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# The interbranchial lymphoid tissue likely contributes to immune tolerance and defense in the gills of Atlantic salmon

Ida Bergva Aas [a], Lars Austbø [a], Knut Falk [b], Ivar Hordvik [c], Erling Olaf Koppang [a, *]

[a] Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, 0454 Oslo, Norway
[b] Norwegian Veterinary Institute, 0454 Oslo, Norway
[c] Department of Biology, High Technology Centre, University of Bergen, 5006 Bergen, Norway

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ABSTRACT

Central and peripheral immune tolerance is together with defense mechanisms a hallmark of all lymphoid tissues. In fish, such tolerance is especially important in the gills, where the intimate contact between gill tissue and the aqueous environment would otherwise lead to continual immune stimulation by innocuous antigens. In this paper, we focus on the expression of genes associated with immune regulation by the interbranchial lymphoid tissue (ILT) in an attempt to understand its role in maintaining immune homeostasis. Both healthy and virus-challenged fish were investigated, and transcript levels were examined from laser-dissected ILT, gills, head kidney and intestine. Lack of Aire expression in the ILT excluded its involvement in central tolerance and any possibility of its being an analogue to the thymus. On the other hand, the ILT appears to participate in peripheral immune tolerance due to its relatively high expression of forkhead box protein 3 (Foxp3) and other genes associated with regulatory T cells (Tregs) and immune suppression.



## 1. Introduction

The interbranchial lymphoid tissue (ILT) of the Atlantic salmon (*Salmo salar* L.), comprise a heterogeneous population of T cells, arranged in a three-dimensional meshwork of epithelial cells characterized by cytokeratin staining and the presence of desmosomes (Aas et al., 2014; Haugarvoll et al., 2008; Koppang et al., 2010). While it was first described at the terminal portion of the interbranchial septum (Haugarvoll et al., 2008), the ILT further extends along the entire primary gill lamellae. These two portions have recently been designated distal and proximal part of ILT (dILT and pILT) (Dalum et al., 2015). Due to its abundance of T cells and its exposed and strategic location, it is reasonable to assume that the ILT functionally constitutes a vital part of the gill-associated lymphoid tissue (GIALT), balancing defense with tolerance towards the external environment.

With no similar homologous tissue organization described in mammals or other species, the ILT is unique in its appearance. Previous investigations of the ILT's function have suggested a possible role in immune regulation, and overall results have largely pointed towards a role as a secondary lymphoid tissue (Aas et al., 2014; Austbø et al., 2014; Dalum et al., 2016), although some structural similarities between the ILT and thymus are notable. Both tissues have a complex three-dimensional arrangement of epithelial cells creating a lattice-like system in which T cells are embedded (Haugarvoll et al., 2008; Koppang et al., 2010; Press and Evensen, 1999). A major anatomical difference is that the thymus contains blood vessels while the ILT does not. On the contrary, the ILT is separated from underlying and adjacent tissue by a prominent basal membrane (Koppang et al., 2010). The thymus and gills of salmon develop from the pharyngeal region (Matsunaga and Rahman, 2001) and the thymus of adult salmon is located in the dorsolateral part of the gill chamber. During early stages of development, a possible connection between the gill tissue and thymus has been identified (Dalum et al., 2016). Detailed knowledge of the development and function of the salmonid thymus and ILT is still lacking, and consequently we cannot exclude that the ILT and

*Abbreviations:* ILT, interbranchial lymphoid tissue; Foxp3, forkhead box protein 3; Tregs, regulatory T cells; GIALT, gill-associated lymphoid tissue; RAG, recombination activating gene; TCR, T cell receptor; Aire, autoimmune regulator; GILT, gamma-interferon-inducible lysosomal thiol reductase; ROR, retinoid-related orphan receptor; dpi, days post infection; ISAV, infectious salmon anaemia virus.

* Corresponding author. Department of Basic Sciences and Aquatic Medicine, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ullevålsveien 72, 0454 Oslo, Norway.
*E-mail address:* erling.o.koppang@nmbu.no (E.O. Koppang).

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thymus have similar or overlapping roles. In mammals, T cells mature through selection and developmental processes in the cortex and medulla of the thymus. Cells enter and leave the thymus through vessels and traffic through the different thymic compartments assisted by the 3D epithelial meshwork (van Ewijk et al., 1999) much like what we find in the ILT.

In the mammalian thymic cortex, recombination activating genes (RAGs) are essential factors required for recombination of the T-cell receptor (TCR) genes (Yamamoto et al., 1992). In two previous studies, transcription levels of RAG-1 in ILT were negative or very weak compared to thymus, suggesting that there are no functional relations to the thymic cortex (Aas et al., 2014; Dalum et al., 2015). From the thymic cortex, immature precursor T cells migrate further into the mammalian thymic medulla where they are subjected to a strict selection process supported by the autoimmune regulator (Aire). The Aire protein is a transcription factor that induces expression of tissue-specific self-antigens in the medullary thymic epithelial cells (mTECs) (Derbinski et al., 2001). Consequently, Aire plays a key role in the regulation of immunological tolerance, a task performed in the early life of T cells by negative selection of potential autoimmune T cells. Not only does Aire ensure that the T cells released into the circulation are tolerant to self-antigens, but it also promotes the establishment of a distinct group of $Foxp3^+$ $CD4^+$ Treg cells, to ensure that tolerance is maintained beyond the thymus' borders (S. Yang et al., 2015). Tregs have proven to be indispensable in maintaining immune homeostasis (Geiger and Tauro, 2012), and create regulatory milieus that promote infectious tolerance (Tang and Bluestone, 2008).

