Acquired Protective Immunity in Atlantic Salmon Salmo salar against the Myxozoan Kudoa thyrsites Involves Induction of MHII+ CD83+ Antigen-Presenting Cells

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Acquired protective immunity in Atlantic salmon *Salmo salar* against the myxozoan, *Kudoa thyrsites*, involves induction of MHII$\beta^+$/CD83$^+$ antigen presenting cells.

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**Running title:** Response to *K. thyrsites* involves MHII$\beta^+$/CD83$^+$ APCs

**Keywords:** *Kudoa thyrsites*; Atlantic salmon; post-mortem myoliquefaction; cytotoxicity; immunohistochemistry; gene expression

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ABSTRACT

The histozoic myxozoan parasite *Kudoa thyrsites* causes post-mortem myoliquefaction and is responsible for economic losses to salmon aquaculture in the Pacific Northwest. Despite its importance, little is known about the host-parasite relationship, including the host response to infection. The present work sought to characterize the immune response in Atlantic salmon during infection, recovery and re-exposure to *K. thyrsites*. After exposure to infective seawater, infected and uninfected smolts were sampled three times over 4275 degree-days. Histological analysis revealed infection severity decreased over time in exposed fish while in controls there was no evidence of infection. Following a secondary exposure of all fish, severity of infection in the controls was similar to that measured in exposed fish at the first sampling time, but was significantly reduced in re-exposed fish, suggesting the acquisition of protective immunity. Using immunohistochemistry, we detected a population of MHIÎ²⁺ cells in infected muscle that followed a pattern of abundance concordant with parasite prevalence. Infiltration of these cells into infected myocytes preceded destruction of the plasmodium and dissemination of myxospores. Dual-labelling indicated a majority of these cells were CD83⁺/MHIÎ²⁺. Using RT-qPCR, we detected significant induction of cellular effectors including macrophage/dendritic cells (mhi/cd83/mcsf), B cells (igm/igt), and cytotoxic T cells (cd8/nkl) in the musculature of infected fish. These data support a role for cellular effectors such as antigen presenting cells (monocyte/macrophage and dendritic cells) along with B and T cells in the acquired protective immune response of Atlantic salmon against *K. thyrsites*.
The Myxozoa are obligate endoparasitic metazoans comprising over 2000 species (1, 2) and are common parasites of fish, invertebrates and occasionally vertebrates in fresh and marine environments. Infections with some myxozoa cause serious economic impact to fisheries and aquaculture such as in whirling disease (*Myxobolus cerebralis*) and soft flesh syndrome (*Kudoa thyrsites*) of salmonids (3, 4). The genus *Kudoa* is comprised of 44 described species distributed throughout marine and estuarine fishes worldwide, within which they infect a range of tissues including ovary, brain, kidney and muscle (3). *Kudoa thyrsites* completes development with the formation of myxospores within plasmodia in the muscle of over 35 species of marine fish and clinical signs are not observed. However, after 38-56 hrs post-mortem, parasite-derived proteases digest the surrounding muscle fibres and produce a pH-dependent myoliquefaction that is proportional to the severity of infection (5–7). The resulting degraded fillet quality causes economic losses to both wild fisheries globally (6), as well as finfish aquaculture in Western Canada (8), and infections have been observed in Ireland (9), Chile (9), and Australia (10). Notably, in British Columbia, Canada, *K. thyrsites* presents a significant challenge to the salmonid aquaculture industry (7), with losses reaching 6 million CAD in 2015 (Marine Harvest Canada, pers. com.).

Most identified myxozoans are known or inferred to possess a biphasic life cycle involving an invertebrate definitive host and a vertebrate intermediate host (11), which becomes infected by exposure to the actinospore stage. Indeed, neither the life cycle of *K. thyrsites* nor other aspects of its host-parasite relationship are well understood. Plasmodia occur in Atlantic salmon myocytes after 9 weeks of *K. thyrsites* exposure (12) and infections typically resolve between 26 and 52 weeks post-exposure (13).
There is no available treatment or prophylactic to prevent infection by \textit{K. thyrsites}. In commercial aquaculture, efforts to minimize impacts of infection rely on early detection (8) or improving site-selection and fish husbandry (14). Some orally-administered compounds have been successful in reducing myxozoan infections in teleosts, including \textit{K. thyrsites} in Atlantic salmon (\textit{Salmo salar}) (15, 16); however, adverse effects have limited commercial applications. Thus, the lack of available options to prevent and/or treat \textit{K. thyrsites} infections necessitates a more thorough understanding of the host-parasite relationship, particularly the host response.

Myxozoan parasites of fish elicit a range of host-specific responses, some cause little or no host cellular response, some occupy immunoprivileged sites to escape host detection, and others elicit inflammation or seroconversion in the host (17, 18). There is an apparent variability in host susceptibility to \textit{K. thyrsites}. For example, among Pacific salmon (\textit{Oncorhynchus} spp.), the parasite has been observed in farmed and wild Coho (\textit{O. kisutch}) salmon (19) but not in farmed Chinook (\textit{O. tshawytscha}) salmon (20). In returning adult wild Pacific salmonids, the parasite was present in the cardiac muscle of coho, Chinook and pink (\textit{O. gorbuscha}) salmon but not in chum (\textit{O. keta}) and sockeye (\textit{O. nerka}) salmon (21).

Differential susceptibility to another myxozoan, \textit{C. shasta}, is well documented among Pacific salmon species and among stocks within salmon species (reviewed in (22)). Similarly, inter- and intraspecific differences in susceptibility to infection have been reported in brown trout (\textit{S. trutta}) and rainbow trout (\textit{O. mykiss}) to the causative agent of proliferative kidney disease, \textit{Tetracapsuloides bryosalmonae} (23), common carp (\textit{Cyprinus carpio}) to \textit{Thelohanellus nikolskii} (24), gilthead sea bream (\textit{Sparus aurata}) and turbot (\textit{Scophthalmus maximus}) to \textit{E. scophthalmi} (25).
Although not well studied, there are multiple examples of cellular and acquired immunity to this group of parasites. For example, Cuesta et al. (26) demonstrated strong cellular responses in the intestinal mucosa of gilthead sea bream to Enteromyxum spp., while Davey et al. (27) showed interferon-stimulated and MHC class II genes were important at local level. In rainbow trout, a large accumulation of IgT+ cells in the intestinal lamina propria has been demonstrated in fish surviving infection with C. shasta (28).

