by far the most abundant in the AK (15.6% - 23.9%), somewhat less abundant in PBLs (9.7% - 10.9%), and least abundant in the spleen (1.6% - 3.0%). Pop. 2 cells were also most abundant in the AK (17.4–33.2%), but had much lower abundance in PBL and SPL (between 1 and 2 %). Pop. 3 abundance was high in SPL (53.8–62.8%) and lower in AK (17.5–19.9%), in agreement with earlier studies (Zwollo et al., 2008). Pop. 4 cell abundance was high in AK (45.8%–51.4%), and low in PBL and SPL (0.87–2.3%). After Fp-challenge, Pop. 4 abundance was reduced in AK, and increased in the SPL.

Correlations between cell abundance and Fp loads.

Next, we determined whether abundance of Pop. 1 and Pop. 2 cells correlated with Fp pathogen loads of each fish, as shown in Table V. Fp-loads had been determined as reported in our earlier study (Zwollo et al., 2017). The majority of correlations between Pop. 1 abundance and Fp loads were negative, in other words, fish with lower Fp-loads typically had higher abundance of Pop. 1 cells (Table V). There was one exception however, in the AK of S-line fish, where IL-1 β ⁺/MPO⁺ cell abundance correlated *positively* with Fp load. In contrast, this correlation was not seen for Q4E⁺/EBF⁺ cells, suggesting that these two cell populations, although both assigned under Population 1 cells, are not identical. For Pop. 2 cells in AK, a significant negative correlation was found between abundance of Q4E⁺/EBF^{low} cells (Pop. 2) and Fp loads but only in R-line fish.

Several significant negative correlations between Pop. 1 cell abundance and Fp-loads were detected in PBL, including for Q4E⁺/EBF⁺ (All fish and FpS fish only), and IL-1 β ⁺/MPO⁺ (FpS group only). Similar (significant) negative correlations were seen in the SPL: higher abundance of IL-1 β ⁺/MPO⁺ cells correlated with lower Fp loads when fish from both lines were included in the analysis. Overall, higher abundance of neutrophil-like cells in immune tissues correlated strongly with lower Fp-loads after challenge.

DISCUSSION

The main goals of the study were to define myeloid-lineage cells in rainbow trout, and then to use this knowledge to determine whether R-line fish differed from S-line fish in their abundance of specific myeloid-lineage populations, either before or after an *in vivo* Fpchallenge. Although we made important progress in the characterization of these cell populations, we were unable to unequivocally name or identify each cell population. From the gene expression study, we learned that R-line fish had higher expression of M-CSFR than Sline fish in their AK and PK tissues (with the caveat that only one M-CSFR gene was detected by our Taqman assay). From the flow cytometric study we learned that the overall abundance of myeloid-lineage immune cells was similar between the two lines if based solely on the staining with the Q4E antibody. However, when Q4E⁺ cells were further

separated based on expression of MPO, Pu1, IL-1 β , and EBF markers, we observed several significant differences between the lines.

Neutrophil-like cells.

In mammalian species, mature neutrophils stain strongly for MPO, while levels of Pu1 protein are low (Hromas et al., 1993), and this pattern was confirmed here. While human neutrophils had the expected MPO⁺/Pu1⁻ phenotype, in rainbow trout the majority of MPO⁺ cells co-stained with Pu1. We hypothesize that these MPO⁺/Pu1⁺ cells are immature neutrophils, which transiently express Pu1, as shown for zebrafish and humans (Bennett et al., 2001) (Friedman, 2007). Of interest is also the report that larval MPO⁺ granulocytes retain their Pu1 expression even when they reach the periphery (Le Guyader et al, 2008), supportive of the unusual phenotype for rainbow trout neutrophil-like cells (Pop. 1 cells).

Whether in teleosts such cells do not fully mature until reaching their site of inflammation, is unknown, but this possibility has been proposed by others (Kilpi et al., 2013) (Nikoskelainen et al., 2006). In cell activation studies in the barramundi (Asian sea bass; Tumbol et al 2009), the authors note that neutrophil-like cells from the AK are fairly insensitive to PMA, compared to those from the peritoneal cavity, presumably because the former are not yet fully mature/activated. Whether our neutrophil-like cells fully mature upon arrival at inflammation sites could be tested in future studies by isolating MPO⁺ cells from infected tissues (eg, muscle) in Fp-challenged fish, and determine whether or not such cells stain for Pu1.