In this study, we seek to expand the knowledge on the ILTs function and its classification in terms of type of lymphoid organ. The presence of immune regulatory mechanisms in the ILT has previously been hypothesized. Hence, this paper will concentrate on the ILTs assumed potential in immune regulation and possible involvement in central and peripheral tolerance. The focus at this stage will be to clarify the presence of Aire transcripts in the ILT. This information will reveal the relationship between epithelial cells in the thymic medulla and the ILT. Further, the functional role of the ILT will be examined in naïve and challenged fish based on transcript levels of relevant genes. Genes (forkhead box protein 3 (Foxp3), interleukin (IL)-2, IL-10, γ-interferon-inducible lysosomal thiol reductase (GILT), retinoid-related orphan receptor (ROR)-γ and GATA-3) were selected were based on their relation to Tregs, anti-inflammatory role and importance in maintaining peripheral tolerance.

## 2. Materials and methods

### 2.1. Fish

Unvaccinated pre-smolt Atlantic salmon of the Sunndalsøra breed, reared at Nofima, Sunndalsøra, were used for the control and infectious salmon anaemia virus (ISAV)-challenged groups. The fish were kept in fresh water tanks and had an average weight of 574 ± 81 g when sampled. Details of the environmental conditions and the ISAV challenge have been described previously (Aas et al., 2014; Austbø et al., 2014). The first sampling was performed 9 days post infection (dpi) and comprised four fish from the control group. The second sampling was performed at 27–33 dpi, and comprised eight individuals from the infected group and four from the control group, respectively. Successful ISAV-infection was confirmed by high levels of virus and classical ISAV pathological findings in fish from the last sampling (Aas et al., 2014; Austbø et al., 2014). Samples were conserved in RNAlater and were collected from the second segment of mid-intestine (Løkka et al., 2013), (hereafter referred to as intestine), gills, and head kidney. In addition, gill samples designated for laser-capture microdissection were snap-frozen in liquid nitrogen for subsequent cryosectioning. Fig. 1 illustrates the division of ILT and the laser dissected area designated for real-time RT-PCR experiments. Thymus samples were collected from 3 remaining excess fish from the control group after the initial sampling, resulting in a higher age of these individuals.

### 2.2. Molecular cloning and sequencing of salmon Aire

Primers were designed based on available EST sequences to cover the full-length salmon *aire* coding sequence (Table 1) and PCR amplification was performed with cDNA from thymus tissue from uninfected fish. The expected PCR product was verified by gel electrophoresis, and cloned with TOPO® TA Cloning Kit for sequencing (Invitrogen Life Technologies) at Eurofins MWG Operon (Ebersberg, Germany).

A phylogenetic tree was constructed by the neighbor-joining method with default settings using the MEGA v.7.0 software (Kumar et al., 2016) based on Muscle multiple alignment with 2000 bootstrap replications.

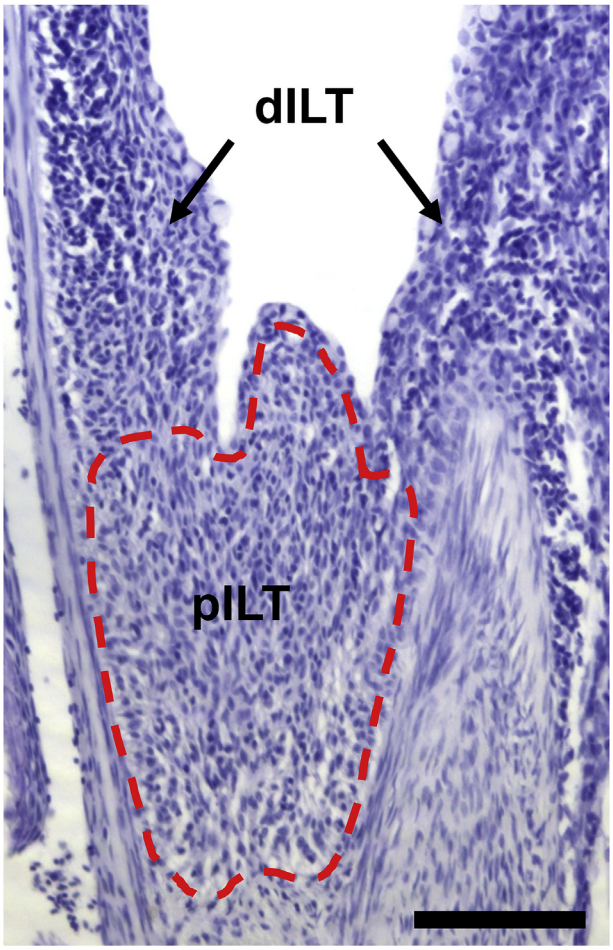


**Fig. 1. Histological micrograph of the ILT**. A transversally sectioned hematoxylin stained gill arch, illustrating the division of ILT and area that were laser-capture microdissected indicated by stapled red line. Scale bar = 100 μm.

