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Nonclassical MHC class I L-lineage genes in Atlantic Salmon (*Salmo salar* L.): An investigation into transcriptional induction and cell type-specific expression patterns of different L-lineage subgroups

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Illustration: Anneke ter Schule

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Many hours, days, and months of hard work have been spent on the following pages and I am joyful to finally submit. But this also implies, heavy-heartedly, that my time as a student at the University of Tromsø is coming to an end.

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Solveig Storegjerde Sætre

Abstract:

The world's population is growing, and so is the world's food demand, underpinning the need for sustainable food production. Over the last few decades, Norwegian aquaculture production of Atlantic Salmon (*Salmo Salar* L.) and rainbow trout (*Oncorhynchus mykiss*) expanded rapidly. Unfortunately, today's fish health situation is challenging due to virus infections, among other reasons. Today's virus vaccine is inadequate and doesn't prevent viral disease outbreaks in the field. Detailed knowledge of how the salmon immune system combats viruses is still limited, and a better understanding is needed to make an efficient vaccine. When a virus infects a cell, the antigen from the virus is presented on a specialised complex called a Major Histocompatibility complex (MHC) class I by the infected cell. The MHC molecule interacts with T-cells that meditate an immune response. There are identified one classical MHC class I gene and several non-classical MHC class I genes in Atlantic Salmon. Compared to a classical MHC gene, the non-classical are not polymorphic and may present a more conserved structure to immune cells during an infection. There are identified six different functionally expressed non-classical MHC class I L-lineage genes in Atlantic Salmon. In this thesis, head kidney leucocytes (HKL) from Atlantic salmon were stimulated in vitro with IFNs and viral mimics, resulting in individual upregulation off L-lineage gene expression. Co-stimulation showed an antagonistic effect on the L-lineage subtypes LIA and LGA. Furthermore, by inhibiting JAK in the JAK/STAT signalling pathway, the gene expression decreased when stimulated in vitro, suggesting that the JAK/ STAT pathways probably have an essential role in regulating the gene expression of non-classical MHC class I L-lineage genes. To further investigate what cells express the different L-lineage genes, an optimised MACS was used to separate Macrophage-like cells, non-B cell lymphocytes and IgM-positive B-cells. In all different cell populations, LIA, LGA and LDA were expressed. However, the MAC-sorting still needs more optimisation before cell type-specific L-lineage gene expression can be implied

Abbreviations

Abbreviations sorted after appearing in the thesis:

RNA	Ribonucleic acid
ssRNA	Single-stranded RNA
dsRNA	Double-stranded RNA
PAMPs	Pathogens-associated molecular patterns
DAMPs	Damage-associated molecular patterns
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
Igs	Immunoglobulins
IgM	Immunoglobulin Ig mu
TCR	T cell receptors
MHC	Major histocompatibility complex
IFN	Interferons
ISRE	IFN-stimulated response elements
STATs	Signal transducer and activates the transcription
TYK2	Tyrosine kinase 2
DC	Dendritic cells
NK	Naturale killer cells
ER	Endoplasmic reticulum
LIA	*Sasa-lia*
LDA	*Sasa-lda*
LCA	*Sasa-lca*
LGA	*Sasa-lga*
LHA	*Sasa-lha*
LFA	*Sasa-lfa*
HKL	Head kidney leucocytes
FBS	Fetal bovine serum
L-15 media	Leibovitz`s L-15 medium
P/S	Penicillin/ streptomycin
RLT buffer	RNeasy Lysis buffer
DTT:	Dithiothreitol
EDTA	Ethylene diamine-Tetra-acetic
FSC	The forward-scatter
SSC	side- scatter
FVD	Fixable Viability dye
PBS	Phosphate Buffered Saline
BSA	Bovine serum albumin
FVD	Fixable Viability Dye

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Introduction

1.1 Norwegian Aquaculture

The world's food production needs to increase by 60% by 2050 due to the expected human population growth (United Nations, 2022). Today food production is responsible for a large share of the total carbon emissions per year. Extreme weather events increasingly impact on agriculture and food availability, highlight the importance of reducing carbon emissions. As the global population grows, it becomes crucial to tap into new sustainable resources to meet the rising food demand. The ocean holds vast untapped potential for food production. Fish and seafood have lower weight CO_2 per produced protein compared to the production of terrestrial protein (beef, chicken and pork). While Norwegian-produced Atlantic salmon emits more carbon than other seafood, it still has a lower carbon footprint, producing only 18% of the emission compared to beef (Ziegler et al., 2022).

In Norway, the Aquaculture industry has rapidly grown into one of the most important national economic industries. In 2021 1,6 million tons of Norwegian Atlantic Salmon (*Salmo salar* L.) and rainbow trout *(Oncorhynchus mykiss*) were sold (Fiskeridirektoratet, 2022). A governmental goal is to expand the aquaculture industry to further contribute to Norwegian industries and global food production (Nærings- og Fiskeriderpartementet 2021). However, over the last few years, the rapid growth in Norwegian aquaculture has declined, due to challenges in production, like virus infections. In addition, the cost per fish produced has increased significantly (Fiskeriderpartementet, 2021), in part due to growing issues of fish health and welfare and high mortality rates. In 2022 16,1% of all salmon and 17,1% of rainbow trout died during sea-phase resulting in a large economical loss (Veterinærinstituttet, 2023). The main reasons for the high mortality rates are associated with delousing procedures and reduced fish health due to uncontrollable outbreaks of fish viral disease (Veterinærinstituttet, 2023). The three dominating virus diseases in Norwegian aquaculture today are Cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI) and Pancreas disease (PD). In 2022 131 aquaculture facilities reported CMS, 147 facilities with HSMI and 98 facilities with PD (Veterinærinstituttet, 2023). To further expand the Norwegian aquaculture industry, fish health must be improved. A key measure to achieving this goal is improving the efficacy of viral vaccines (Veterinærinstituttet, 2023).

High-density populations, rapid growth and a life cycle that does not ensure natural pathogen exposure in early life or realistic training for the innate immune system make farmed salmon vulnerable to infections (Malik et al., 2021). Vaccination of Atlantic salmon was a turning point for the industry in the 1980this. Vaccines against bacterial infections effectively improve fish welfare and eliminate antibiotic use in Norwegian aquaculture. Today, all Atlantic salmon in the sea phase are vaccinated with vaccines with multi-antigen vaccines. Compared to bacterial vaccines, anti-viral vaccines have been shown to be less effectively (Adams, 2019; Veterinærinstituttet, 2023). A key difference between viruses and bacteria is that while most bacteria are extracellular and relatively easily targeted by antibodies produced by B cells, viruses are intracellular pathogens; they live and replicate inside the host cell where they can "hide" from neutralizing antibodies. Accordingly, T cell-mediated immune responses are key to combating viral infections. (Abbas et al., 2016). Most of today's commercial fish vaccines consist of inactivated pathogens or parts of the pathogen that provide protection against bacteria, but fail to induce protection against intracellular or complex pathogens (Adams, 2019). Effective ways to induce protection from intracellular antigens are needed and more knowledge about the teleost immune system is therefore demanded.

1.2 The immune system

The immune system is divided into two main parts: the innate and the adaptive immune system. Innate immunity is the first line of defence against pathogens; it includes mechanical barriers like skin or mucus but also a spectrum of specialised cells. These include neutrophils, monocytes/ macrophages, dendritic cells (DC), mast cells, innate lymphoid cells, natural killer cells (NK) and lymphocytes with limited diversity. In addition, the complement system and other plasma proteins play important roles in the innate immune response (Abbas et al., 2016). Microbes that enter the host can be recognised by structures that are shared by various classes of microbes so-called pathogens-associated molecular patterns (PAMPs). PAMPs or damage-associated molecular patterns (DAMPs), molecules that are produced by or released from damaged and dying cells, are recognised by conserved pattern recognition receptors (PRRs). A big family of PRRs are Toll-like receptors (TLRs) (Abbas et al., 2016). In humans, there are identified 13 different TLRs, while there in teleost, modern bony fish, many more have been

discovered (Sahoo, 2020). TLR3 and TLR7 are intracellular TLRs located in endosomes and are essential in viruses recognision. TLR-3 recognise double- stranded (ds) sdRNA, and TLR-7 recognises single-stranded (ss) RNA. By ligand binding to TLRs, transcription factors, including NFκB and interferon regulatory factors (IRFs) are activated. NFκB increases the gene expression of inflammatory cytokines and various cytokines, and IRF stimulates interferon gene expression. Antiviral cytokines stimulate gene expression of antiviral proteins and may also stimulate DC, T cells and B-cells (*Figure 1*).