It is likely that cell abundance in a tissue by itself is not a good indicator for resistance to BCWD. For example, higher MPO⁺/IL-1 β ⁺ cell abundance in the spleen correlated strongly with lower Fp-loads in challenged fish (both R-line and S-line fish). This would suggest a protective role for neutrophil-like cells in the spleen, but this is not reflected in their cellular abundance, as R-line fish have lower abundance than S-line fish. Further, strong (negative) correlations were seen between abundance of Q4E⁺/EBF⁺ cells and Fp-loads in AK, although abundance of this cell type is the same in both (mock-challenged) lines. After challenge, R-line fish retain that same abundance, while in S-line fish this population is strongly reduced.

Studies on whole body gene expression using 1.1 gr fry (Marancik et al., 2014a) showed changes in expression of the MPO-like eosinophil peroxidase-like gene (EPO-like; GSONMT00079314001) between the genetic lines, as well as after Fp-challenge: EPO expression in R line fish was higher than in S line fish, when comparing mock-challenged animals. In Fp challenge experiments, the authors observed increases in expression of EPO in R-line fish (from day 1 to day 5), but not in S-line fish. These changes are, as our data, genetic line-dependent. However, the transcriptome study is very different from ours in terms of methods, tissues analyzed, and age/size of the fish. However, the stronger increase in EPO seen in their R-line fish is important information, and suggest that future studies should focus on additional sites (eg, not just AK, SPL, and PBL), including sites of inflammation, where neutrophil-like cells are expected to accumulate after challenge.

Population 2 cells.

In AK, R-line fish had a higher abundance of Pop. 2 cells compared to S-line fish, and further, higher Pop. 2 cell abundance correlated with lower Fp-loads, suggesting it is protective. Unfortunately, the Pop. 2 cells are highly heterogeneous, and low/intermediate staining of MPO has been observed in a number of different cell types, including during hematopoiesis. In zebrafish, myeloblasts (neutrophil precursors) and monocytes stain moderately positive for MPO, while in mammals, (low levels of) MPO have been detected at the Hematopoietic Stem Cell, Common Myeloid Progenitor, and Granulocyte/Monocyte Progenitor stages (Miyamoto et al., 2002). Therefore, it is possible that in the AK, MPO^{low/int} cell populations include monocytes, early progenitor cells, and/or precursor neutrophils (Nikoskelainen et al., 2006)(Tumbol et al., 2009).

A 2013 study (Kilpi et al., 2013) on resistance to Fp in the rainbow trout also described two distinct populations of myeloid-lineage (“phagocytic”) cells in blood: monocytes and neutrophils. The authors conclude that blood monocytes play more significant roles in *in vivo* resistance to Fp-infection than neutrophils, because trout neutrophils are relatively late in becoming fully functional phagocytes compared to blood monocytes (as was noted by Tumbol et al 2009). However, the authors did not identify their cell populations using MPO or Q4E, and assigned monocytes, not neutrophils, the FSC^{low}/SSC^{high} phenotype, making their data difficult to compare to ours.

Our gene expression studies using M-CSFR, a monocyte/macrophage marker, showed that its expression was higher in the AK of R-line fish compared to S-line fish (although only for YC13). If we speculate that Pop. 2 cells include the monocyte population, then R-line fish may have relatively more monocytes/macrophages in the AK than S-line fish, and the observed negative correlation between abundance of Pop. 2 cells (Q4E⁺/EBF^{low}) and Fp-loads in R-line fish supports this. Hence, it is possible that monocytes/macrophages play roles in protection from BCWD.

Intensity of immune response to Fp-challenge differs between the lines.

Upon Fp-challenge, S-line fish had a much higher induced immune response (based on changes in cell abundance) compared to R-line fish. This likely means that R-line fish, which also had much lower pathogen loads than S line fish after Fp-challenge (Zwollo et al., 2017) respond faster, and/or more efficiently to infection, and consequently, have only minor cellular changes (by day 5). Although the intensity of the response differed between R-line and S-line fish, the *direction* of the changes was similar. They included 1). reduction in abundance of Pop. 1 cells in all three tissues (AK, PBL, and SPL); 2). reduction in Pop. 2 cell abundance in the AK; 3). increase in Pop. 2 cell abundance in the spleen. This observation of similarity in direction of response shared by both genetic lines is in agreement with a hypothesis based on a histopathologic study on the same genetic strains (Marancik et al., 2014b) that differential survival after Fp-challenge results from a divergence of disease magnitude, not altered disease course.

It remains unclear whether the changes in cell abundance after Fp-challenge reflect migration of cells away from (storage) sites or towards infection sites, and/or whether cell

populations expand (through cell division), or differentiate into phenotypically distinct effector cells. Hence, use of flow cytometry, which measures relative population abundance for a given tissue, is unable to distinguish between these various possibilities.

The identification of EBF expressing cells.