**Table 1**
Gene assays used for quantitative real-time RT-PCR.

| Gene | Gene sequence (5'→3') | GenBank Accession No. | Ct value Mean ± SEM |
|---|---|---|---|
| $EF1A_B^*$ | F-TGCCCCTCCAGGATGTCTAC<br>R-CACGGCCCACAGGTACTG<br>P-FAM-AAATCGGCGGTATTGG-MGB | 100136485 | 20.6 ± 0.2 |
| Aire | F-GGTGATTGATGATCCCTTCC<br>R-CCCTCTCCAGGGTAT**CC**TTC<br>P-CAGCATCATCAGCGACCAAATTCT | 106568810 | Thy: 35.6 ± 0.5 |
| Aire sequencing | F-TGAACACCACGCATATTAACTG<br>R-GGATTGCGCTTGTGTGAG | | |
| Foxp3 | F-CCCACCATCTACTCCCAGAT<br>R-TCTTATCAGGCAGGCGTAGG<br>P-FAM-TACAACAACATCCGGCCTCCGTA-BHQ | 100526661 | 30.6 ± 0.2 |
| IL-2 | F-TGATGATTGATAAGGACAACAGC<br>R-TTGTCCCAATTACGTAATGTTCC<br>P-AAGTCCTTGAAAGACTTTTCCAACCGC-BHQ | EG762969 | 33 ± 0.2 |
| IL-10 | F-ACGAAGGCATTCTACACCAC<br>R-AAAGTGGTTGTTCTGCGTTC<br>P-AAGTTCTACCTCGACACGGTGTTGC-BHQ | 106594794/<br>106566262 | 36 ± 0.5 |
| GILT | F-TGGAGGCCTCCACTGATG<br>R-CTTCACACAGGTCATGATGC<br>P-GCTCTATGACCCAAACACCAAGTGG-BHQ | 106599011/<br>106568950 | 26 ± 0.2 |
| ROR-γ | F-CCAATGCCATCCAGTACG<br>R-GCACATCCAGACATCCTGC<br>P-AGATCCAGGAATCCAGTGATGCGC-BHQ | 106584392/<br>106613816 | 30.6 ± 0.2 |
| GATA-3 | F-ACCCAAGCGACGACTGTC<br>R-TCTTCGCCACAGTGTTGTC<br>P-TCATGTGCAAACTGTCAAACGAGC-BHQ | 106609266/<br>106576211 | 23.7 ± 0.2 |

Gene assays used for quantitative real-time RT-PCR with relevant accession numbers (National Center for Biotechnology Information database: http://www.ncbi.nlm.mih.gov). Primers and probes were designed to span intron sections. Asterisk * indicates assay designed by others, $EF1A_B$ (Olsvik et al., 2005). Samples with no detectable expression within 40 cycles are not included in the calculation, as this would result in erroneous average value. Mean Ct values ± SEM are from control gills as representative tissue (except Aire, exclusively expressed in thymus).

### 2.3. Quantitative real-time PCR

Real-time quantitative PCR (qPCR) analysis was performed as described previously (Aas et al., 2014). Primers and probes were designed to span intron sections. To exclude any sequence polymorphism that could interfere with binding of primers and probe, all available salmonid sequences (annotated, expressed sequence tags, salmon genome and in-house RNAseqs) were considered. The ROR-γ primers in this study were designed based on a study that characterized ROR-γ in rainbow trout (*Oncorhynchus mykiss*) (Monte et al., 2012). Like mammals, rainbow trout also possess two isotypes of ROR-γ (ROR-γa1/ROR-γa2) and the same is true also for zebrafish (*Danio rerio*) (ROR-γa/ROR-γb) (Flores et al., 2007). As in the work by Monte et al., the design of two primer pairs identifying each of the isotypes was prevented by the high sequence identity in the coding region, even though alignment and homology mapping indicated that the trout homologues (ROR-γa1/ROR-γa2) are more alike the mammalian splice variant ROR-γt (Monte et al., 2012).

The primer and probe sequences for $EF1A_B$, Aire, Foxp3, IL-2, IL-10, GILT, ROR-γ, and GATA-3 transcripts are listed in Table 1. The expression levels were measured with relative quantification using elongation factor 1A, $EF1A_B$ (Løvoll et al., 2011; Olsvik et al., 2005) as reference gene, and gene expression was calculated from cycle threshold (Ct) values using the ΔΔCt method (Schmittgen and Livak, 2008). In short, real-time qPCR was carried out in 13 μl reactions with cDNA corresponding to 15 ng RNA added to each reaction. To compensate for lower template concentration, the laser-dissected cDNA samples were only diluted 5 times instead of 10. Each quantification target was amplified in triplicate samples (whole tissue) or duplicate samples (laser-dissected tissue) with additional negative controls, lacking the template, for each master mix. Statistical differences in gene expression in control and infected tissue samples were evaluated using the non-paired *t*-test. Differences in expression between the control and challenged group were considered significant with values of probability $p < 0.05$ ($\alpha = 0.05$). In addition, Bonferroni correction was performed to account for multiple tests, using the formula α/number of tests performed. All analyses were performed using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego CA, USA, www.graphpad.com).