Pathological responses associated with K. thyrsites infections occur in mahi mahi Coryphaena hippurus (29) and Pacific hake Merluccius productus (30) and inflammation coincided with resolution of infection in Atlantic salmon (31). In Atlantic salmon, sexual maturation increases the likelihood of infection severity (14), however, the innate mechanisms of resistance including host recognition, cellular targeting and/or destruction of parasite stages remain unknown. Experiments conducted by Jones et al. demonstrated that following resolution of K. thyrsites infections, Atlantic salmon are protected against subsequent infections (32). To investigate the mechanisms responsible for resolution and the ensuing protective immune response, previously unexamined histological samples from the latter study were probed either with histochemical stains or with monoclonal antibodies targeting cellular effectors and flash-frozen samples were screened de novo for immune-related transcript expression. The objectives for the current study were to characterize the cellular immune response of Atlantic salmon during resolution of the initial infection and subsequent protection against a secondary exposure.

RESULTS

K. thyrsites infection resolves over time and surviving fish are protected against reinfection.
The severity of *K. thyrsites* infection was measured in control and infected groups at T1 (1985 dd), T2 (3500 dd), T3 (4275 dd), and T4 (6225 dd) (Fig 1A). The severity of infection (mean plasmodia/mm² ± standard deviation) at T1, T2, and T3 was 1.42 ± 2.29, 0.93 ± 1.66, 0.12 ± 0.19, and 1.81 ± 1.68, 1.58 ± 1.88, 0.08 ± 0.11 following brief and long exposures, respectively. There was no significant difference in mean severity between brief and long exposure times at any time; therefore, the two groups were pooled at each time for subsequent analysis. We did not detect plasmodia in naïve control fish until after secondary exposure (T4). At this time, the mean severity in controls was comparable to infected fish at T1 and significantly different from re-exposed infected fish at T4 (1.22 ± 1.86, *p* < 0.001). There was no evidence of re-infection in previously exposed fish at T4 (Fig 1A). We observed a decreasing trend over time (T1→T4) in the abundance of plasmodia in infected fish, despite a secondary exposure with RSW at 4275 dd (*p* < 0.001).

The presence of *K. thyrsites* was also quantified by measuring the abundance of *r18S* (*kt18S*) transcript in control and infected salmon muscle by RT-qPCR (Fig 1B). There was significantly higher abundance in infected fish at T1 (*p* < 0.0001) and T2 (*p* < 0.0001) compared to control fish. At T2, 3 fish in the control group showed presence of *K. thyrsites* 18S mRNA (data not shown). We excluded these animals from subsequent analysis. At T4, *kt18S* transcript abundance in the control fish (naïve to infection) was significantly elevated (*p* < 0.0001) and reached levels comparable to those observed in the infected groups at T1. In contrast, there was a significant decrease in *kt18S* expression at T4 in the infected group (re-exposed group) compared to the control group (*p* < 0.0001). Moreover, there was a significant decrease in *kt18S* expression in infected fish over time (T1→T4; *p* < 0.001).
Antiparasitic response involves activation of MHII$\beta^+$, CD8$^+$, CD83$^+$, and IgM$^+$ cells

The monoclonal antibody Sasa MH class II $\beta$ F1-4 ($\alpha$-MHII$\beta$) detected cells that were either large with an amorphous nucleus, diffuse cytoplasm and dendritic-like projections, or those with morphological features more similar to lymphocytes. In the musculature of *K. thyrsites*-infected salmon, these cells were associated with infected myocytes in a pattern divisible into four major stages (Fig 2): initial detection of the infected myocyte, which was completely surrounded by MHII$\beta^+$ cells (Stage 1; Fig 2A); infiltration of the infected myocyte by MHII$\beta^+$ cells (Stage 2; Fig 2A); and complete degradation of the myocyte by MHII$\beta^+$ cells (Stage 3; Fig 2A); and complete infiltration and degradation of the plasmodia and engulfment of myxospores by MHII$\beta^+$ cells in the immediate area, or distal to the myocyte (Stage 4; Fig 2A). MHII$\beta^+$ cells were also scattered throughout the musculature of naïve control fish. In these fish at T1 to T3, we did not observe stages of myocyte infiltration by MHII$\beta^+$ cells; however, after exposure of control fish to RSW, a pattern of MHII$\beta^+$ staining and infiltration was observed similar to that of infected fish at T1.

Plasmodia were classified as positive (+plas) for immune detection if a myocyte was surrounded entirely by or infiltrated with MHII$\beta^+$ stained cells, or if the myocyte was disintegrated with observable myxospores. If a myocyte contained plasmodia but no MHII$\beta^+$ cells then it was considered negative or not recognized by the immune system (-plas). The ratio of +plas to -plas was calculated for each group and served as a proxy for the host response to *K. thyrsites*. There was a shift in the ratio of +plas to –plas over time (Fig 2B). After secondary exposure, the

There were significantly more MHII$\beta^+$ cells in the musculature of infected fish compared to control fish from T1 to T3 ($p < 0.001$; Fig 2C).
number of labelled cells in control fish increased significantly to levels comparable to infected fish at T1 ($p < 0.001$). In contrast, the number of labelled cells did not increase after secondary exposure in previously infected fish ($p = 0.695$), and was not significantly different from controls prior to exposure ($p = 0.959$). There was a significant positive correlation between severity of infection (plasmodia/mm$^2$) and abundance of MHII$\beta^+$ cells ($p = 0.002$; Pearson’s $r = 0.30$; Fig S1).