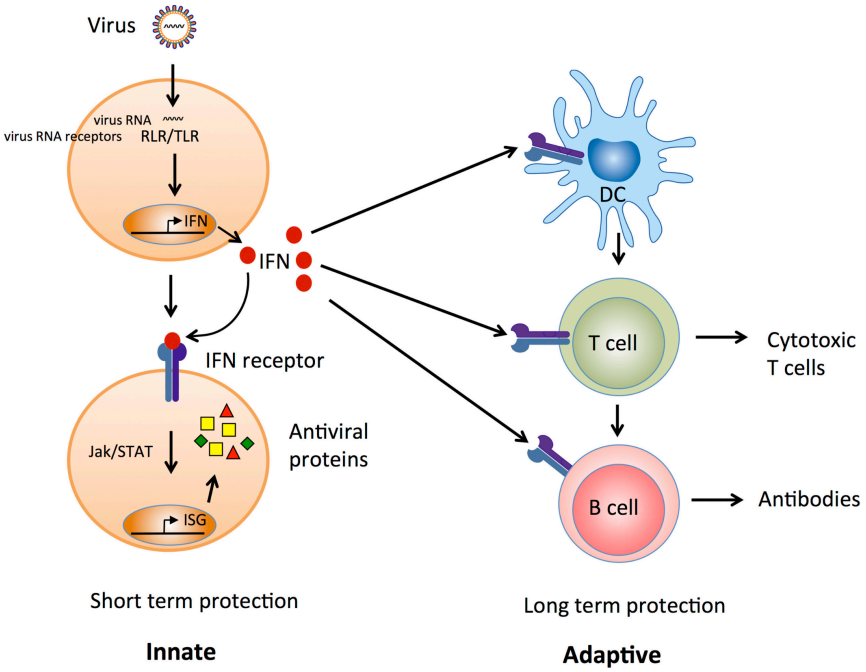


*Figure 1:**Schematic overview of type I IFN in innate and adaptive response.** The host cell recognises the virus through various TLRs . that induce IFNs gene transcription. IFN binds to IFN receptors, this binding initiates a signalling cascade involving janus kinases (JAKs) and* signal transducer and activator of transcription proteins (STATs), *that induce transcription of antiviral genes termed interferon stimulated genes (ISGs) that activate immune cells; DC, T-cells and B-cells in the adaptive immune system (Robertsen, 2018).*

While the innate immunoreceptors are coded in the organism's genome and stay the same throughout the life of the organism, the adaptive immune system is formed by exposure to different pathogens (Abbas et al., 2016). An organism has an adaptive immune system if these

immunity components are included: Immunoglobulins (Igs), T cell receptors (TCR) and major histocompatibility complex (MHC).

The adaptive immune system can be divided into humoral immunity and cell-mediated immunity. Humoral immunity includes B- cells, while cell-mediated immunity includes T-cells. B and T cells have somatically rearranged and clonally selected surface receptors, making the array of molecules they can recognise immense (Yamaguchi et al., 2019). Humoral immunity is mediated by antibodies that neutralise and eliminate extracellular microbes and microbial toxins. Activating of naïve B lymphocytes results in proliferation and differentiation into plasma cells that secrete antibodies that target and disarm the antigens (Abbas et al., 2016). Cell-meditated immunity is meditated by T-lymphocytes that work as a defence mechanism against microbes that are taken up by a cell by phagocytose or infection. The infected cells present molecules from the intracellular pathogen on a major histocompatibility complex (MHC) class I molecules that interacting with a cytotoxic T-cell with a CD8+ coreceptor. T-cells mostly respond to peptide antigens and sometimes to other small molecules. T-cells can't recognise the whole pathogen, unlike B-cells that can produce antibodies with the ability to bind to polysaccharides and lipids directly on pathogens.

1.3 Major Histocompatibility Complex (MHC) class I and class II

The antigen receptor for T- cells (TCR) can only recognise peptide antigens that are presented bound to a Major Histocompatibility Complex (MHC) molecule. MHCs are membrane-bound proteins with a characteristic antigen-binding clef that bind and present peptides. Classical MHC class I and II are highly polymorphic, and codominant expressed exist in two or more variants (Adams & Luoma, 2013). The classical MHC gene alleles are inherited from both parents and co-dominantly expressed. Class I MHC are presented on all nucleated cells, while class II are expressed only on dendritic cells, macrophages and B lymphocytes (Abbas et al., 2016). In the different class I and II peptide loading pathways, MHC class I present intracellularly derived peptides from microbes located in the cell cytosol. MHC class II present extracellularly derived peptides taken up by the cell from the surrounding environments. Thus, MHC allows the host to distinguish between and respond to extracellular and intracellular microbes differently(Abbas et al., 2016). The range of peptides that can activate T-cells is

immense because of the TCRs' high variability due to somatic rearrangement and the polymorphic nature of classical MHC genes (D'Souza, Adams et al. 2019).

MHC class I is constructed of a α chain forming three extracellular immunoglobulin-like domains followed by a transmembrane and cytoplasmic tail and is associated with a β2-microglobulin (Figure 2). (MHC class I can present 8 to 9 amino acids long peptides. CD8+ coreceptor of T- cytotoxic cells binds to the MHC αdomain while the peptide and the parts of α1 and α2 domain bind to the TCR Effector T-cytotoxic cells are activated by binding to an MHC class I presenting a "matching" peptide to the T- cell receptor (TCR). This binding activates the T cell and leads to the killing of the antigen-expression target cell by releasing cytokines that lead to apoptosis of the target cell, by perforin that makes holes in the cell membrane and the granzymes that enter the cell and kill it (Abbas et al., 2016).

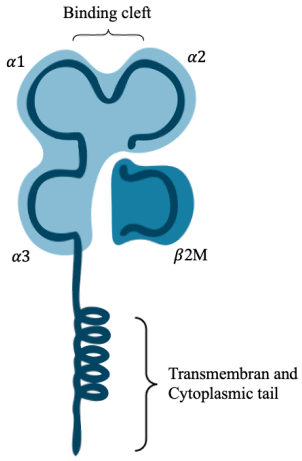


Figure 2: ***Illustrated MHC class I.*** *The MHC consist of a alpha chain made up of three, and one β2 microglobulin extracellular domain and the transmembrane and cytoplasmic tail. The binding cleft is associated with the α1 and α2 domains.*

Compared to Classical MHC class I, classical MHC Class II consists of two transmembrane chains (α and β) and presents longer peptides, between 10 to 30 amino acids. MHC class II α2 domains and β2 binds to the CD4-receptor on, T- helper cells while the TCR binds to the peptide in the binding clef and α1 domain and β2. Activation of T- helper cells lead, among other things to activation of macrophages and B- cells antibody secretion.

1.4 Regulation of classical MHC class I expression

The transcription of MHC class I and MHC class II is tightly regulated by various transcriptional factors (Van Den Elsen, 2011). During viral infection in mammals, type I interferons (IFN) and type II IFN rapidly induce MHC class I transcription (Jørgensen et al., 2006). Induced upregulation of classical MHC class I leads to more antigen presentation. This increases the chance of activation of T-cells and meditates an adaptive immune response. The peptides that are presented by in MHC class I molecules are derived from cytosolic proteins that have gone through the ongoing the ubiquitin-proteasome protein degradation pathway. Cells exposed to inflammatory cytokines, change the enzymatic composition of the proteasome change and resulting in a more efficient cleaving of cytosolic and nuclear proteins into peptides suited for presentation on MHC class I molecules.