## 3. Results

### 3.1. Identification and characterization of salmon Aire

At the start of this study, no annotated salmon transcript sequences for *aire* were available in the NCBI database (http://blast.ncbi.nlm.nih.gov). By using human and zebrafish sequences, a tBLASTn search in the salmon genome was performed, which identified only one genomic variant. Subsequent PCR amplification of salmon thymus cDNA and cloning confirmed several potential transcript isoforms of salmon Aire. This finding was verified by the recent availability of the updated salmon genome (Lien et al., 2016) (ICSASG_v2, annotation release ID: 100) locating salmon *aire* (Gene ID: 106568810) on chromosome 14, NC_027313.1 (10836382.10846720, complement). Like other fish species, the salmon *aire* gene consists of 13 exons, while mammals have 14. Fish lack the mammalian counterpart of exon 7 (Fig. 2).

The composition of the sequenced clones did not display a characteristic isoform variation, for example addition/exclusion/rearrangement of an exon. The variations present were all manifested as differences in exon length (varying from 1 to 13 aa) located at exon intron boundaries, appearing to be a result of additional splice-sites without disrupting the reading frame.

Besides one amino acid substitution in two of the clones, our 18 sequenced *aire* clones all showed a random combination of the exon alterations present in the 5 predicted transcript isoforms (XM_014139476.1, XM_014139478.1, XM_014139477.1, XM_014139474.1 and XM_014139473.1). Table 2 shows an overview of exon variance and frequency in sequenced clones. A more detailed overview of the exon variations and alignment to other species are presented in Supplementary Fig. 1.

### 3.2. Phylogenetic analysis

A phylogenetic tree was constructed based on available vertebrate RefSeq Aire protein sequences (Fig. 3). All the Atlantic salmon transcript isoforms clustered together with Northern pike (*Esox lucius*) as the closest related neighbor. Exon numbers varied between 10 and 14, with 13 as the most common.

### 3.3. Transcription of genes related to immune regulation and tolerance

Real-time qPCR analysis was performed on laser-dissected ILT, gills (including primary and secondary lamellae), intestine and head kidney from eight control individuals and eight from the challenged group sampled on days 27-33 p.i. Thymus samples from three control individuals were also included.

The real-time RT-qPCR showed low variance between the triplicates and within the groups. For the laser-dissected ILT samples, the expected RNA yield was too low to measure quantitatively. To ensure the presence of sufficient template in the laser-dissected samples, a Ct threshold of 28 was set for the reference gene $EF1A_B$. Samples exceeding this Ct were harvested and isolated again so that all analyzed samples were below this threshold. Mean Ct-values ± SEM for control gills are given in Table 1.

*aire* transcripts were only found in the thymus. Except for *aire*, transcripts corresponding to all the investigated molecules were present in all examined organs. Fig. 4 shows the transcript levels in the control group. Transcript levels including significant alterations for the individual genes for both control and infected fish are shown in Fig. 5, which also shows significance after application of Bonferroni correction.

Transcript levels of Foxp3, IL-2 and IL-10 were higher in the ILT than in the other investigated tissues (6-fold, 63-fold and 70-fold higher than control gills respectively), and the levels remained unchanged in the infected group. In intestine, significant increases of Foxp3, IL-2 and IL-10 transcript levels were observed at 27–33 dpi. In head kidney, significant increases of IL-2 and IL-10 were observed at 27–33 dpi. A significant increase in the IL-10 transcript level was seen in the gills at the last sampling.

**Table 2**
Overview of exon variations in the Atlantic salmon *aire* transcripts.

| | exon 3 | exon 5 | exon 9 | exon 10 | exon 14 |
|---|---|---|---|---|---|
| *aa exon length long/short* | 47/44 | 39/38 | 25/12 | 45/40 | 27/26 |
| **NCBI predicted isoforms of salmon aire long (L) and short (S)** | | | | | |
| ***X1**: XP_013994948* | L | S | L | L | L |
| ***X2**: XP_013994949* | L | S | L | L | S |
| ***X3**: XP_013994951* | S | S | L | L | L |
| ***X4**: XP_013994952* | L | S | L | S | L |
| ***X5**: XP_013994953* | L | S | S | L | L |
| **Frequency of the exon alterations in the 18 sequenced clones** | | | | | |
| *Long exon variant* | 15 | 16 | 6 | 15 | 8 |
| *Short exon variant* | 3 | 2 | 12 | 3 | 10 |

The intestine and ILT had the highest transcript levels of GILT, and the transcript level remained unchanged in the infected group. In gill and head kidney, a significant (**) up regulation was observed in the infected group.

The highest transcript levels of GATA-3 was seen in the gills, and remained stable in the infected group. In the ILT, a significant (**) increase of GATA-3 transcript level was observed in the infected group. Thymus had the same expression level as the ILT in the control group. In intestine, a significant (**) increase in transcript level was observed by 27–33 dpi.

The highest transcript levels of ROR-γ were observed in the intestine and thymus. The expression of ROR-γ in ILT, gill and head kidney remained unchanged in the infected group, although a decrease (*) in transcript level was seen in the intestine by day 27–33 dpi.

## 4. Discussion

The results presented here strongly indicate a role for the ILT in maintaining immune tolerance and homeostasis in the gills. Immunological tolerance, with the aim of avoiding continual immune stimulation by innocuous antigens, is a hallmark of the mucosal immune system (Kim et al., 2012). The aquatic environment exposes the mucosal surfaces of fish to many diverse antigens, and the ILT, with its abundant number of T cells, very likely plays a central role in the GIALT.