The monoclonal antibody Sasa CD8$\alpha$ ($\alpha$-CD8$\alpha$) detected cells associated with infected myocytes in all stages of infection. The CD8$\alpha^+$ cells either surrounded or infiltrated infected myocytes, or were present in lesions of cellular aggregates (Fig 3A). These cells presented variable morphology; some were small and round as previously described (33), while others were larger and diffuse with large nuclei and a dendritic-like morphology (Fig 3A). CD8$\alpha^+$ cells were not observed in the musculature of non-infected fish (data not shown).

Dual labeling of tissue sections with $\alpha$-MHII$\beta$ revealed a CD8$\alpha^+$/MHII$\beta^+$ population of cells in inflammatory lesions and associated with infected myocytes (Fig 3B).

The monoclonal antibody Omy CD83 14-55-12 ($\alpha$-CD83) detected a population of cells associated with later stages of infection and predominantly in areas of disintegrated myocytes. These cells were large with a diffuse and irregularly shaped nucleus, and often contained myxospores (Fig 4A). When co-labeled with $\alpha$-MHII$\beta$, CD83$^+$/MHII$\beta^+$ cells were involved in all 4 stages of detection (see above) and were present in inflammatory lesions (Fig 4B).

The monoclonal Sasa IgM ($\alpha$-IgM) detected cells in the musculature of uninfected and infected Atlantic salmon. Positive cells resembled lymphocytes with a round and
compact nucleus. More of these cells were counted in infected fish (data not shown) and were observed between myocytes in *K. thyrsites*-infected tissue, and associated with lesions in later stages of infection (Fig S2), but were never observed infiltrating infected myocytes.

*Kudoa thyrsites* infection induces gene expression of several cellular effector molecules. Transcript abundance of several cellular effectors and immune response markers were characterized in control and infected fish muscle at T1, T2, and T4. We first explored the dataset using an unsupervised partitioning clustering analysis (R-package ‘cluster’) with expression profiles of all sample-gene combinations to identify patterns of similar expression profiles. Despite significant variability in the data, *k*-means clustering analysis revealed 3 distinct expression profiles grouped according to infection status (Fig 5). This analysis indicated that naïve fish had similar expression profiles to infected fish that were protected against secondary infection, while infected fish early in the challenge were similar in gene expression to control fish after initial exposure. The final cluster was comprised of infected fish later in infection and re-exposed infected fish.

We then performed hierarchical clustering and calculated Pearson’s correlation coefficients (r) for all genes to understand the relationship in expression of the different cellular markers. There was significant correlation among the expression profiles of macrophage/DC markers (*mhiib, mcsf, cd83*), T cell markers (*cd8, nkl*) and B-cell markers (*igt, igm*) (Table S1; Fig 6A-B).
Macrophage/dendritic cell markers highly expressed during *K. thyrsites* infection.

In concordance with protein abundance of MHIIβ determined by immunohistochemistry (IHC), transcript abundance of *mhiιβ* was significantly higher in infected fish compared to controls T2 (*p* < 0.001) and this expression decreased over time (T2→T4; Fig 7). After secondary exposure (T4), expression of *mhiιβ* was significantly higher in control fish than in re-infected fish. In the latter group, expression was comparable to uninfected controls.

Similar to *mhiιβ* transcript abundance of *cd83* and *mcsf* was higher in infected fish at T2 (*p* < 0.0001) and in control fish at T4, following the secondary exposure (Fig 7). In the re-exposed fish, there was a significant decrease in expression of *mcsf* and *cd83* (*p* < 0.005, *p* < 0.001, respectively). There was a significant correlation (*r* > 0.80, *p* < 0.0001) among the expression of *mhiι, cd83* and *mcsf* indicating co-expression of these transcripts (Fig 6B).

There was a significant difference in transcript abundance of c-type lectin *clec4m* in infected fish compared to control fish at T2 (Fig S3; *p* < 0.001), but this response was not observed after re-exposure. There was no increase in *clec4m* expression following exposure of control fish.

Evidence for a cytotoxic T cell response to *K. thyrsites*.

Expression of *cd8* and *nkl* was significantly induced in infected fish compared to controls at T1 (*p* = 0.026, *p* = 0.002 respectively), and at T2 (*p* < 0.05, *p* < 0.001 respectively; Fig 8). However, we failed to detect significant differential expression of *cd4* (data not shown).

After secondary exposure, expression of *tcr, cd8* and *nkl* increased in controls while in re-exposed infected fish there was sustained expression of *nkl* only. In contrast, transcript abundance of *tcr* and *cd8* decreased after re-exposure (*p* < 0.01, *p* < 0.05, respectively) and was lower than controls (*p* < 0.01). The expression of both *nkl* and *tcr* positively correlated...
with \( \text{cd8} \) (\( r = 0.88 \) and \( r = 0.80 \), respectively; \( p < 0.0001 \)) (Fig 6B), as was the expression of \( \text{tcr} \) and \( \text{cd83} \) (\( r = 0.79, p < 0.0001 \)).

**B cell markers activated in response to \textit{K. thyrsites}**

There was significant induction of both \( \text{igt} \) and \( \text{igm} \) expression in infected fish compared to controls at T2 (\( p < 0.00001 \); Fig 9). After secondary exposure, the abundance of both \( \text{igm} \) and \( \text{igt} \) was significantly induced in control fish (\( p < 0.001 \)), with sustained expression in re-infected fish. There was a strong positive correlation between \( \text{igt} \) and \( \text{igm} \) (\( r = 0.86, p < 0.0001 \), Fig 5C), indicating co-regulation of these transcripts.

**Interleukins**

There was significant but transient up-regulation of \( \text{il12} \) in infected fish compared to control fish at T1 (\( p < 0.001 \); Fig 10). After secondary exposure, transcript abundance was significantly induced in control fish at T4 (\( p = 0.007 \)) to levels comparable with infected fish observed at T1. We failed to detect reliable expression of \( \text{il4} \) (data not shown).