IFN induces MHC class I gene expression through the JAK/ STAT pathway. JAK/ STAT signalling pathway is a chain of interactions of proteins which transduce extracellular signalling to intracellular gene transcription of molecules like classical and non-classical MHC. The pathway consists of the enzyme Janus kinase (JAK) that phosphorylates tyrosine proteins, signal transducer and activates the transcriptional proteins (STATs) and the belonging receptors for molecule signals. Type I and type II IFNs target the JAK/ STAT pathway (Seif et al., 2017).

Type I IFNs bind to a class I IFNs receptor on the cell surface. The type I IFNs receptor is composed of two subunits, IFNAR1 and IFNAR, which are associated with the Janus-activated kinases (Jaks) tyrosine kinase 2 (TYK2) and JAK1(Figure 3)(Platanias, 2005). Upon binding of IFN to the receptor, the kinases are activated and phosphorylate the signal transducer and activator of transcription proteins STAT1 and STAT2, which then dimerise, translocate into the nucleus and associate with IRF-9 to form the transcription factor complex ISGF3. The ISGF3 binds to interferon-stimulated response elements (ISRE) in the promoter of interferon-stimulated genes and activates transcription (Samuel, 2001).

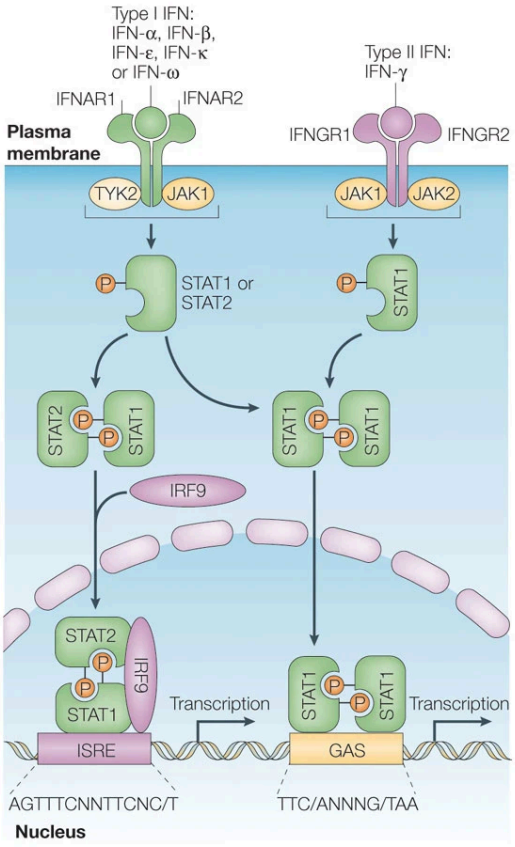


Figure 3 A schematic illustration of IFN type I and type II inducing transcription through human cells' JAK/ STAT signalling pathway. By binding to the IFN receptor, STAT is phosphorylated. IFN type II results in the formation of the ISGF3 complex consisting of STAT 1-STAT2-IRF9. This complex is translocated to the nucleus, binds IFN-stimulated response elements (ISRE), and induces gene transcription. Type II and type I IFN induce the formation of STAT 1-STAT 1 homodimer that translocates into the nucleus and binds to the GAS and induces transcription (Platanias, 2005).

IFNγ binds to receptors different from the IFN type I receptor and mediates signalling through a different but overlapping JAK/STAT pathway (Robertsen 2006). Type II IFN receptors bind IFN_{γ} and are composed of IFNGR1 and IFNGR2, associated with JAK1 and JAK2 (Platanias, 2005). IFN type II, induces activation by STAT1 homodimer complex, which binds to the *cis*-acting gamma activation site (GAS) (Decker et al., 1991). In Mammals, several types of type I Interferons (IFN), including, IFNα, IFNβ and IFNω, and one type II IFN, IFNγ, are identified (Pestka et al., 1987).

1.5 Non-classical MHC class I genes

"Non-classical MHC" (ncMHC) describes typically non-polymorphic MHC class I genes encoded within or outside the classical MHC gene region. In mammals, there are identified various ncMHC with the ability to present lipids, glycolipids, and other molecules in addition to peptides (D'Souza, Adams et al. 2019). Further, specific populations of T-cells recognise lipids and other nonpeptides presented by these nonpolymorphic MHC molecules (Adams & Luoma, 2013). Some ncMHC class I genes are highly conserved among diverse species suggesting that these ncMHC play a similar role in the immune system as pattern recognition receptors. They may present conserved antigens that are recognised by T-cells with limited diversity. The limited polymorphic and conserved antigen presentation of the ncMHC makes it attractive vaccine targets that could be recognised in genetically diverse populations.

18 nonclassical class I genes have been identified in the human genome (D'Souza, Adams et al. 2019). Among these, the CD1 gene family is one of the best-studied ncMHC. CD1 molecules (including CD1a, CD1b, CD1c and CD1d) are found expressed on specific cells (Grimholt, 2016). Human CD1a, CD1b, CD1c and CD1d molecules bind lipids and present them to distinct populations of T cells. CD1dproteins present lipids to specific T cells expressing a well-conserved semi-invariant T cell receptor (TCR) called NKT cells. A self-lipid antigen methyl lysophosphatidic acid is described, overexpressed in cells that stimulate CD1c self-reactive T cells. This CD1c-self-reactive T cell control leukaemia growth in vitro and in vivo. CD1e do not bind to T- cells but assist the lipid-processing and loading of other CD1 molecules (D'Souza, Adams et al. 2019). Group 1 CD1 molecules, CD1 a-c, are predominantly expressed on dendritic cells (DC) and thymocytes. Langerhans dendritic cells in the skin highly express the CD1c and CD1a isoforms. B-cells and hemopoietic cell types mainly express CD1d. Intestinal epithelial cells have high CD1d levels in mice and humans (Dougan et al., 2007). Since there is no evidence of a CD1 gene in fish, it is hypothesised that CD1 evolved from a duplication of the MHC class II locus in the primordial reptilian ancestor (Adams & Luoma, 2013).

The loading of antigen lipids to the CD1 binding groove occurs in endoplasmic reticulum (ER), endosomal, lysosomal, and is supported by lipids and lipids binding transfer proteins (Ogg et al., 2019).

1.6 Regulation of non-classical MHC class I genes in mammals

In endoplasmic reticulum (ER) newly synthesised CD1 molecules get loaded with self-lipids. Dendritic cells (DC) and B cells are suggested to have a highly regulated subcellular pathway for internalising exogenous lipids in the endosomal sub-compartment for loading onto CD1 proteins (Prigozy et al., 1997). There is established that various cytokines regulate CD1 expression. Treatment with GM-CSF and IL-4 leads to dendritic cell differentiation, resulting in increased group 1 CD1 expression. (Kasinrerk et al., 1993). For example, infection with *Mycobacterium leprae* shows induction of CD1a, CD1b and CD1c on DCs (Sieling et al., 1999). There is evidence that myeloid DCs can express all human CD1 isoforms selectively to certain activating stimuli (Porcelli et al., 1992). In a human patient with common variable immunoglobulin deficiency, CD1a, CD1b, and CD1c expression was downregulated after restoring immunoglobulin (Ig) to physiologic levels. This indicates that Ig is necessary and sufficient for control of CD1 expression on circulation monocytes (Smed-Sörensen et al., 2008). Virus infection has been shown to down-regulates cell surface expression of CD1d (Sanchez et al., 2005). Toll-like receptors have been found to modulate the functions of CD1-restricted T cells through different mechanisms, and the aspect of CD1 functions seems to be delayed and regulated in the innate immune system (Moody, 2006). Furthermore, transcription of the individual isoforms for the CD1 molecules in the locale inflammatory response differs from each other (Moody, 2006).