In the mammalian thymus, potential autoimmune cells are eliminated through an Aire-dependent process that involves presentation of tissue-specific self-antigens (TSAs) to developing T cells (Gardner et al., 2009). In our work, the characterization of the salmon *aire* gene was central as it, unlike RAG-1 in the thymus cortex, could serve as a specific transcript marker for thymic medulla as in mice (Zuklys et al., 2000). In this paper, the salmon *aire* gene was identified through its homology to known *aire* sequences

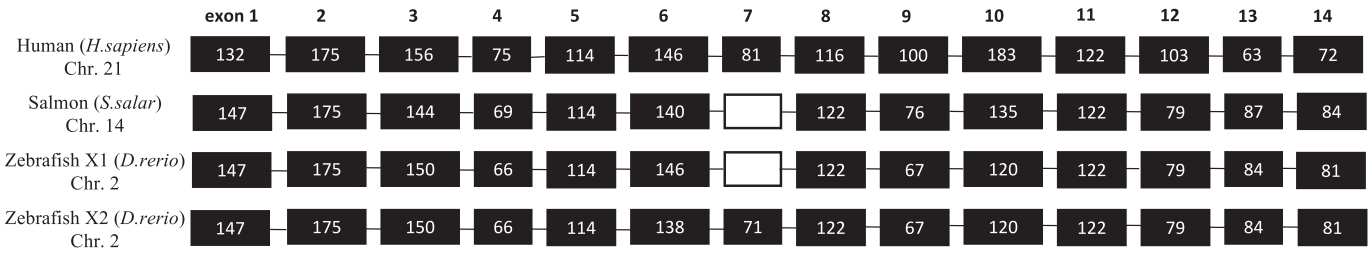


**Fig. 2. Diagram of the *aire* gene structure**. The observed transcript isoforms (X1-X5) in Atlantic salmon all include 13 exons. Differences between the isoforms are due to shorter (3–39 bp) variations in exon length of exon 3, 5, 9, 10 and 14. The Atlantic salmon aire gene structure is compared to human aire (14 exons), zebrafish X1 (13 exons), zebrafish X2 (14 exons). The lack of exon 7 in Atlantic salmon and zebrafish X1 is illustrated by an empty exon box to facilitate comparison. Black boxes represent exons, numbered according to size (bp). Introns are indicated with a line. Boxes and lines are not to scale. The aire gene organization were derived from sequences X1: XP 013994948, X2: XP 013994949, X3: XP 013994951, X4: XP 013994952 and X5: XP 013994953 (*S.salar*), NP_000374 (*H.sapiens*) and XP_00929678.1, NP_001103484 (*D. rerio*) and does not include complete UTR's.