**Cellular proliferation**

There was a significant induction of \( \text{pcna} \) over time (T1\( \rightarrow \)T2) in infected fish (\( p < 0.00001 \)), and at T2 expression in infected fish was higher than control fish (\( p < 0.001 \)). After secondary exposure \( \text{pcna} \) expression increased in control fish (\( p < 0.0001 \)) and was significantly higher than re-exposed infected fish (\( p < 0.01 \)) (Fig 10).

**DISCUSSION**

The present study demonstrated that the response of Atlantic salmon to myocytes infected with \textit{K. thyrsites} involves infiltration of the lesion with MHII\( \beta^\text{+} \), MHII\( \beta^\text{+}/\text{CD83}^\text{+} \), MHII\( \beta^\text{+}/\text{CD8}\alpha^\text{+} \), CD83\textsuperscript{+}, and IgM\textsuperscript{+} cells. This response is further characterized by up-regulation of M\( \phi/\text{DC} \) (\textit{mhii}, \textit{cd83}, \textit{mcsf}), cytotoxic T cell (\textit{cd8}\alpha, \textit{tcr}), and B cell (\textit{igm}, \textit{igt}).
genetic markers. Furthermore, we provide evidence for cytotoxic activity in the significant expression of nkl and its upstream inducer, il12. The expression of these markers was positively correlated with both the abundance of K. thyrsites 18S rRNA and infection severity. Taken together, these results indicate a cell-mediated immune response is involved in the resolution of K. thyrsites infection in Atlantic salmon, and further that CD8α+ cytotoxic T cell killing is a candidate mechanism for protection upon re-exposure to the parasite.

We observed a population of MHIIβ+ cells whose abundance was proportional to infection severity and which progressively infiltrated infected myocytes and appeared to be associated with disintegration of the infected cell. MHIIβ+ cells were first observed surrounding intact infected myocytes while infiltration of the infected myocyte by these cells was then later observed. As the myocyte became degraded, the plasmodium gradually lost integrity and myxospores were eventually released and subsequently ingested by phagocytes including MHIIβ+ cells. The presence of macrophages (Mϕ) within infected salmon myocytes has been previously reported at a low level (34). Here, infiltrating MHIIβ+ cells were frequently observed within plasmodia and, as the infection progressed, most plasmodia had deteriorated and MHIIβ+ cells were observed with engulfed myxospores. We propose that this population of cells is directly involved in parasite detection and clearance. Additionally, the persistence of these cells in fish demonstrating protection against re-infection suggests they also play a role in the acquired protective immune response. This work challenges an earlier paradigm in which stages of the infection prior to myocyte rupture went undetected by the host (3, 34).
Dual labelling of infected muscle showed that many of cells involved in detection of *K. thyrsites* were MHI\(\beta\)^+/CD83^+. Thus concordant up-regulation of mhiI\(\beta\) with cd83, a hallmark marker for mature dendritic cells (DCs) (35, 36), together with immunohistochemical data confirms a large proportion of these cells are DCs. A recent study described a population of cells in rainbow trout possessing all of the characteristics of DCs (37), and subsequent studies have identified DCs in zebrafish (38), salmon (39) and trout (40). In the latter study, the authors describe a CD8\(\alpha\)^+/MHI\(\beta\)^+ population of DCs in the skin of trout with potent phagocytic and cross-presentation abilities, while Haugland et al. described a population of phagocytic cells expressing cd83 and mh class II in Atlantic salmon as progenitor DCs (39). Thus, current evidence supports the existence of DCs in teleosts, which is intuitive given the importance of DCs in vertebrate innate immunity (41) and the repertoire of mammalian cellular equivalents already confirmed in teleosts (42–46).

Our data suggested a heterogeneous population of leukocytes is present in the musculature of *K. thyrsites*-infected salmon which act in concert to eliminate the parasite. For example, in addition to mhiI\(\beta\) and cd83 we also observed concordant up-regulation of mcsf, a macrophage/monocyte marker, and igm and igt, surface immunoglobulins of teleost B cells which also possess antigen processing capabilities (43). IgM\(^+\) cells were also in the same lesions as the MHI\(\beta\)^+ , CD8\(\alpha\)^+ and CD83^+ cells. Lymphocyte-mediated responses have been described as important determinants of immunity in other myxozoan infections of fish. For example, a strong IgM\(^+\) B cell response was observed in turbot infected with *E. scophthalmi* (47) and gilthead sea bream infected with *E. leei* (48). In the present study, up-regulation of both igm and igt in response to infection with *K. thyrsites* and IgM\(^+\) cells was...
associated with later stages of myocyte detection. Generally, up-regulation of IgM is associated with a switch from T<sub>H1</sub>- to T<sub>H2</sub>-like cytokine profiles which is regulated by anti-inflammatory cytokines, including interleukin-4 (49). Paradoxically, despite a significant induction of *igm* in the muscle of *K. thyrsites*-infected salmon, we did not detect differential expression of the T<sub>H2</sub> marker *interleukin-4* at any time point throughout the infection, indicating that either the typical T<sub>H2</sub>-associated pathways are not induced during infection with this parasite, or that transient transcription of this marker was missed due to sampling design.