1.7 Immune responses in Fish

The innate immune system is evolutionarily older than the adaptive immune system and can be found in all multicellular organisms (Smith et al., 2019). On the other hand, the adaptive immune system is assumed to have arisen about 450 million years ago and is restricted to jawed vertebrates. Teleost (modern bony fish), like mammals, have all essential elements of adaptive immunity (i.e. highly variable B and T cell receptors and MHC molecules) and like mammals, express recombinational- activating genes (RAG1 and RAG2), which are essential in recombination of T and B- cell receptors (Flajnik, 2018).

A difference between teleosts and mammals are the primary and secondary immunological organs. Primary lymphoid organs are the site of lymphocyte development and education, while secondary lymphoid organs constitute lymphocyte activation and proliferation sites. The different immunological organs for fish are illustrated in Figure 4. Fish lack bone marrow which in mammals functions as a primary lymphoid organ and the site for B cell development. The

thymus is the primary immunological organ for bony fish, while the head kidney is designated as both a primary and a secondary lymphoid organ. Similar to the situation in mammals fish T cells develop in the thymus. The bone marrow equivalent in fish is the head kidney The Head kidney contains endocrine cells, hematopoietic tissue, and immune cells (Bjørgen & Koppang, 2022). In the kidney, there are present B-cells and B- cells precursors (Zwollo et al., 2008). Both mature and immature B cells, T-cells and detection of viruses and bacteria after systemic infection are found in the Head Kidney (HK), implying B-cell development. But the fact that there are detected other immune cells, viruses and bacteria implies that it is a vital immunological organ that it functions as both a secondary and primary immunological organ. CD4+ and CD8+ transcription are present, indicating that T helper cells and cytotoxic T cells are present, as well as identified αβ and ϒδ T cells. The secondary lymphoid organs for fish are the spleen and kidney and the mucosal-associated lymphoid tissues (MALT) (Press & Evensen, 1999).

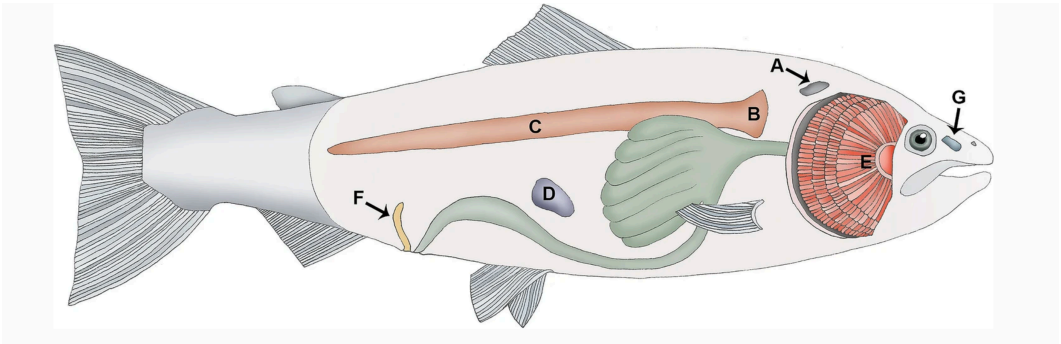


Figure 4: Schematic illustration of the essential lymphoid in fish. Organs and structure. A: Thymus, B Head kidney, C: kidney, D: Spleen, F: Bursa, G: nose-associated lymphoid tissue (Bjørgen & Koppang, 2022).

In the spleen, adaptive immune responses are generated (Flajnik, 2018). Since fish lacks lymph nodes, blood filtration in the spleen may be more important for fish than for mammals. Teleost B- cells do not proliferate in follicles or germinal centres like mammalian B-cells. There is speculated that melanoma- macrophages centres in the fish spleen and Head kidney have similar functions (Flajnik, 2018; Bjørgen & Koppang, 2022). So far, there is no established thymic medulla cortex in salmonid Thymus (Bjørgen & Koppang, 2022). The blood of rainbow trout contains numerous B cell populations where 40- 80% is resting mature B cells. No plasma cells

were found (Zwollo et al., 2008; Zwollo, 2018). How and where antigen presentation to and activation of T and B- cells are still unknown. Teleosts have fewer isotypes of Immunoglobulin Ig mu (IgM) (Zwollo, 2018).

Mammal B cells mature in the bone marrow and evolve in the follicles Marginal-B cells zone found within the lymph node and spleen. Mammallian T-cells progenitors migrate from the bone marrow to the thymus (Abbas et al., 2016). In the mammal thymus cortex αβ T cells undergo random rearrangement for T- cell receptors (TCR), followed by positive selection (Barraza et al., 2020). Finally, the selected CD8+ or CD4+ cells migrate to the medulla, where they are presented to self -antigen by MHC class I and class II molecules, respectively (Abbas et al., 2016).

1.8 Classical and non-classical MHC class I genes in fish

MHC genes are found in all groups of jawed vertebrates studied to date, while awless fish and invertebrates do not have MHC genes (Flajnik & Kasahara, 2010).

Most fish possess both MHC class I and MHC class II genes. However, some fish species including Atlantic Cod (*Gardius morhua*) have lost their MHC II molecules during evolution. (Yamaguchi & Dijkstra, 2019). Extensive classical MHC gene polymorphism is a common feature among jawed vertebrate species (Maccari et al., 2017; Yamaguchi & Dijkstra, 2019). Unlike mammals, where all classical MHC class genes are encoded within a large genomic region in, classical MHC class II genes in teleosts are not linked with the classical MHC class I gene (Yamaguchi & Dijkstra, 2019). Atlantic cod has many non-classical MHC class I genes that have been suggested to compensate for this species' lack of MHC class II (Grimholt et al., 2015).

The MHC class I genes in teleost are structurally similar to those in mammals. Like mammals, teleost fish possess at least one polymorphic classical MHC class I and several nonpolymorphic nonclassical MHC class I (Grimholt et al., 2015). Based on phylogenetic analyses and lineages' characteristic motifs, five different lineages, categorised as U, L, Z, P and S MHC class I, have been described in teleost (Grimholt et al., 2015). More recently, a sixth lineage, termed H lineage, was identified (Grimholt et al., 2019). Teleost Z and U -lineage genes are found in all

studied species, while the S, L, and P lineages were not found in all studied species. There are defined both classical MHC and ncMHC genes within the U lineage. The non-classical U-lineage in salmonids UCA, UDA, UEA, UFA, ULA, UGA, and UHA genes have mostly retained the conserved residues known to bind peptides, have limited polymorphism and a mainly a more restricted expression pattern. (Shum et al., 2001; Grimholt et al., 2015). In Atlantic salmon there are found six Z lineages that have dominating gene expression in different organs and probably binds peptides (Grimholt et al., 2015). S lineage molecules are suggested to have non-peptide or no ligands in their antigen-binding groove (Grimholt et al., 2015). The H-lineage have an unusually cytoplasmatic tail that is expected to have more important conserved function than the ectodomain, which may indicate a role in intracellular signalling (Grimholt et al., 2019).

1.9 Non-classical MHC class I L- Lineage genes

Among the different non-classical MHC class I lineages, the L-lineage poses the highest hydrophobicity of the different lineages (Dijkstra et al., 2007). A hypothesis is that L-lineage has similar functions to mammal CD1 (Grimholt et al., 2015), because of the CD1's ability to bind and present lipids. The L-lineage most likely binds hydrophobic ligands and can be traced way back before the third whole genomic duplicate events in Atlantic salmon (Grimholt et al., 2015). In Atlantic salmon, six functionally expressed L-lineage genes are identified: *Sasa-lia* (LIA), *Sasa-lda* (LDA), *Sasa-lca (*LCA), *Sasa-lga (*LGA), *Sasa-lha (*LHA) *and Sasa-lfa* (LFA) (Svenning et al., 2019*).*

L-lineage gene expression is relatively low in healthy fish but is elevated in response to viruses and bacterial infections within a short period (Svenning et al., 2019). In a study by Svenning (Svenning et al., 2019)) LIA, LDA, LCA, LGA and LHA were found to be expressed in primary and secondary lymphoid organs as well as the gill and gut-associated tissues. Overall, the relative expression of the L-lineage gene was lower compared to classical MHC class I (Svenning et al., 2019). LGA and LHA have the highest expression across all tissues, while LIA and LCA have significantly lower baseline expression. LGA and LIA expressions resembled that of UBA, the classical MHC class I gene, and was predominantly observed in the gills and gut. LDA was highest in the gill, kidney, and liver while LHA expression was observed at equivalen levels in most tissues. *Ica* expression pattern and transcripts levels were

higher in the spleen and head kidney compared to all other tissues examined. *Ifa* expression was low in all organs (Svenning et al., 2019).