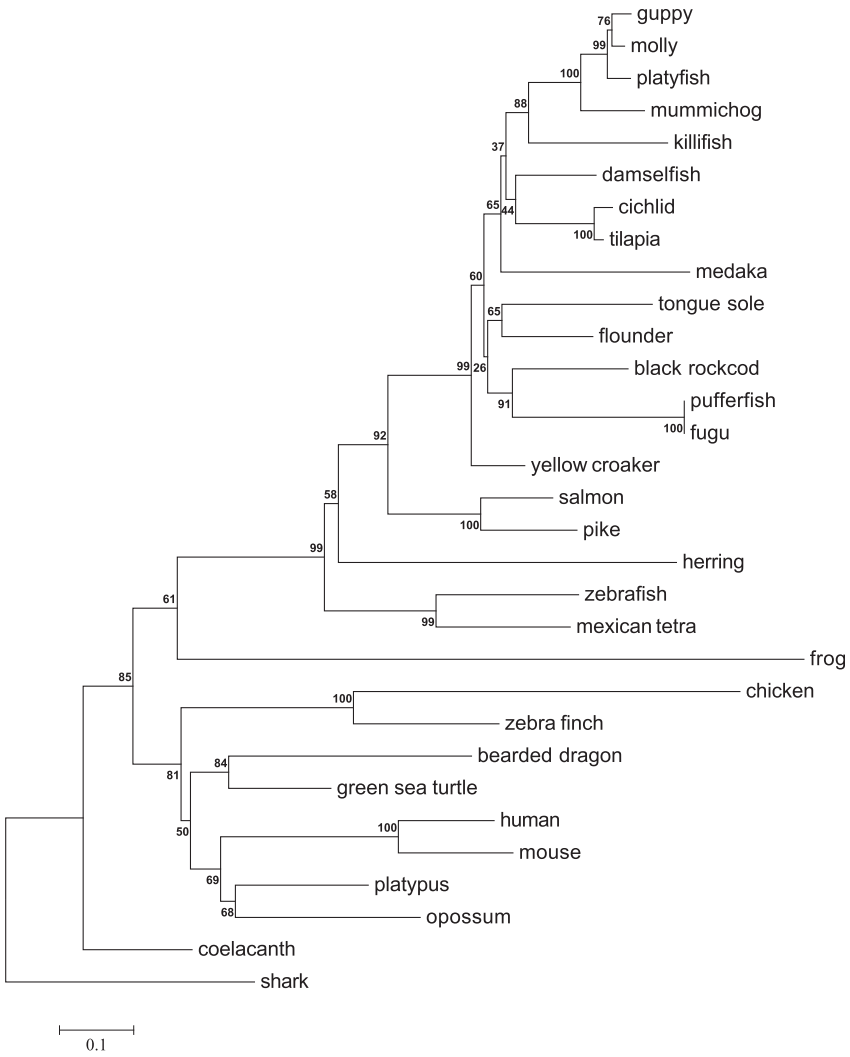


**Fig. 3. Phylogenetic comparison of Aire in different species**. The phylogenetic tree was constructed using the neighbor-joining method in the MEGA program based on sequences from selected vertebrate species with available genomes and identified *aire* sequences to show the relationship between them. Accession no.: guppy (*Poecilia reticulata*) - XP_008432373.1, molly (*Poecilia formosa*) – XP_007567088, platyfish (*Xiphophorus maculatus*) - XP_014328269, mummichog (*Fundulus heteroclitus*) - XP_012734428, killifish (*Austrofundulus limnaeus*) - XP_013886813, damselfish (*Stegastes partitus*) - XP_008283989, cichlid (*Haplochromis burtoni*) - XP_005912859, tilapia (*Oreochromis niloticus*) - XP_013124273, medaka (*Oryzias latipes*) - XP_011485226, tongue sole (*Cynoglossus semilaevis*) - XP_008332262, flounder (*Paralichtys olivaceus*) - XP_019959496.1, black rockcod (*Notothenia coriiceps*) - XP_010783868, pufferfish (*Takifugu rubripes*) - XP_011614747, fugu (*Takifugu rubripes*) - XP_011614747.1, yellow croaker (*Larimichtys crocea*) - XP_010741958, salmon isoform X1 (*Salmo salar*) - XP_013994948, northern pike (*Esox lucius*) - XP_010900119, atlantic herring (*Clupea harengus*) - XP_012682171, zebrafish (*Danio rerio*) - XP_009296781, mexican tetra-like (*Astyanax mexicanus*) - XP_007245227, frog (*Xenopus tropicalis*) – XP_012826738.2, chicken (*Gallus gallus*) - ABW24495.1, zebra finch (*Taeniopygia guttata*) - XP_012431231.1, bearded dragon (*Pogona vitticeps*) - XP_020643033.1, green sea turtle (*Chelonia mydas*) - XP_007067449.1, human – NP_000374, mouse (*Mus musculus*) – ADZ48462, platypus (*Ornithorhynchus anatinus*) - XP_007667891.1, opossum (*Monodelphis domestica*) - XP_007480436.2, coelacanth (*Latimeria chalumnae*) - XP_014348180.1, shark (*Rhincodon typus*) - XP_020384593.1. Bootstrap values are shown at the branches. Branch length in terms of genetic distance is indicated below the tree.

from different species and later verified by the sequencing and annotation of the salmon genome. In line with the predicted isoforms, our cloned Aire transcript confirmed the deletion of exon 7, a feature common to many teleosts. In man, exon 7 is a 27 amino-acid-long sequence, partly coding for the last part of the SAND-domain (truncated SAND-domain). Previous investigations have shown that the SAND-domain is poorly conserved. Consequently, its function may differ among species, suggesting that this domain is not crucial for the function of Aire (Saltis et al., 2008). The sequenced clones also confirmed six transcript isoforms manifested as shorter deletions of the exon (3–39 bp) appearing in random combinations in the intron-exon boundaries. As none of the deletions disturbed the frameshift, one can speculate on what functional advantage this might have for a gene that only exists as a single copy in the salmon genome.

The exclusive expression of *aire* in salmon thymic tissue corresponds to results from zebrafish (Saltis et al., 2008), indicating that Aire has the same role during T cell development in the thymus of both species. Importantly, this supports previous investigations indicating that the teleost thymus has similar functions to that of mammals (Chilmonczyk, 1992; Ellsaesser et al., 1988; Picchietti et al., 2015). It is notable that the teleost thymus, like the mammalian, undergoes age-dependent involution (Bowden et al., 2005) and it is expected that the transcript level of Aire would be higher in younger fish.

Aire has been reported to promote the establishment of a distinct subset of Foxp3$^+$ CD4$^+$ Tregs in mice (S. Yang et al., 2015), showing that Aire indirectly contributes to peripheral immune tolerance.

In mammals, Foxp3 is a transcription factor that controls the

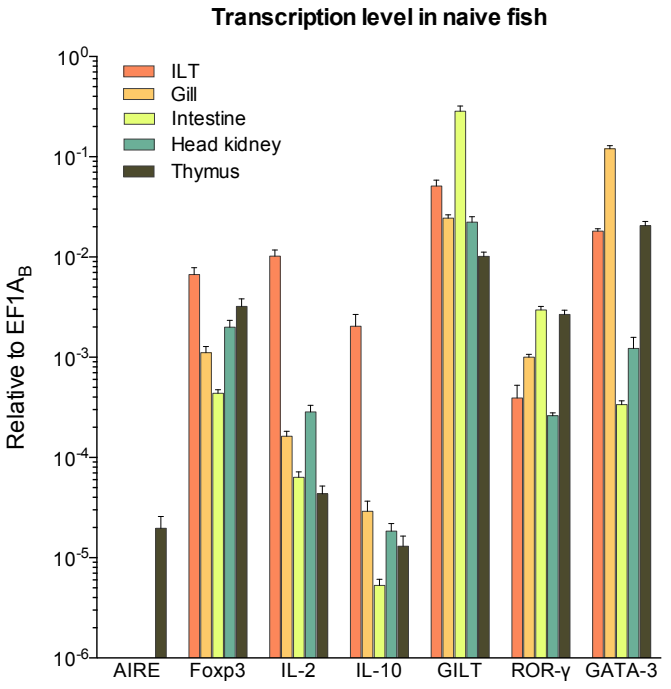


**Fig. 4. Basal transcription level in naïve fish**. Transcript levels of genes related to immune regulation in control group, representing naïve fish (n = 8). Transcript levels are normalized with EF1A$_B$ and displayed with mean ± SEM.