In contrast, up-regulation of *il-12* indicated a T<sub>H1</sub>-type response in *K. thyrsites*-infected muscle. Interleukin-12 is produced by activated phagocytes (e.g., M<sub>ϕ</sub>, DCs, B cells) in response to intracellular parasites in early phases of detection (50, 51). In mice, resistance to acute protozoan infections is characterized by early synthesis of IL-12 by CD8<sup>α<sup>+</sup></sup> DCs (52), which permits host survival in low-level chronic infections (53). IL-12 acts as a growth factor for activated T and NK cells and enhances CTL and NK cell killing by activating transcription of cytolytic molecules including perforin and granzymes (54). In the present study, *il-12* expression declined over time, suggesting a positive correlation between severity of *K. thyrsites* and *il-12* production. Resistance to *Ceratovia shasta* infections in Chinook salmon is associated with overexpression of IFN-γ (49). Although the latter study did not assess levels of IL-12, the IL-12/IFN-γ regulatory pathway has been thoroughly characterized in vertebrates, including teleosts (55, 56). The absence of *il-12* induction after re-exposure of salmon to *K. thyrsites* suggests protection against subsequent infection cannot be explained by the presence of IL-12, as has been described for other intracellular parasites (51, 57, 58). Instead, the data suggest an important role for
IL-12 early in the host response to *K. thyrsites* infections. Based on the importance of this cytokine during infection with other intracellular parasites, future studies should investigate a comparable mechanism during *K. thyrsites* infections in Atlantic salmon. CD8+ DCs play a pivotal role in antigen presentation and T-cell priming against intracellular pathogens, including *Listeria monocytogenes* (59), *Salmonella typhimurium* (60), and *Plasmodium* spp. (61). Up-regulation of *cd83* transcription was observed in a CD8α+/MHIIβ+ population of leukocytes in the skin of rainbow trout following poly I:C stimulation, further substantiating this population as a DC subset in teleosts (40). Here we show significant up-regulation of *mhiiβ, cd8a* and *cd83* in the Atlantic salmon in response to *K. thyrsites*. Furthermore, application of salmon-specific markers revealed a population of spore-phagocytic leukocytes in skeletal muscle co-expressing MHIIβ and CD8α or CD83 and with dendritic-like morphology. We propose this cell population is similar to the dendritic-like cells described by Granja et al. (40).

It is likely that some of the CD8α+ population of cells observed in this study were cytotoxic T lymphocytes (CD8α+/CD83−/MHIIβ−/−). Cytotoxic T lymphocytes use both secretory and non-secretory killing mechanisms to protect the host against infected and transformed cells (42), and a subset of CD8+ T cells (known as effector memory cells) migrate to sites of infection and display immediate effector function (62). Activation of CD8+ T cells or adoptive transfer of these cells can induce protective immunity against protozoan parasites (63). In mice, persistent activation of CD8+ T cells following infection with viral, bacterial or parasitic infection has been associated with increased protection against subsequent infection of *Trypanosoma cruzi* (64). Furthermore, NK-lysin released from activated CD8+ T cells has been shown to directly lyse *T. cruzi* infected cells (65).
We observed a protective effect in Atlantic salmon after secondary exposure to *K. thyrsites* concomitant with persistent up-regulation of \( \text{cd8}\alpha \) and presence of CD8\( \alpha \)\(^+ \) cells, suggestive of a comparable mechanism in fish. The presence of NK-lysins has been confirmed in teleosts (66–70), and recent work has shown that rainbow trout (*O. mykiss*) CD8\( \alpha \)\(^+ \) T cells express granulysin/NK-lysin and are the dominant cytolytic cell population in teleost fish (71). Interestingly, we detected significant and persistent up-regulation of \( \text{nk-lys} \) in response to *K. thyrsites*, despite decreasing parasite severity over time. Further to this, the pattern of \( \text{nk-lys} \) expression was highly correlated with \( \text{cd8}\alpha \), implying co-regulation of these two genes. Thus, infection with *K. thyrsites* in Atlantic salmon appears to elicit a cytotoxic T cell response characterized by up-regulation of *il12*, *cd8* and *nkl*.

Although our data indicate an activated immune response in the musculature of infected Atlantic salmon, the parasite stage targeted during re-exposure is unknown. A putative actinospore stage may interact with a resident M\( \phi \) or DC at the proposed infection site of *K. thyrsites* in the mucosa (72, 73) prior to establishing infection in striated muscle. Migratory (extrasporogonic) stages within teleost circulatory systems have been described in several myxozoans (74, 75). Indeed, in Atlantic salmon infected with *K. thyrsites*, weakly infective circulating extrasporogonic stages have been detected (13, 34), which may interact with APCs to activate the host immune response. There is evidence of a stage-specific surface antigens in *K. thyrsites* (76), raising the possibility of differential stimulation of defence responses during early and late stages of parasite development.

The current study demonstrated significant transcriptional induction of a c-type lectin (*c-type lectin family m*) associated with later stages of *K. thyrsites* infection. Either secreted as soluble proteins or expressed on the surface of DCs and M\( \phi \), c-type lectins are
known to play an important role in host-parasite interactions (18, 77), including fish parasites (78–82). More importantly, the presence of carbohydrate terminals specifically detected by lectins on the surface of the myxozoans, *Tetracapsuloides bryosalmonae*, *Myxobolus cerebralis* and *E. scophthalmi* suggest a role in host–parasite interactions (83–85). Activation of this gene during the response to *K. thyrsites* may represent a comparable host-parasite interaction. However, *clec4m* expression was only associated with later stages of infection after a large proportion of infected myocytes had been disintegrated by the host response. Thus, it is unlikely that this molecule is involved in the initial host-parasite interaction, but may instead be important during the acquired protective response reported here.

**CONCLUSION**

In summary, this study provides evidence for an acquired cell-mediated immune response in Atlantic salmon infected with the myxozoan histozoic parasite, *K. thyrsites*. The proteomic and transcriptomic data indicate a heterogeneous population of leukocytes are involved in the processing and protective response of Atlantic salmon to *K. thyrsites*. Specifically, an APC-mediated (Mφ, DCs) cytotoxic T cell response is involved in the resolution of infection and in the protective response against subsequent infection.

**MATERIALS AND METHODS**

**Fish husbandry**

Juvenile Atlantic salmon *S. salar* from a single commercial hatchery stock were maintained in a research aquarium at the Pacific Biological Station, Nanaimo, British Columbia, as described previously (32). Temperature ranged from 8.2 to 13.6°C and mean salinity was 29.2 PSU. The fish were smoltified in 6500 L flow-through tanks supplied with UV-treated
seawater (UVSW) and acclimated for a minimum of 2 weeks prior to experimentation.

Salmon were fed a commercial pelleted diet (EWOS) at a daily rate of 1% biomass.

Husbandry protocols followed guidelines of the Canadian Council on Animal Care. The fish were exposed to *K. thyrsites* by holding them in raw seawater (no-UV treatment; RSW) as described below and in Jones *et al.* (32). The duration of all exposure events was expressed in degree-days (dd), which are the sum of daily water temperatures.