Transcriptional factors control the expression of classical MHC genes. The classical MHC class I promotors consist a SXY sequence module and additional MHC I promotors elements, including interferon-stimulated response elements (ISRE) and GAS, which are recognised by interferon response factors IRF1 and STAT1/ STAT2 binding. Some MHC class I also has other motific that can be activated by NF-κB (Grimholt et al., 2020). The SXY sequence module binds to various transcriptional factors suggesting transcriptional enhanceosome controls (Grimholt et al., 2020). No SXY motifs were found in the promoter region of any of the MHC class I L-lineages genes. However, ISRE elements are found in LIA, LGA, LHA and LFA, but not in LDA and LCA. Lia contains two identical ISRE elements. In LHA and LFA, a GAS element is found in the promotor (Svenning et al., 2019). The identified IFN type I and type II promotor elements can imply that – L-lineage genes are induced in response to interferon. The L- lineage MHC for salmon may be induced through the JAK/ STAT signalling pathway, but a further examination of the signalling pathway is required. LIA had a transcriptional induction in response to stimulation with type I IFNa and infection the virus SAV3 but not the bacterial *P. salmonis*. LGA expression responded moderately to IFNa and SAV3 and was up-regulation by *P. salmonis infection*.

1.10 Regulation of classical MHC class I expression by Interferons

As in mammals, type I and type II IFNs induce classical MHC class I gene expression in teleosts (Robertsen, 2006; Grimholt et al., 2020). There is established that teleost fish possess an innate IFN system similar to mammals (Robertsen, 2008). Type I IFN and IFNγ have been cloned from several fish species (Robertsen, 2006). Secondary structure analyses suggest that fish type I IFNs have similar conformation as mammalian type I IFNs despite a relatively low per of cent sequence identity. (Robertsen et al., 2003; Robertsen, 2008). This is because IFNs in fish cannot be classified as IFNα and β because mammalians' IFNα and β genes have evolved after separations of the sarcopterygians (lobe-finned fish) from the actinopterygians (bony fish) (Lutfalla et al., 2003). Atlantic Salmon has multiple type I IFNs: IFNa, IFNb, IFNc and IFNd (Svingerud et al., 2012; Robertsen, 2018). By recognising viral nucleic acid type IFNs are increased by the host cell and protect other cells by inducing antiviral proteins like Mx proteins.

Salmons express IFNs in response to Poly (I:C) and other TLR ligands. IFNa and IFNc have the most robust relative transcriptional levels in Atlantic salmon in the Head kidney (Robertsen, 2008). A key interferon stimulated gene family are the Mx proteins, these are antiviral GTPases that play an important role in the immunity of vertebrates. (Verhelst et al., 2013; Haller et al., 2015; Robertsen et al., 2019). IFNs, dsRNA and virus infections typically induce Mx. Mx proteins are found in most vertebrates, localised in the cytoplasm and are highly conserved among species (Verhelst et al., 2013; Haller et al., 2015; Robertsen et al., 2019). Nine different Mx genes are found in fish compared to mammals with1-3 Mx genes (Verhelst et al., 2013; Solbakken, 2016; Robertsen et al., 2019). The different Mx genes have different expression properties in response to type I and type II IFNs. Atlantic salmon Mx1 is more strongly induced by IFNa than IFNγ, while Mx8 is more strongly induced by IFNγ compared to IFNa (Robertsen et al., 2019). In mammals, Mx is induced by IFN type I through the JAK/ STAT pathway. The IFN type I receptor binding results in phosphorylation and dimerisation of STAT 1 and STAT 2 proteins which interact with IRF9 to form transcriptional factors ISGF3 and activate transcription by binding to ISRE. IFNγ is signalling through another heterodimeric receptor resulting in the dimerization of STAT 1 (Stark et al., 1998; Robertsen et al., 2019).

In mammals IFNγ is produced by CD4+ T helper 1 (Th1) lymphocytes and CD8+ cytotoxic T lymphocytes in response to MHC-presented antigens and is more important later in the immune response. In addition, IFNγ is a crucial activator of macrophages for increasing the killing of bacteria (Robertsen 2006).

Salmonoids and carp experience a fourth whole genome duplication, adding gene complicity to the species (Lien et al., 2016; Chen et al., 2019; Grimholt et al., 2020). I Atlantic salmon many of the gene duplicates have evolved into functional copies. In a study done by (Grimholt et al., 2020), the transcription of immune genes in Atlantic salmon focused on the gene duplicates in the MHC pathway with IFNγ regulation was investigated. In the study four JAK1 genes and three JAK2 genes were found. ssJAK1.La was the highest expressed of the JAK1 genes. The master regulator in the classical MHC class pathways induced by IFNγ was found to be IRF1 and not the enhanceosome as seen in mammals (Grimholt et al., 2020).

2 Objective

The Norwegian aquaculture needs a more efficient virus vaccine to improve fish health and reduce morbidity and mortality. Therefore, in-depth knowledge about the Atlantic Salmon immune system is essential. An efficient virus vaccine needs to activate both the humoral and the cell-mediated immune system. Activation of cell-mediated immunity relies on MHC moleucles. Non-classical MHC, L- lineages genes are shown to have a well-conserved and potentially important role in antiviral response.

To increase the current understanding of the function of the various L- lineage genes and their, potential roles in the immune system, the signalling pathway that induces gene expression and what cell types that express the different L-lineage genes need to be examined.

Aims:

- Map the basal gene expression of ncMHC L and U lineage genes in primary HKL from adult Atlantic salmon.
- Establish which stimulations induce gene expression of the different L-linages genes.
- Examine if L- lineage gene expression is mediated through the JAK/STAT signalling pathway.
- Separate different HK cell populations and optimise magnetically activated cell sorting of B cells
- Investigate if L-lineagegenes are expressed in IgM+ B cells

3 Materials and methods

3.1 Experimental animals and tissue collection

Adult Atlantic salmon (*Salmo salar L.)* individuals 1-2 kg in size used in the study were obtained from the Tromsø Aquaculture Research Station in Kårvika, Norway. Fish were kept at natural temperature, around 8°C on a salinity at 33 pptm under natural light and feed with commercial dry feed (Skretting, Stavanger, Norway Spirit Throut 4,5mm). All fish were treated in accordance with relevant guidelines and regulations given by the Norwegian Animal Research Authority (Forskrift om bruk av dyr i forsøk, 2015). During the experiments, there was a concern of fish pox virus infection in the facility. During the time of the experiment, there was a confirmed outbreak of fish pox virus at the research station, but not in the same facility or in the sea face as the fish used in this experiment. Accordingly, fish used in these experiments were tested routinely for the presence of the virus, and only negative was used.

3.2 Tissue collection

Fish were humanely killed with a quick blow to the head and circulating blood was removed from the caudal vein using heparinzed vacutainer tubes 0,8x25 mm BD Vacutainer® PrecisionGlide™ needles and BD VacutainerÒ collecting tube and used for other purposes. Head kidneys (HKs) were aseptically collected within 10 minutes post sacrifice, transferred to 50 ml Falcons with 15-20 ml of ice-cold transport media (TM, Leibovitz`s L-15 medium, supplemented with 10U/ml penicillin, 10μl/ml streptomycin, 2% fetal bovine serum (FBS), 20 U/ml heparin), and stored on ice under transport and until processed further. Gills were collected in 1,5 ml microcentrifugation tubes with 500 μl RNA later (Thermo Fisher Scientific) and stored in case of unresolved disease suspicion.