differentiation and function of Tregs, known for their suppressive function and crucial role in maintaining immune homeostasis (Geiger and Tauro, 2012). Previous studies have shown that mechanisms of peripheral tolerance exist in zebrafish and are a feature of early vertebrate evolution (Quintana et al., 2010). Foxp3 was characterized in the Atlantic salmon in 2011, and has its highest level of expression in the thymus (Z. Zhang et al., 2011). This corresponds to results in grass carp (*Ctenopharyngodon idellus*) (M. Yang et al., 2012). In contrast, our results showed that the ILT, followed by the thymus, had the highest transcript level of Foxp3. The importance of Foxp3$^{+}$ Tregs in maintaining peripheral tolerance indicates that the ILT is a central organ in immune homeostasis, and considering its exposed location, that it has an important protective function in the gills.

Concurrent with Foxp3, the highest transcription levels of the two cytokines IL-10 and IL-2 were also observed in the ILT, in each case at least 50-fold greater than in other tissues examined. It has been shown that fish IL-10s, like their mammalian counterparts, are important in dampening exaggerated immune responses by acting as a suppressor (Zou and Secombes, 2016). In mammals, IL-2 has been shown to play a key role in regulation of T cell growth and development, and on the other side, to have an essential role in the maintenance of peripheral tolerance (Bachmann and Oxenius, 2007). This cytokine is primarily produced by T cells, and has a crucial role in generation and maintenance of Tregs (Lan et al., 2007; Malek and Bayer, 2004), thereby exerting an immunosuppressive function (Bachmann and Oxenius, 2007).

While most of the tissues examined showed an up-regulation of the two cytokines with infection, the transcript levels in the ILT remained stable.

Previous studies in fish have shown variations in the expression level of IL-10. In zebrafish and sea bass (*Dicentrarchus labrax*), IL-10 was slightly expressed in healthy tissues, with up-regulation following LPS stimulation (D.-C. Zhang et al., 2005; Pinto et al., 2007).

The relatively high and stable transcript levels of Foxp3, IL-10 and IL-2 in the ILT indicate a role in peripheral tolerance, and strongly imply a presence of Tregs within the tissue. The cytokine transforming growth factor β-1 (TGF-β1) is known to be released by Tregs (Zou and Secombes, 2016) and is involved in maintaining immune homeostasis due to its influence on T cell development, differentiation, activation and proliferation (Letterio and Roberts, 1998). Analysis of the original TGF-1b (Brown et al., 2016; Lilleeng et al., 2009) were initially included but were evaluated as incomplete quantifications and not included in the results, as the taqman assay did not detect both recently identified paralogues (Maehr et al., 2013). The quantification showed highest transcript levels in head kidney and thymus (both having primary lymphoid organ function), while ILT had higher levels than intestine and gill, in line with the postulated functions of the ILT. Transcripts corresponding to GATA-3 and GILT were present in all tissues examined. Both are involved in immune regulation, although by different mechanisms. GILT through a negative effect on T cell activation (Lilleeng et al., 2009) and GATA-3 by being a master regulator of Th2 and counteracting proinflammatory responses (Takizawa et al., 2011; Wang et al., 2010). A higher baseline transcript level of GATA-3 in exposed and vulnerable tissues such as gills, brain, intestine and skin of rainbow trout has been speculated to protect these vital tissues from inflammatory responses (Wang et al., 2010), and the significant up regulation observed in ILT of infected fish might be a strategy to limit inflammatory insult in the ILT.

ROR-γ has been shown to play an essential regulatory role in T cell homeostasis and lymphoid organogenesis (Kurebayashi et al., 2000), and in teleosts, to be involved in Th17-type responses (Monte et al., 2012). In our study, ROR-γ was expressed across all tissues examined, although the thymus and intestine had the highest transcript levels.

Taken together with previous results showing the absence or very weak transcript levels of RAGs in the ILT (Aas et al., 2014; Dalum et al., 2015), and now the confirmed absence of *aire*, we can conclude that the ILT does not share key characteristics of a mammalian primary lymphoid tissue. Transcripts corresponding to salmon Aire were exclusively expressed in the thymus and in no other tissues investigated. This suggests that a negative selection occurs during T cell development in the salmonid thymus like that seen in mammals.

In mammals, tolerance is detrimental to maintain immune homeostasis and to avoid inflammation-associated tissue damage, of utmost importance in organs with vital and delicate functions such as the lungs (Snelgrove et al., 2011). In fish, the gills fall arguably into the same category, and we have previously speculated on the immune regulatory mechanisms in the ILT (Aas et al., 2014; Austbø et al., 2014). The possible presence of cells tailoring the mucosal immune response in the gills of rainbow trout has been suggested in previous studies (Castro et al., 2014), and our findings agree with these results. The high and stable transcription levels of especially Foxp3, IL-10 and IL-2 give us reason to assume that ILT is equipped with mechanisms of tolerance and counter-regulation.

Despite the morphological similarities and developmental associations, the present work largely put an end to the hypothesis of ILT as a thymus analogue, indicated further by the lack of medulla-specific factors in the ILT. However, the levels of genes related to Tregs suggest an involvement in maintaining immune homeostasis.