**Experimental design and sampling procedure**

Fish (85 g) were randomly allocated into 6 2500 L tanks ($n = 150$ /tank) with flow rates of 40 L UVSW/min. Duplicate tanks were assigned to each of 3 treatment groups: UVSW only (naïve controls), RSW (infected) for 440 dd (brief) and RSW for 950 dd (long). All fish were maintained in UVSW following the RSW exposure. Muscle sample collection was performed as described previously (32). Samples were collected from 20 fish/tank at 1985 dd (T1) and 3500 dd (T2) following the onset of exposure and from 13–20 fish/tank at 4275 dd (T3). Immediately following T3 sampling, all remaining fish were exposed to RSW for 530 dd and then maintained in UVSW until 6225 dd (T4) when final samples were collected ($n = 25$–30/tank). For sampling, fish were sedated in 0.25mg L$^{-1}$ Aquacalm (Syndel Laboratories, Canada) and euthanized in 250 mg L$^{-1}$ MS-222 (Syndel Laboratories). Three skeletal muscle samples were dissected from the same locations on each fish immediately after euthanasia and fixed in 10% neutral buffered formalin (NBF) for 24–48 h then transferred to 70% isopropanol. A subset of samples ($n = 20$ from T1, T2 and T4) were flash-frozen in liquid nitrogen (LN$_2$) and stored at -80°C until RNA extraction.

**Histology and Immunohistochemistry (IHC)**
NBF-fixed samples were processed for histology as previously described (32). Serial sections (5 μm) were obtained (n = 15 fish per treatment, per time) using a Leica RM2135 microtome (Leica Microsystems, Germany) and placed on SuperFrost UltraPlus (Menzel-Gläser) positively charged glass slides to dry overnight at 40°C. After de-paraffinization in xylene and rehydration in graded isopropanol, the sections were either stained with hematoxylin and eosin (H&E) for routine histopathology or probed with monoclonal antibodies against immune cell markers (Table 1).

Antigen retrieval and immunolabelling was performed as previously described (87). After de-paraffinization and rehydration, sections were heated to 100°C in antigen retrieval buffer (Table 1), cooled to room temperature for 10-min in phosphate-buffered saline (PBS) then washed twice in Tris-buffered saline + 0.2% Tween-20 (TBS-T; pH 8.0) for 5-min with gentle agitation. For MHIIβ and mIgM (membrane-bound IgM) detection, sections were blocked in protein blocker for 10-min before gentle rinsing with TBS-T. The sections were incubated with primary antibody in TBS-T and 1% bovine serum albumin (BSA, Sigma) overnight at 4°C in a humid chamber. After incubation, the sections were washed in TBS-T (2 × 5-min), incubated in a mouse-specifying reagent (EXPOSE Mouse/Rabbit Specific HRP/DAB kit, Abcam) for 10-min followed by a 10-min incubation in hydrogen peroxide blocker. Labelled cells were detected after a 15-min incubation in a goat anti-rabbit HRP conjugate followed by 10-min with 3,3′-diaminobenzidine (DAB) in PBS with 0.015% H₂O₂. For detection of CD83 and CD8α, an alkaline phosphatase (AP) detection kit (EXPOSE Rabbit/Mouse Specific AP kit, Abcam) was used according to manufacturer's instructions. Blocking, incubations with primary antibodies and washings were as described above. Sections were incubated in biotinylated goat anti-rabbit for 15-min,
washed in TBS-T (2 × 5-min) then incubated in streptavidin AP for 15-min. After a final wash (2 × 5-min) the sections were developed using the StayRed chromogen following manufacturer’s instructions (Abcam).

All sections were counterstained in 1% Alcian blue (3-min) and Mayer’s hematoxylin (diluted 1/20, 30 sec), dehydrated in graded ethanol, cleared in xylene and cover-slipped (Permount). Sections treated with irrelevant antibodies served as negative controls while sections known to contain the target molecules served as positive controls.

Positively labeled cells were quantified by counting the total number of cells in 10 fields of view (FoV) in an “S” pattern in each section at 400X magnification (FoV = 0.25 mm²). If a FoV included a plasmodium, it was disregarded and the next FoV was considered to avoid bias due to exaggerated cellular abundance in these areas.

Infection severity for each time point was determined from H&E-stained sections by quantifying number of plasmodia and the total area of each muscle section (ImageJ V2.0.0-rc-43/1.51h). An infection severity index for each fish (n = 15 fish per treatment, per time) was calculated as the arithmetic average number of K. thyrsites plasmodia/mm² from the three muscle sections.

Antibody preparation and validation

Antibodies against trout Oncorhynchus mykiss CD83 were raised in mice (NMRI) by immunizing with a synthetic peptide representing the c-terminal end of the amino acid residues from 141-154 (C-LESTDQSEERDTI) of CD83. The antigen was mixed with GERBU adjuvant (GERBU Biotechnik GmbH) in accordance with the manufacturer’s procedure.

Mice received 2 subcutaneous injections at least 14 days apart, with 25 μg of synthetic peptide coupled to diphtheria toxoid via the end cysteine (Statens Serum Institut,
Immunised mice received an intravenous boost with 25 μg of the antigen, administered with adrenalin, 14 days later. The fusion of spleen cells and selection was done 3 days after the boost as described by Kohler and Milstein (88), however, the SP2/0-AG14 myeloma cell line was used as fusion partner. Positive clones were selected by screening against the immunized peptide coupled to BSA in ELISA. Cloning was performed by limited dilution, and single clones were grown in culture flasks in RPMI + 10% FCS.

Immunohistochemical characterization of the antibodies was done comparing the staining pattern in various tissues. Here another trout CD83 antibody, made against a different determinant (the N-terminal part of CD83 Ig like domain), was used as reference antibody. The CD83 Mab 14-54-04 was considered CD83-specific when it produced the same pattern of stained cells as the reference.