3.3 Primary leucocytes isolation

Head kidney leucocytes (HKL) were obtained by generating a single-cell suspension, followed by separation on two to three replicate Percoll (GE Healthcare) gradients per sample. Percoll separate cells based on their density. By adding different consecrations of percoll in the same tube there are created gradients of the colloidal silica particles. Leukocytes will be collected in between 25% and 54% Percoll gradient while debriding cells and tissues will stay in the 25%

Percoll gradient. Erythrocytes will, with their high density, be collected as a pellet in the lower layer of the 54% Percoll. The tissues were homogenised by passing through a 100-µM cells strainer using a syringe plunger and collected in a 50ml Falcon tube containing a total volume of 20-30 ml transport media. The resulting cell suspensions were layered on 25/54% discontinuous Percoll gradients prepared earlier the same day in 50 ml Falcon centrifuge tubes. The gradients were prepared by adding 10 ml of 25% Percoll solution to each tube and layering 8 ml of 54% Percoll at the bottom, using a sterile glass pipette tip. 10 ml cell aliquots were carefully layered on top of the gradients and centrifuged at 400 x g for 40 minutes at 4°C. Leucocytes were collected from the interface of the Percoll gradients using a sterile plastic pipette in approximately 5 ml volume and pooled into a single 50 ml centrifuge tube, then topped with transport media to a total volume of 40 ml. The cells were centrifuged at 600 x g for 10 minutes, followed by the removal of the Transport media. The resulting pellets were washed by resuspending in 10 ml L-15 media and centrifuged for 600xg for 10 minutes. The washing step was repeated. Finally, the pellets were dissolved in culture media (L-15:41ml, 5ml P/S and FBS 5%), and the cells were counted using an automatic cell counter (Countess II by Thermo Fisher Scientific). The viability of living cells were always over 80% or the fish was discarded. Briefly, a 10 µl cell suspension was transferred to an Eppendorf tube, mixed 1:1 with Trypan blue stain (0.4%) and transferred to a cell counting chamber slide. The cell number and viability were recorded, and the concentration of leukocytes was adjusted for each experiment. The concentration of FBS in culture media was adjusted from 5% to 1% as required.

3.4 *In vitro* cell Stimulations and Reagents

Leucocyte isolation from the head kidney (HKL) were done according to the previously described method, counted and diluted. A total of 3 million cells in 1ml culture media with or without added stimuli were seeded in 24 well plates (Nunclon Delta Surface, Thermo Scientific). Cells were stimulated with either 400 units of recombinant Atlantic salmon IFNa1, 400 units of recombinant Atlantic salmon IFNc, 10ng/ml recombinant trout IFNγ, 10ug/ml Poly(I:C) (Invitrogen), 2 ug/ml R848 (Invitrogen, tlrl-r848); or combinations of the different stimuli's as follows (Robertsen et al., 2003); 400 unitsIFNa+ 10ng/ml IFNγ. 400 units IFNA+400 units IFNC, 10ng/ml Poly(I:C) + 400 units IFNA, 10ng/ml Poly(I:C)+ 400

units IFNC , 10ng/ml Poly(I:C) + 10ng/ml IFNγ, 10ng/ml Poly I: C+ 2 ug/ml R848, 2 ug/ml R848+ 400 units IFNA, 2 ug/ml R848+400 units IFNC, 2 ug/ml R848+10ng/ml IFNγ.

Recombinant interferon was available from previous work. Recombinant IFNa (rIFN-a) and IFNc (rIFN-c) were produced in HeK293-cells, previously described in (Svingerud et al., 2012). IFNγ was produced in E. coli. described in (Robertsen et al., 2019). Polyinosine polycytidylic (Poly(I:C)) mimic viral double RNA (dsRNA), while the imidazo quinoline R848 mimic single-stranded RNA (Svingerud et al., 2012; Robertsen, 2018). Poly I: C induces IFN type I in fish through TKLR22 and TLR3 (Matsuo et al., 2008). IFNb through MDA5 and TLR3, while R848 induce IFN-a production through TLR 7 pathway in mammals.

The cells were collected 16h post-stimulation and stored in RNeasy Lysis (RLT) buffer (Qiagen) supplemented with 20µl dithiothreitol DTT to 1ml RLT buffer at -20 C until further processing. The use of RLT buffer with (DTT) during freezing is a common method for preserving RNA intergrity. Freezing the cells in RLT buffe with DTT breaks the cell membrane and allows for lysis of the cells, while DTT helps protect the RNA from degradation by reducing disulfide bonds (Figure 5).

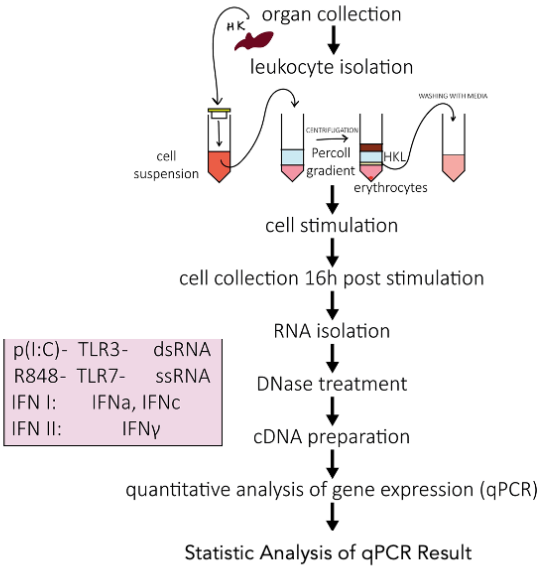


Figure 5: Schematically figure of collecting and in vitro stimulations of primary head kidney leucocytes from adult Atlantic salmon.

3.4.1 Inhibition of JAK/STAT pathway using JAK I inhibitor

The effect of JAK/STAT pharmacological inhibition on stimulation induced transcriptional induction in primary HKLs was performed using an ATP-competitive inhibitor of Janus protein tyrosine kinases (JAKs) with potent activity against TYK2 and JAK2 as well as JAK1 and JAK3. In mammalian systems, the IC50 of this inhibitor 1nM for inhibition of Tyk2 and Jak2, 5nM inhibiting JAK3 and 15nM for murine Jak1 and (Thompson et al., 2002). The inhibitor inhibits Jak3 with the concentration of 5nM, Tyk and Kal2 with 1nM and murine Jak1 with 15nM, 1nM JAK 2, 1 nM Tyk 2 and JAK2 in 1nM. 5nM inhibiting JAK3 in mammals (Thompson et al., 2002).

Leucocyte isolation from HK was done according to the previously described method (section 1.2), counted and diluted to a concentration of 4 x 10^6 cells/ml and seeded in 1ml Culture media supplemented with 5 % FBS, in 12 well plates (Nunclon Delta Surface, Thermp Scientific). The cells were stimulated with or without the presence of InSolutionTM JAK Inhibitor JAK I inhibitor (Calbiochem **COMPANY CAS 457081-03-7)** as follows: 400 Units of recombinant Atlantic salmon type I interferon-a (Sasa-IFNa1), 400 units of recombinant Atlantic salmon type I interferon c (Sasa-IFNC) (Svingerud et al., 2012), 10-100 ng/ml recombinant Rainbow trout IFNγ (Robertsen et al., 2019) , 10ug/ml poly(1:C), 2ug/ml R848 or left unstimulated (media alone). The concentration of the inhibitor ranged from 15 to 150 nM. Recombinant Atlantic salmon IFNa1 (GeneBank accession no. DQ354152.1) and IFNc (GenBANK accession no.JX524153), were produced by transfection of HEK-293 cells with IFN expression plasmids as described (Svingerud et al., 2013).

Primary leukocytes JAK/STAT inhibition trial was repeated three times according to the description below. Adjustments based on the results from the first trial were implemented in the second and the third trial.

Cells were collected at 12h and 24h in Eppendorf tubes and centrifuged at 600xg for 3 minutes at 12°C. The supernatant was removed and resulting pellet was resuspended in 350*ml* RLT buffer (120*m* DDT to 60ml RTL) and stored at -20 until RNA isolation.