We also show that mechanisms of central tolerance, as present in mammals, are found in the thymus of Atlantic salmon. Identification of transcripts corresponding to Foxp3, a marker of Tregs, suggests the involvement of Tregs in maintaining peripheral tolerance in the ILT.

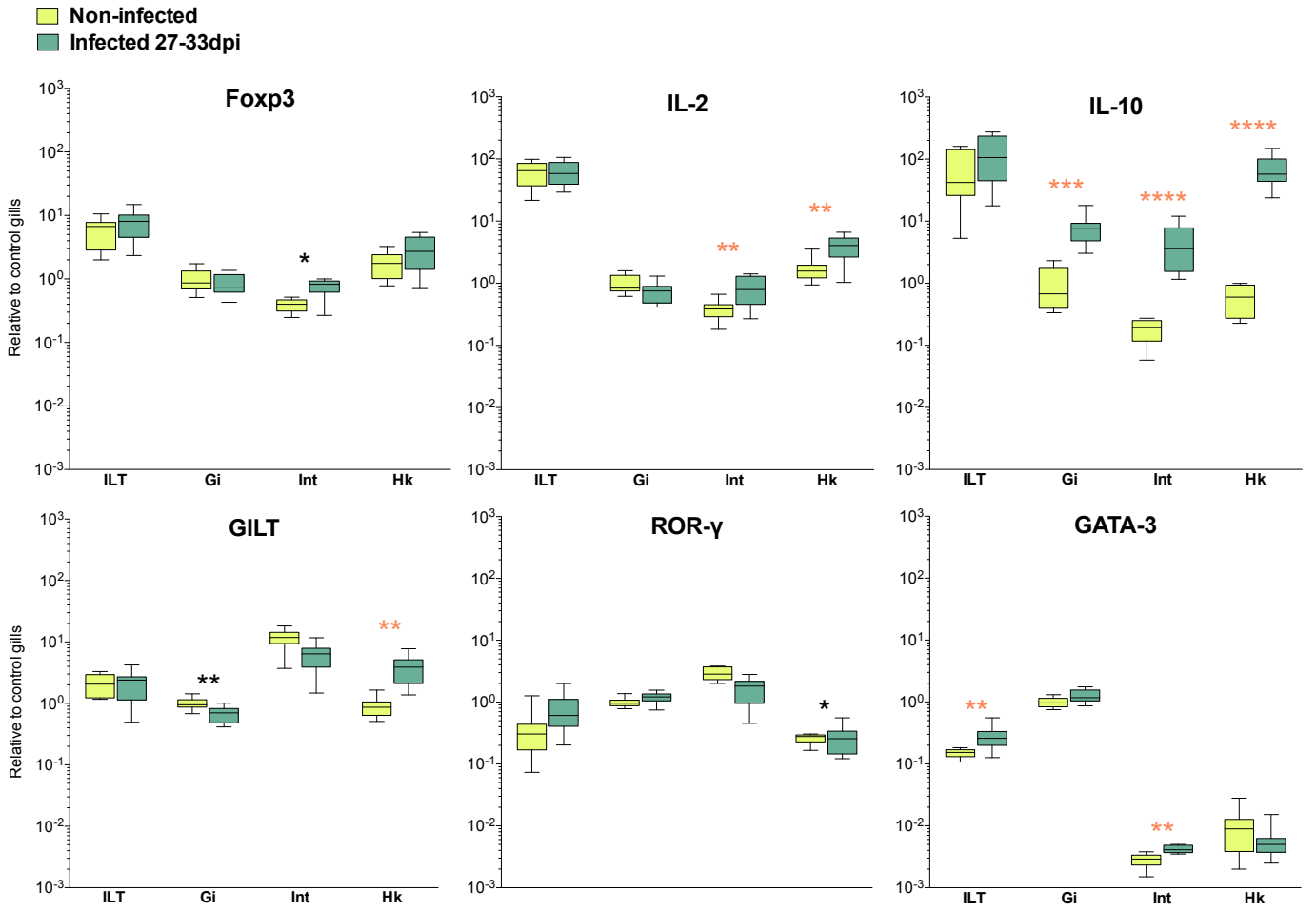


**Fig. 5. Transcription level in ISAV-challenged fish**. Transcript levels of genes related to immune regulation in ISAV-infected ($n = 8$) compared to control group ($n = 8$). Transcripts in laser-dissected ILT, gill, intestine and head kidney quantified by real-time qPCR. Significant changes in transcription level by day 27-33 p.i: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$. Stars colored red indicates significance at $p < 0.005$ according to Bonferroni correction, performed to account for multiple tests. The transcript levels are displayed with mean ± SEM. Transcript levels are normalized with $EF1A_B$ and displayed relative to the mean of control gills. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## The authors' contributions

I.B.A. participated in study design, collection of material, performing the experiments, interpretation of results, statistical analysis, and writing of the manuscript; L.A. managed the study design, the experimental challenge and sampling, primer and probe design, interpretation of results and writing of the manuscript; K.F. designed, participated in execution, and was responsible for the infection experiment and for cultivation of virus; I.H. participated in study design and interpretation of results; E.O.K. supervised the study; all authors read and approved the manuscript.

## Disclosures

The authors have no financial conflicts of interest.

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## Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2017.06.013.

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