We unexpectedly found that a significant population of EBF⁺ cells co-stained with Q4E. EBF is a transcription factor that plays diverse roles in cell development. Previously, we had used the EBF antibody to identify early developing B cells (Zwollo et al., 2015) (Zwollo et al., 2010) which express EBF1. However, in this study it was revealed that Pop. 1 cells also stained strongly for EBF. Further, the majority of EBF⁺ cells also stained strongly with IL-1 β and Pu1 antibodies. As discussed in our original study, the EBF antibody is likely reactive with all four EBF proteins (EBF1–4; Zwollo et al, 2010). A literature search revealed that family member EBF3 is expressed in several myeloid-lineage cells (Jimenez et al., 2007), but very little is known about this gene within the context of immune cell development. Hence, it is possible that myeloid-lineage cells express EBF3, but this needs further investigation.

In conclusion, we have described two populations of Q4E⁺ myeloid-lineage cells in immune tissues of the rainbow trout which differ in MPO staining. Further, we provide evidence for differences in myeloid-lineage cell abundance between the two genetic lines, both before and after *in vivo* Fp-challenge, and negative correlations with Fp-loads are present for both Populations, but more strongly so for neutrophil-like cells. Further, qualitatively, both genetic lines responded similarly to *in vivo* Fp-infection, while quantitatively, S-line fish responses were much more intense, most likely the result of higher Fp-loads in S-line fish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

A line of Rainbow trout resistant to BCWD differed significantly from a susceptible line in abundance of myeloid-lineage populations in anterior kidney and spleen, but not PBLs.

After *in vivo* challenge with *Flavobacterium psychrophilum*, rainbow trout had a reduced abundance of neutrophil-like cells in anterior kidney, PBL, and spleen, and BCWD-susceptible fish responded more strongly than BCWD-resistant fish.

Those Fp-challenged fish with higher abundance of neutrophil-like cells in their anterior kidney or PBLs had significantly lower Fp-loads.

Figure 1.

Gene expression of M-CSFR in 4 rainbow trout immune tissues (AK, PBL, SPL, and PK) from YC13 and/or YC15, using RT-qPCR in a Taqman assay. M-CSFR fold-change values relative to the S AKYC13 average are indicated on the Y-axis. R-line fish (R) are shown in grey, S-line fish (S) in black. Average $\pm$ SE. *: $p < 0.05$; ** $p < 0.01$. N values: AK YC13: 8R, 8S; AK YC15: 7R, 7S; PBL YC15: 12R, 11S; SPL YC15: 13R, 15S; PK YC13: 10R, 10S.

Figure 2.

Examples of contour graphs from two-color flow cytometry, using samples from mock R-line fish. Q4E on the Y-axis and Pu1, IL-1 β , EBF, Pax5.PD, or MPO on the X-axis. Boxes label the populations of interest using numbers 1–5, as defined in Table I. **A.** AK. **B.** PBL.

Figure 3. Contour graphs comparing cell size (FSC, Y-axis) and cell abundance of MPO⁺ and Pu1⁺ stained cells between naïve trout and human wbc from blood, using flow cytometry. Left top and bottom contours: Percoll-purified (50%–60%) trout PBLs. Middle top and bottom contours: Percoll-purified (50%–60%) human PBLs. Right top and bottom contours: purified human neutrophils. Percentage of cells in each quadrant are indicated by numbers. Circled populations: MPO⁺, neutrophil-like populations. Boxed populations: Pu1⁺ populations.

Table I.

Antibodies used in the study and staining pattern for each cell population.

Population	Q4E	MPO	IL-1 β	Pu1	EBF	Pax5.PD	FSC	SSC	Potential cell types:
1	+	+	Int to + $\frac{1}{2}$	Int to + $\frac{1}{2}$	+	–	low	high	(immature) neutrophil
2	+	Low-Int	Int.	Int.	Low	–	high	high	monocyte, macrophage, progenitor
3	–	–	Int.	Low	Low	+	low	low	B lymphoid
4	+	–	Low	+	+	Low to + *	high	high	(B/myeloid) progenitor
5	Int/+	–	Int.	Int.	Low	++	high	high	Act/Phagocytic B

$\frac{1}{2}$ intermediate in AK, positive (+) in PBL and SPL.

* Low to intermediate in AK, positive (+) in PBL and SPL.

Cellular abundance of each cell population in **anterior kidney**. The populations (Pops.) are defined in Table I, and are shown here on the left. Numbers are average percent cells $\pm$ SE. Numbers in bold with grey background indicate significant differences.

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Cellular abundance of each cell population in **PBLs**. The populations (Pops.) are defined in Table I, and are shown here on the left. Numbers are average percent cells $\pm$ SE. Numbers in bold with grey background indicate significant differences. ND, no data.

Table III.