Trial 1:

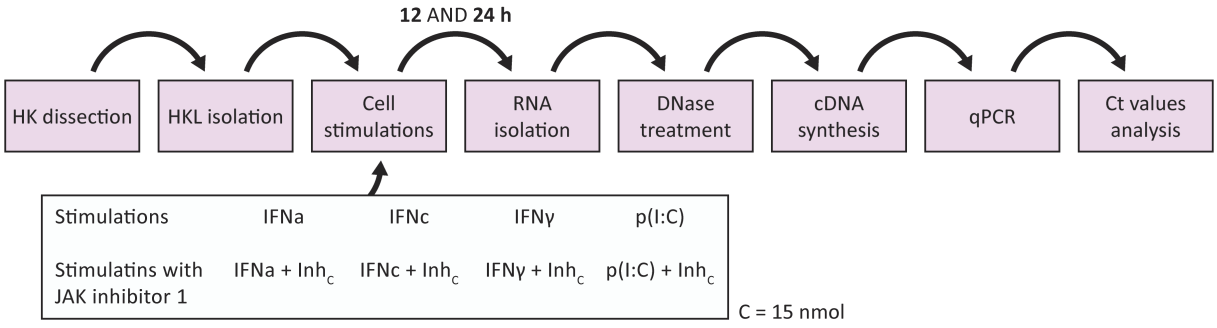


Figure 6: Schematically figure of the experiment trail 1: in vitro stimulation with inhibition of primary head kidney leucocytes from adult Atlantic salmon at two time points.

Primary HKL was isolated from collected tissue and incubated with IFNa, IFNc, IFNγ and Poly(I:C) with and without the JAK1 inhibitor (Calbiochem) in the concertation 15nM for two different time points: 12 and 24h. The stimulation was done accreting to described method with the exception of 100ng/ml IFNγ of RNA was collected and analysed using quantity qPCR (Figure 6).

Trial 2:

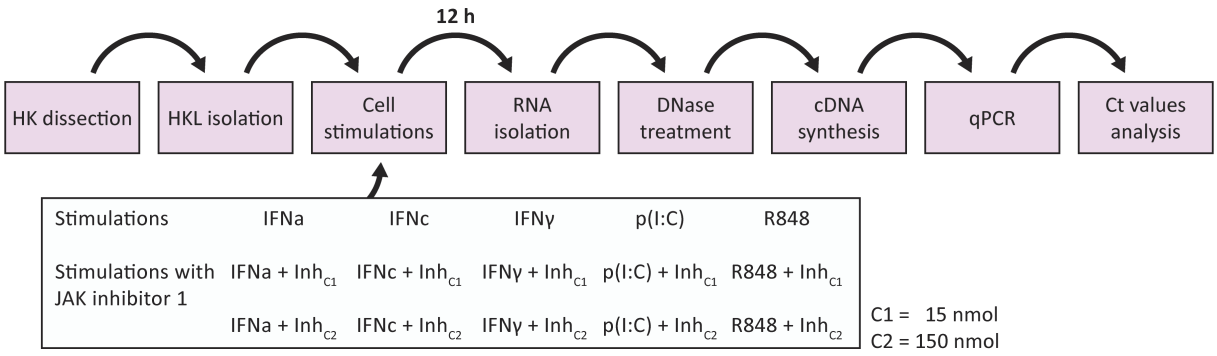


Figure 7: **Schematically figure of the experiment trail 2 and 3**: in vitro stimulation with inhibition at two consetrations of primary head kidney leucocytes from adult Atlantic salmon.

The leucocytes were also stimulated in triplicates and with two different concentrations of inhibitor: IFNa, IFNc, IFNγ, Poly (I:C) and R848 with and without 15nm JAK inhibitor and 150nm JAK inhibitor **I; CAS 457081-03-7** and RNA isolated at timepoint 12h. The

concentration of IFNγ was in trial 100ng/ml and adjusted to 10ng/ml in trial 2 and trial 3 (Figure 7).

Trial 3

Trail 3 was done exactly as trail 2.

3.5 Cell separation based on cell surface protein's ability to adhere

Cell separations were repeated three times according to the description below. Adjustments based on the results obtained in the first trial were implemented in the second and third trial. When incubating leucocytes in plastic bottles over time, some cells will attach to plastic, while others will not. This makes it possible to divide cells into two groups based on the ability of the cells to adhere to the plastic. Accordingly, to separate suspension cells from adherent cells HK from eight fish was harvested and primary head-kidney leukocytes were isolated according to the previously described method (3.3). HKLs from 4 fish, were incubated at 20°C, for 48 or 72h in culture media (1% P/S, 5% FBS) in 50 or 200ml cell culture flasks. After 72h the cells in suspension were transferred to a 50 ml falcon, while the adherent cells were gently washed with Phosphate Buffered Saline (PBS), which washes the FBS away and removes residual non-adherent cells as well as floating dead cells. 1ml Trypsin- Ethylene diamine-Tetra-acetic (EDTA) solution (Sigma-AldrichÒ) was added to the flask with adherent cells. Trypsin is an enzyme that inhibits surface proteins on the cells that are adherent, which allows them to detach from the plastic. When the cell loosened from the flask walls, 10ml culture media (0,5 Heparin, 5% FBS) were added to inhibit the trypsin and adherent cell suspension was immediately transferred to a falcon tube. The cells were counted and diluted in culture media (1% or 5% FBS). The two populations, suspension and adherent cells were analysed using flow cytometry analysis to assess the degree of separation as described below.

3.6 Flow cytometry

Flow cytometry is a method where cells in a heterogeneous mix are analysed based on specific light scattering and fluorescent characteristics. It is based on the passage of a single cell

suspension through a laser where forward-scatter, side- scatter and fluorescent data are collected. The forward-scatter (FSC) indicates the size of the cell, while side- scatter (SSC) indicates the complexity of the cell based on the reflection of the laser. The cells can also be stained with specific antibodies targeting cell surface proteins. These antibodies can be indirectly or directly conjugated to fluorescent molecules that will be detected by the laser. In these experiments, an primary antibody anti-trout IgM monoclonal antibody specific for Atlantic salmon IgM IgM mAb (IgF1-18(6-1-18); 1:200) (Hedfors et al., 2012) and with isotype-specific secondary antibody (IgG-RPE; 1:400 dilution; Jackson ImmunoResearch). In contrast to polyclonal antibodies that are produced by multiple immune cells, monoclonal antibodies are generated by identical immune cells and recognize only a single epitope of an antigen which makes it extremely specific. The IgF1-3 anti-trout are shown to bind to both IgM-A and -B isotypes of Atlantic salmon B cells. Attach to the secondary antibody there are detected whit fluorescent molecules that absorb light that will be detected by the flow cytometry (Figure 8). Cell- count per sample was 10 000.

Cell staining for Flow cytometry

Total HKL cell populations as well as adherent and suspension cells from the same cells (separated as described above) were stained with and /or anti-IgM and Fixable Viability dye (FVD) to identify the proportion of living and dead cells and B-cells.