**RNA extraction and qPCR**

Total RNA was isolated from frozen muscle samples (*n* = 20 fish per treatment, per time) using a modified phenol-chloroform extraction method as previously described (89) followed by an on-column purification (RNeasy RNA purification kit, QIAGEN). Potential contaminating genomic DNA was eliminated using Turbo DNase (Ambion) following manufacturer’s instructions for a routine digestion. Resulting RNA was quality-checked using the automated electrophoresis (Experion HighSens kit, BioRad) and an RQI-cutoff of >7.5. High-quality RNA (2 μg) was synthesized into cDNA using the iScript Reverse Transcription Supermix (BioRad) with a mix of random hexamers and oligo (dT) primers, following manufacturer’s instructions in 40 μl reactions.

Following cDNA synthesis, an aliquot from every sample was pooled prior to being diluted 3-fold. Primer efficiencies were determined from a 5-fold, 6-point serial dilution of
the pooled cDNA sample. All assay efficiencies were between 90-105%, and specificity was

determined by melt-point analysis and sequencing of PCR amplicons (Table 2).

qPCR amplification was performed using Sso-Fast Advanced qPCR kit (BioRad) as

per manufacturer's instructions for the CFX thermal cycler (BioRad) and using the

following thermal regime: 95°C for 30 secs (1 cycle), 95°C for 15 secs followed by 60°C for

30 secs (40 cycles), followed by a melt curve (65°C to 95°C reading fluorescence at 0.5 sec

increments). Samples were plated in triplicate using the Aurora automated plate dispenser

run by VersaWare, and technical replicates were accepted only with a deviation of <0.5 Ct.

No-template (NTC) and no-reverse transcriptase (NRT) controls were run for every gene.

Raw expression profiles were imported into qBASE+ (Biogazelle) and calibrated

normalized relative quantities (CNRQs) were calculated. Elongation factor 1-α (ef1α),

eukaryotic initiation factor-3 subunit-6 (eif3,6), ribosomal protein 40S (rps40), β-actin, and

glyceraldehyde phosphate dehydrogenase (gapdh) were selected as candidate normalizer

genes. Of these, ef1α, eif3,6 and β-actin showed the highest stability (GeNorm M-value and

coefficient of variation of 0.592 and 0.235, respectively) and were therefore chosen for

CNRQ analysis (95).

Resulting log2-transformed CNRQs (log2CNRQ) were tested for significance by two-

way ANOVA and post hoc Tukey HSD in R (R Development Core Team 2012, v3.3.13) with

time and infection status as the two explanatory variables. Distance matrix analysis,
correlational matrices, hierarchical clustering and clustering analysis were also performed
in R using the ggplot2 package and log2CNRQ values of all sample-condition combinations
(n = 247).

ACKNOWLEDGEMENTS
The authors would like to thank Carter Van Iderstine, Heather Wotton, Kaitlin Fitzpatrick and Brittany Ng for their excellent help with sample preparation. Holly Hicklin is gratefully acknowledged for fish care. This research was funded in part by the Fisheries and Oceans Canada Aquaculture Collaborative Research and Development Program, Marine Harvest Canada, Cermaq Canada, and Novartis/Elanco Animal Health. LMB was funded by an NSERC post-doctoral fellowship during this study. The authors are grateful to Jordan Poley for valuable comments on an earlier version of this manuscript, and to two anonymous reviewers for their excellent insight and comments that greatly improved the final manuscript.

FIGURE DESCRIPTIONS

Fig 1. (A) Infection severity index of *K. thyrsites* measured by plasmodia/mm² in Atlantic salmon muscle samples at 1985 dd (T1), 3500 dd (T2), 4275 dd (T3), and after secondary exposure at 6300 dd (T4) in control (red) and infected (blue) fish. (B) RT-qPCR of *K. thyrsites* 18S rRNA in control and infected fish. Bars represent the mean Ct-value (±SD). Differences among groups were determined using a two-way ANOVA followed by a post-hoc Tukey’s HSD, with a cut-off of *p* < 0.01. Significant differences between groups are denoted by lower-case letters. The arrow represents the secondary exposure, where both control and infected fish were exposed to infective salt water for ~550 dd.

Fig 2. Immune detection of *K. thyrsites* by MHIIβ⁺ cells. (A) Photomicrographs of stages of cellular response. In Stages 1 and 2, MHIIβ⁺ cells surrounding and infiltrating an infected myocyte; In Stage 3, MHIIβ⁺ cells recruited to the infected myocyte and surrounding the plasmodia (p) while disintegrating the myocyte; In Stage 4, degraded plasmodia (star), and released spores engulfed by MHIIβ⁺ cells (red arrowhead). (B) Proportion of stages of
detection by MHII$\beta^+$ cells in infected fish at T1-4 (InfT1, InfT2, InfT3, InfT4, respectively) and in control fish at T4 (CntT4). (C) Number of MHII$\beta^+$ cells in the musculature of infected salmon over time. The arrow represents the secondary exposure, where both control and infected fish were exposed to infective sea water for ~550 dd. Significance was determined using a two-way ANOVA followed by post hoc Tukey’s HSD with $p < 0.05$. Lower-case letters denote differences between groups at each sampling time, while asterisk denotes differences over time in each group.

**Fig 3.** Photomicrographs of *K. thyrsites*-infected muscle tissue (T2; 3500 dd) probed with monoclonal antibody Sasa CD8$\alpha$. (A) CD8$\alpha^+$ cells (brown) associated with myocytes containing intact plasmodia (p) as well as with disintegrated myocytes. Positive cells were observed with engulfed myxospores (light blue; arrowhead), (b) infiltrating infected myocytes (arrowhead), or (c) associated with fibrinolytic lesions (asterisk). There appeared to be two morphologies of CD8$\alpha^+$ cells, dendritic-like (d) and lymphocyte-like (e).

(B) Dual staining with $\alpha$-CD8$\alpha$ (red) and $\alpha$-MHII$\beta$ (brown). Dual-labelled cells associated with fibrinolytic lesions (a, asterisk), or infiltrating infected myocytes (a-b, arrowheads).