Pop.	Phenotype	Mock challenge				Differences after challenge			
		MR	MS	p	MR	FpR	p	MS	FpS
1	Q4E+/EBF+	10.9 $\pm$ 1.7	10.7 $\pm$ 1.4	0.95	10.9 $\pm$ 1.7	8.9 $\pm$ 3.3	0.56	10.7 $\pm$ 1.4	2.8 $\pm$ 0.95
2	Q4E+/EBF low	2.0 $\pm$ 0.38	1.54 $\pm$ 0.36	0.41	2.0 $\pm$ 0.38	5.7 $\pm$ 3.0	0.11	1.5 $\pm$ 0.36	0.89 $\pm$ 0.23
1	IL-1 β +/MPO+	10.2 $\pm$ 1.9	9.7 $\pm$ 1.2	0.90	10.2 $\pm$ 1.9	12.7 $\pm$ 3.4	0.47	9.7 $\pm$ 1.3	2.9 $\pm$ 0.67
2,3	IL-1 β int/MPO low	15.3 $\pm$ 5.0	20.4 $\pm$ 5.2	0.37	15.3 $\pm$ 5.0	32.3 $\pm$ 0 2.5	<0.001	20.4 $\pm$ 5.2	14.8 $\pm$ 3.6
3	Q4E-/Pax5.PD+	55.9 $\pm$ 4.1	63.8 $\pm$ 4.4	0.23		ND			ND
4	Q4E+/Pax5.PD+	2.3 $\pm$ 0.71	0.87 $\pm$ 0.22	0.12		ND			ND
1, 2	Q4E+/Pax5.PD-	11.6 $\pm$ 2.0	10.6 $\pm$ 1.6	0.72		ND			ND

Cellular abundance of each cell population in **spleen**. The populations (Pops.) are defined in Table I, and are shown here on the left. Numbers are average percent cells +/- SE. Numbers in bold with grey background indicate significant differences.

Table IV.

Pop.	Phenotype	Mock challenge				Fp challenge			
		MR	MS	p	MR	FpR	p	MS	FpS
1	Q4E+/EBF+	3.04 +/- 0.49	2.43 +/- 0.42	0.34	3.04 +/- 0.49	1.34 +/- 0.30	0.02	2.43 +/- 0.42	1.88 +/- 0.27
2	Q4E+/EBF low	1.21 +/- 0.66	0.93 +/- 0.23	0.64	1.21 +/- 0.66	1.76 +/- 0.38	0.55	0.93 +/- 0.23	2.15 +/- 0.34
1	IL-1 β +/MPO+	1.63 +/- 0.25	3.51 +/- 0.50	0.04	1.63 +/- 0.25	1.83 +/- 0.36	0.69	3.51 +/- 0.50	0.88 +/- 0.19
2,3	IL-1 β int/MPO low	50.1 +/- 4.9	52.7 +/- 3.0	0.64	50.1 +/- 4.9	25.2 +/- 5.4	0.004	52.7 +/- 3.0	4.5 +/- 0.86
3	Q4E-/Pax5.PD+	62.8 +/- 3.4	53.8 +/- 3.4	0.09	62.8 +/- 3.4	34.9 +/- 6.4	<0.001	53.8 +/- 3.4	17.0 +/- 3.7
4	Q4E+/Pax5.PD+	1.38 +/- 0.41	1.64 +/- 0.28	0.60	1.38 +/- 0.41	2.23 +/- 0.41	0.21	1.64 +/- 0.28	4.8 +/- 0.78
1, 2	Q4E+/Pax5.PD-	1.37 +/- 0.25	2.51 +/- 0.55	0.08	1.37 +/- 0.25	3.46 +/- 1.29	0.03	2.51 +/- 0.55	2.98 +/- 0.57
									0.580

Table V.

Significant correlations between immune cell populations and Fp-load.

Pop:	Phenotype:	Tissue:	R:	N	R:	N	R:	N
Pop. 1	Q4E+/EBF+	AK	-0.88 ***	28	-0.88 ***	15	-0.67 *	13
		PBL	-0.84 **	19		9	-0.90 **	10
		SPL		21		9		12
Pop. 1	IL-1 β + / MPO+	AK		29		15	0.86 **	14
		PBL		24		13	-0.89 ***	11
		SPL	-0.69 ***	21		9		12
Pop. 2	Q4E+ / EBF low	AK		28	-0.66 *	15		13
		PBL		19		9		10
		SPL		21		9		12
Pop. 4	Q4E+/PD+	AK		28		15		13
		SPL		18		8		10

*
p<0.05**
p<0.01***
p<0.001

R values for each sample set (either all fish (All), only Rfish (R), or only S-fish (S), are indicated, with N-value for that set, in columns.