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Short communication

Visualization of CCL19-like transcripts in the ILT, thymus and head kidney of Atlantic salmon (*Salmo salar* L.)

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1. Short communication

The interbranchial lymphoid tissue (ILT), so far described in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), is located at the terminal extension of the gill interbranchial septum, in the cleft formed at the site where the primary gill lamellae divide. Histologically, the ILT appears as a classic lymphoepithelial organ, consisting of reticulated epithelial cells forming niches containing predominantly T cells and covered with flat, non-keratinized squamous epithelial cells and occasional mucus cells [1,2]. Of note, no vessels have been identified in the ILT, and being attached from the underlying tissue with a prominent basal membrane, the structure appears strictly intraepithelial. A recent investigation allowed a differentiation of the ILT into two portions; *i.e.* the distal ILT (dILT) located to the trailing edge of the primary gill lamellae in addition to the originally described portion now termed the proximal ILT (pILT) [3].

Since its discovery, several studies have addressed aspects of the nature of the ILT and possible functions (reviewed in [4]). The absence of transcripts corresponding to recombination-activating gene (RAG) 1 and 2 [5] and autoimmune regulator (Aire) [6] suggests that the pILT is not a primary lymphoid organ. Secondary immune organ functions in this structure have so far not been revealed.

As the ILT is rich in T cells, factors attracting them to this site should be highly expressed. In this respect, the chemokines CCL19 and CCL21 are central. In mammals, their expression is un-regulated but rather constitutive and further restricted to a variety of stromal cells in primary and secondary lymphoid organs. Among other leukocytes, naïve T cells express the chemokine receptor CCR7 that promotes migration towards CCL19 and CCL21 (reviewed in [7]). Through transcriptome profiling of the pILT, it became apparent that one of the most strongly expressed genes was CCL19 [8]. In salmonids there are six *CCL19-like* genes classified in trout as chemokines: *CK10a/b, CK12a/b* and *CK13a/b* [9]. While the *CK12a* seems to be important for NALT in rainbow trout [9], our transcriptome data (unpublished) indicated that it is the Atlantic salmon homologues to *CK13a/b* corresponding to the zebrafish *CCL19b* that is the most prominent form in the ILT.

In this study, we aimed to visualize CCL19 expression in the ILT. We performed *in situ* hybridization experiments targeting a sequence for the most abundantly expressed form; however, cross-hybridization with other CCL19 transcripts is also likely. Gills from a population of unvaccinated Atlantic salmon kept at Matre Research Station and sampled at an average weight of 56 g (in fresh water); 58 g (transferred to salt water and onwards); 150 g; 300 g; and 1.2 kg over a period of 18 months, three individuals at each sampling. All wet-lab samplings were performed at the Institute of Marine Research, Matre Research Station (60° N, 5° E, Western Norway) which is authorized for animal experimentation (Norwegian Food Safety Authority, facility 110) and in accordance with international guidelines. In addition, gills from three sexually matured salmon, average weight 5 kg, River Drammenselven (sampling described in [1]) were included in the investigation, altogether 24 individuals. From the 150 g fish (three individuals), thymi, head kidneys and livers were also included. The material was formalin-fixed and paraffin-embedded and sections produced following standard procedures. The gills were sectioned in both dorsal and transverse projections to allow analysis of both the pILT and the dILT (procedure described in [3]). The *in situ* hybridization procedure was performed using RNAscope® 2.5 HD Assay - RED (Advanced Cell Diagnostics (ACD), Newark, CA) according to the manufacturer's instructions. A custom 7ZZ RNAscope® probe was constructed, targeting bp 134–444 (coding sequence) of the NCBI Reference Sequence XM_014128861, the Atlantic salmon homologue to rainbow trout (*Oncorhychus mykiss*) *CK13a/b* [9]. A probe targeting Peptidylpropyl Isomerase B (PPIB) in Atlantic salmon was used as a reference target gene to test the RNA integrity. A negative control probe (targeting DapB gene of *Bacillus subtilis*, universal negative control probe) was used to assess cross reactivity. Both positive and negative control probes were provided by the manufacturer (Table 1).

The described findings were similar across all groups and fish investigated. In the pILT, positive cells showed a morphology and location consistent with that of reticulated epithelial cells, forming niches in

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Table 1
Target and control probes for *in situ* hybridization.

	Probe	Accession no.	Target Region (bp)
Target	CCL19	XM_014128861	134–444
Control	PPIB	NM_001140870	20–934
	DapB	EF191515	414–862

which non-epithelial cells were embedded (Fig. 1A and B). This pattern is in concordance with previous studies showing that pILT consists of T cells embedded in a meshwork of epithelial cells [2].

Positive cells were also detected in both the dILT (trailing edge) and in the leading edge of the primary lamellae (Fig. 1C). In the dILT, abundant positive cells occurred, seemingly forming a similar lymphoepithelium consisting of reticulated epithelial cells with embedded leukocytes as in the pILT. In the leading edge, scattered positive cells occurred. The distribution within the lamellae thus reflected the amount of T cells observed at these two different locations, with an abundancy in the trailing edge compared with that of the leading edge [3]. In the secondary lamellae, positive signals were only rarely detected. Positive cells were always confined to the epithelium. Cells beneath the basal membrane throughout the gills with connective tissue including bone and cartilage, nervous tissue and vasculature containing peripheral blood cells were without exception negative (Fig. 1A–C).