**Fig 4.** Photomicrographs of *K. thyrsites*-infected muscle tissue (T2; 3500 dd) probed with monoclonal antibody Omy CD83 (red). (A, a-b). Positive cells were observed associated with later stages of infection after plasmodia were disintegrated (asterisk), and often with engulfed myxospores. (B) Dual labelling of CD83 (a; red) and MHII$\beta$ (b; brown) revealed most cells were CD83$^+$/MHII$\beta^+$ (c-e; red + brown). Dual labelled cells were observed at high densities within Stage 4 lesions and associated with free myxospores or with engulfed myxospores (arrowheads).
Cluster analysis of all sample-gene combinations. (A) The optimal number of clusters was calculated by the \( k \) means gap statistic (\( k \)). (B) Cluster plot of all samples using \( k \). This analysis showed 73.4\% of the variability in the dataset is explained by the time of infection with *K. thyrsites*. Cluster 1 is comprised uninfected controls (27.4\% and 22.5\% for T2 and T1, respectively), re-exposed infected fish (34.3\%, T4), and infected fish (15.7\%, T2). Cluster 2 was comprised of infected fish later in the infection (48.4\%, T2) and infected fish after re-exposure to *K. thyrsites* (19.4\%, T4), control fish after secondary exposure (16.1\%, T4), and infected fish early after primary infection (16.1\%, T1). Cluster 3 was comprised of fish early after primary exposure (T1 infected fish, 37.5\%; T4 control fish, 25\%), and infected fish after re-exposure (14.1\%, T4).

Fig 6. (A) Correlational matrix of expression profiles for all genes of interest. Coloured scale shows degree of correlation ranging from \( r = -1.00 \) (orange) to \( r = 1.00 \) (purple). The size of the coloured circle indicates significance. The reader is referred to Table S1 for associated \( r \)- and \( p \)-values. (B) Hierarchical clustering of log\(_2\)CNRQs for cellular markers with high positive correlation. Gene names are labeled on the base of the figure while hierarchical clustering of the columns based on individual samples shows similar expression profiles of genes associated with particular cell types.

Fig 7. Expression profiles of macrophage/dendritic cell markers (*mahii*, *mcsf*, and *cd83*) in control and infected salmon at T1 (1985 dd), T2 (3500 dd) and T4 (6225 dd). Prior to sampling at T4, both control and infected fish were re-exposed (dotted line, arrowhead). Expression differences are represented by log\(_2\) calibrated normalized relative quantities (CNRQ) with boxplots and whiskers showing the median expression and 95\% confidence intervals, respectively. Statistical differences were detected with a two-way ANOVA.
followed by a post-hoc Tukey's HSD for pairwise comparisons ($p < 0.05$). Differences between groups are denoted by lower-case letters, while over time, differences are denoted by asterisk (*).

**Fig 8.** Expression profiles of T cell markers ($cd8$, $tcr$, and $nkl$) in control and infected salmon at T1 (1985 dd), T2 (3500 dd) and T4 (6225 dd). For interpretation, see description for Fig 7.

**Fig 9.** Expression profiles of B cell markers ($igm$ and $igt$) in control and infected salmon at T1 (1985 dd), T2 (3500 dd) and T4 (6225 dd). For interpretation, see description for Fig 7.

**Fig 10.** Expression profiles of cytokines ($il4$ and $il12$) and marker of cellular proliferation ($pcna$) in control and infected salmon at T1 (1985 dd), T2 (3500 dd) and T4 (6225 dd). For interpretation, see description for Fig 7.

**TABLE DESCRIPTIONS**

**Table 1.** Monoclonal antibodies used in immunohistochemistry indicating dilutions, antigen retrieval methods, and the original reference.

**Table 2.** Primers used for RT-qPCR, indicating the forward and reverse sequences, expected amplicon size and original reference.

**REFERENCES**


23. Grabner DS, El-Matbouli M. 2009. Comparison of the susceptibility of brown trout (Salmo trutta) and four rainbow trout (Oncorhynchus mykiss) strains to the myxozoan Tetracapsuloides bryosalmonae, the causative agent of proliferative kidney disease (PKD). Vet Parasitol 165:200–206.


Tsutsui S, Tasumi S, Suettake H, Suzuki Y. 2003. Lectins homologous to those of monocotyledonous plants in the skin mucus and intestine of pufferfish, *Fugu*
832 Fish Dis 26:627–646.
834 Tetracapsuloides bryosalmonae using lectin histochemistry and immunogold electron
839 carbohydrate terminals in the enteric parasite Enteromyxum scophthalmi (Myxozoa)
840 and possible interactions with its fish host Psetta maxima. Parasitol Res 102:1257–
841 1267.
842 86. Pettersen FE, Fyllingen I, Kavlie A, Maaseide NP, Glette J, Endresen C, Wergeland HL.
843 1995. Monoclonal antibodies reactive with serum IgM and leukocytes from Atlantic
846 salmonis include a Th2-type response at the louse-salmon interface. Dev Comp
848 88. Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of
851 exposure induces metabolic and stress-related gene expression in copepodid salmon
852 lice (Lepeophtheirus salmonis). Comp Biochem Physiol - Part D Genomics Proteomics
855 responses of Atlantic salmon (Salmo salar L.) to infection with the salmon louse
857 91. Tadiso TM, Lie KK, Hordvik I. 2011. Molecular cloning of IgT from Atlantic salmon,
858 and analysis of the relative expression of tau, mu and delta in different tissues. Vet
860 92. Fast MDD, Muise DMM, Easy REE, Ross NWW, Johnson SCC. 2006. The effects of
861 Lepeophtheirus salmonis infections on the stress response and immunological status
863 93. Sutherland BJG, Hanson KC, Jantzen JR, Koop BF, Smith CT. 2014. Divergent
864 immunity and energetic programs in the gills of migratory and resident
869 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric
870 averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.
Table 1. Monoclonal antibodies used in immunohistochemistry indicating dilutions, antigen retrieval methods, and the original reference.

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Table 2. Primers used for RT-qPCR, indicating the forward and reverse sequences, expected amplicon size and original reference.

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