In the head-kidney, scattered positive cells were seen. The morphology was difficult to assess, and the nature of the positive cells was therefore not possible to determine (Fig. 1D).

In the thymus, positive cells were in general confined to a layer between the epithelial capsule and the underlying tissue. Some scattered positive cells could also be seen deeper within the tissue, particularly in association with blood vessels (Fig. 1E and F). Our results indicate that the outer layers of the thymus, with a considerable number of epithelial cells, are the most active CCL19-producing cells within this organ. In this location, reticulated epithelial cells form intercellular spaces or niches where T cells are embedded [10] with an appearance similar to that found in the ILT [2]. The distinction between thymic medulla and cortex has not been fully established in salmonids, and we will therefore not speculate on a further anatomical definition regarding the location of the positive cells within this organ. It is however noteworthy that in the mammalian thymus, CCL19 expression has in particular been noted in medullary thymic epithelial cells (mTECs) [7].

In the liver, no positive cells were seen (data not shown). This is consistent with the expression of CCL19 in mammals, which is restricted to primary and secondary lymphoid organs [7], and the liver is neither. Positive controls were positive and negative controls were negative (data not shown).

In this report, we have established evidence for CCL19 transcription in reticulated epithelial cells in the ILT and in the thymus. Additionally, cells in the head kidney were positive, whereas liver tissue proved negative. The thymus is a primary lymphoid organ whereas the head kidney is regarded as both a primary and secondary lymphoid organ in fish. In mammals, CCL19 expression is confined to primary and secondary lymphoid organs and mostly to reticulated epithelial cells. In the ILT, this seems also to be the case, and our results therefore support our previous assumption that the ILT is a lympho-epithelial organ.

Conflicts of interest

The authors declare that they have no competing interests.

Authors' contributions

HB: Planning and performing laboratory experiments, sampling, validation of results, writing manuscript.

OML: Performing laboratory experiments, validation of results, writing manuscript.

IBA: Responsible for the initial transcriptome study identifying CCL19, a homologue to the trout CK13a/b, as highly expressed in the salmon ILT.

TH: Planning and performing wet-lab experiments, sampling.

PGF: Planning performing wet-lab experiments, sampling.

LA: Responsible for the initial transcriptome study identifying CCL19, a homologue to the trout CK 13a/b, as highly expressed in the salmon ILT.

EOK: Planning experiment, sampling, validation of results.

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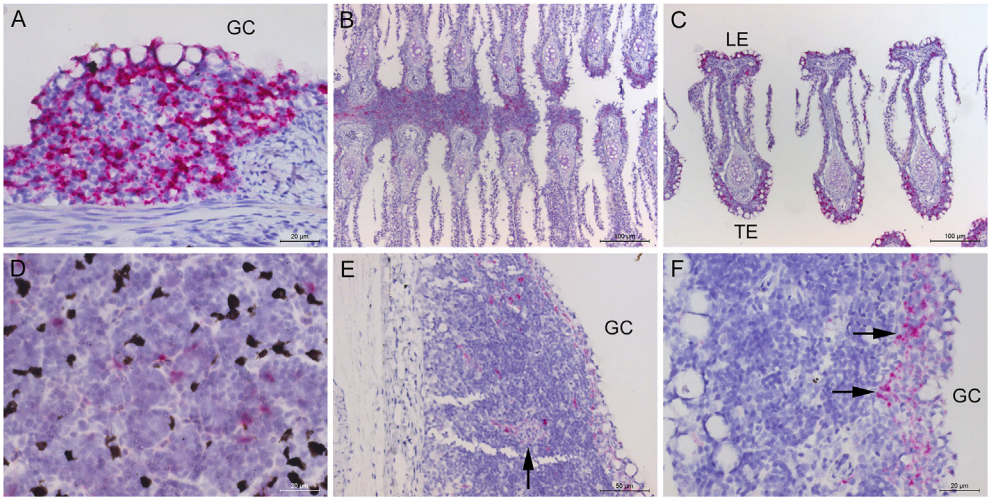

Fig. 1. *In situ* hybridization for CCL19 transcripts A) A representative transverse section of the pILT from a 150 g Atlantic salmon. Note strong signal (red) in reticulated cells throughout the tissue. B) A representative longitude section of the ILT with strong signals (red) in the sectioned structure. C) A transverse section of primary lamellae with a strong signal in the TE (trailing edge containing the dILT) and a weaker signal in the leading edge (LE). D) Head kidney with scattered positive cells (red). Note also the scattered melano-macrophages in the tissue (black). E) Thymus from a 150 g individual. The gill chamber (GC) is marked. Note scattered positive cells (red) in the thymic parenchyma, in particular in association with blood vessels (arrow) and strong signals in the capsular region. F) A magnified image of the capsular region demonstrates strong signals, in particular in cells forming a band (arrows) in the subcapsular tissue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

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