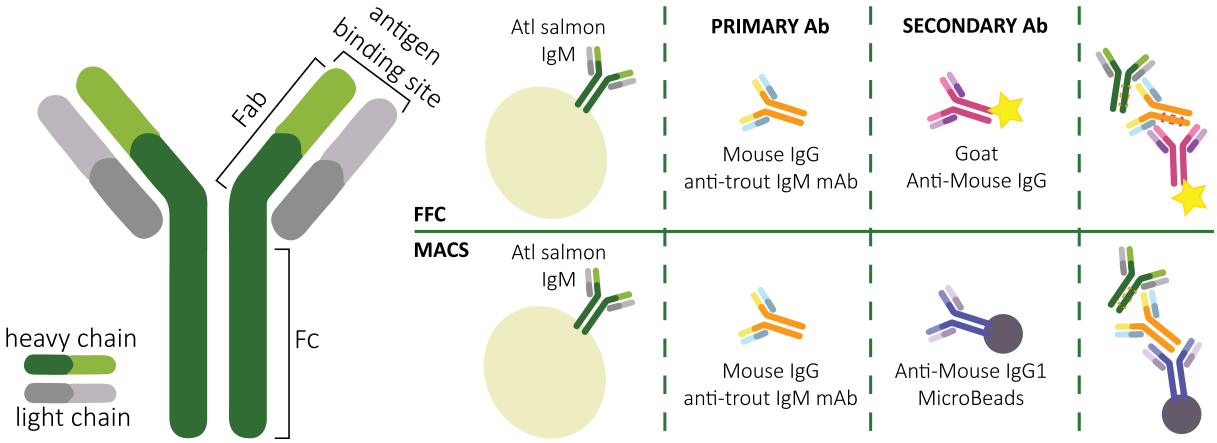


Figure 8: **Illustration of the stainig of primary and secondary antibody for flow cytometry and MACS. (**Illustrated Agata Teresa Wyrozomeka).

Staining with anti-IgM: Suspension with 600 000 HKs per tube was transferred to 2ml Eppendorf tubes and spun at 500 x g for 5 min at 4° C. Supernatant where discarded and the pellet was washed with 200 µl staining buffer (PBS with 1% Bovine serum almumin (BSA)). BSA is included to bind nonspecific binding sites, to increase the chance for antibodies to bind only to the antigens of interest.

The cells were dispersed and 100ul Anti-IgM (IgF1-18(6-1-18); 1:200) was added and incubated for 20 minutes at 4° C protected from light. Following incubation 100µl staining buffer was added and the cells were spun down at 500 x g 4° C for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 200 µl staining buffer. 100 µl anti-trout, secondary antibody, Goat Anti- Mouse IgM (IgG-RPE; 1:400 dilution) was added and incubated for 20 minutes at 4° C protected from light. 100µl staining buffer was added and the cells were spun down as described above and followed by washing described above. After the second wash, the cells were resuspended in 100µl staining buffer. The Goat Anti- Mouse IgM was labelled with fluorochrome be detected in the flow cytometry (Figure 8).

Staining with Live-or-Dye Fixable Viability: The staining was done according to the protocol Thermo Fischer Scientific: eBioscience™ Fixable viability Dye eFluor™ 780, Protocol A: Standard staining in tubes The cells were washed 2 times in azide- free and protein- free PBS. The cells were resuspended in 100ul aside- free and serum/protein-free PBS. 1ul Fixable Viability Dye (Thermo Fisher Scientific) (FVD) per mL of cells was added. The samples were vortexed and incubated for 30 minutes at 4° C, protected from light. Live-or-Dye Fixable viability stain dead cells that have compromised mambran integrity and allow intracellular fluorescenc signaling. With flow cytometry dead cells can be excluded from the analysis. The stained cells were covered in aluminium foil and left on ice until run through FACS after calibration of the experiment.

3.7 Magnetic activating cell sorting (MACS) of IgM+ B cells

To distinquish the IgM+ positive B-cells from the suspension the method of Magnetic activating cell sorting (MACS) was used. The target protein of the cells was IgM, and was targeted by anti-IgM antibody (aIgM-mAb) that further gets targeted with secondary antibody specific for the anti-throuth IgM coupled with microbeads, Anti- Mouse IgG1 Microbeats (Milteny biotec). Microbeads are small magnetic spheres coated with a secondary antibody that will attach to a magnet column. The sorting was performed according to the company`s recommendation (Miltenyi Biotec). The incubation with anti-trout IgM antibody followed by incubating with anti-Mouse IgG1 coated microbeads and small magnets will make the B-cells attach to a magnetic column(Figure 8) .

The washes and incubations were done using MACS sorting buffer (SB; PBS with 0.5% bovine serum albumin and 2mM EDTA) and centrifuged at 450xg at 4°C for 5 minutes. After one wash with SB, 4mln for the first MACS and 3 mln for trail 2 and 3. Cells were incubated with a 1:200 dilution of mouse anti-trout IgM mAb at 4°C for 30 minutes. After the second wash the cells were incubated in 100µl SB and 25 µl anti- Mouse IgG1 microbeads (Miltenyi Biotec) for 15 minutes at 4°C. After activation of the magnetic column, cells were added, and the flow through was collected. The columns were then removed from the magnetic separator to a new collection tube and flushed with 1ml SB. Sorted IgM+ and IgM-cells were centrifuged, and the pellets were immediately resuspended in 300ul RLT + βmerc and frozen for RNA isolation. A fraction of the IgM- cells were resuspended in 50µl PBS for flow cytometry, for the MACS sorting quality check on FACS (Figure 9).

1 million HKL per sample, were stained with and /or anti-torut-IgM-mAb and FVD, described in section Fluorescented- activated cell sorting and run through Flow Cytomerty. RNA was isolated from both the sorted IgM+ and the IGM- populations according to the previously described protocol and checked for quality and quantity on NanoDrop.

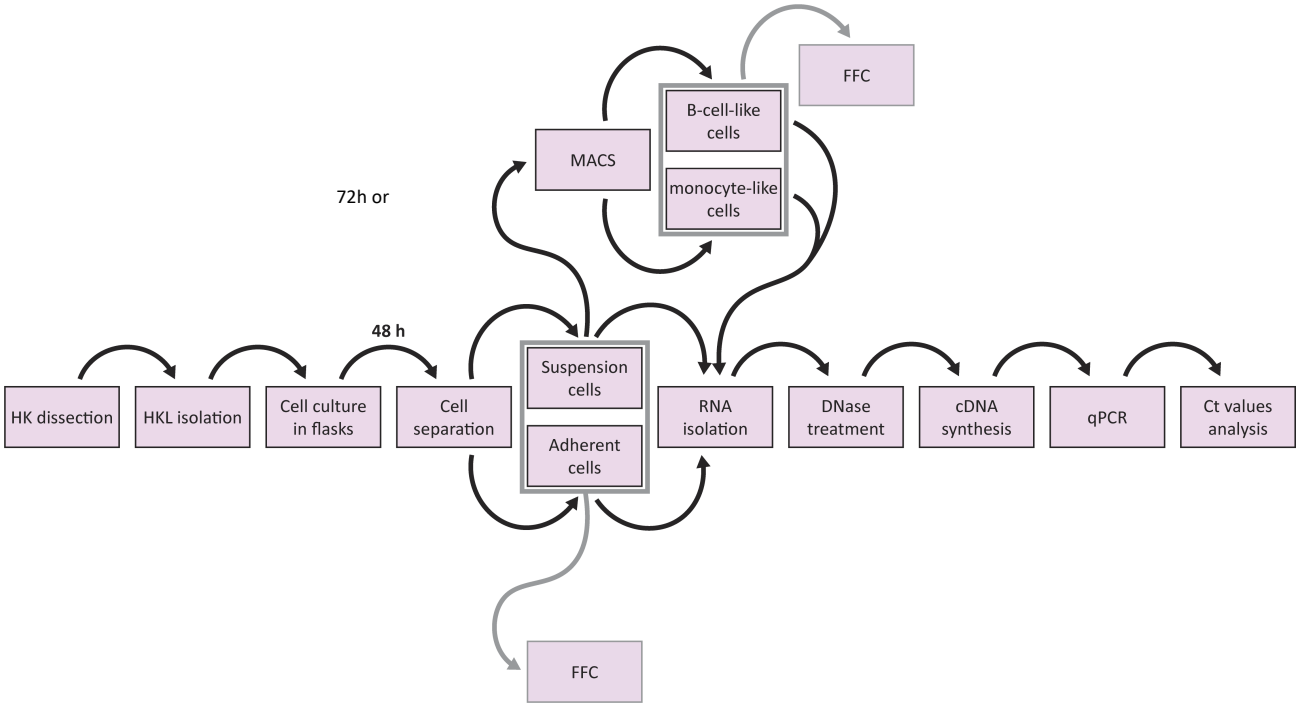


Figure 9: ***Schematically figure of the MACS- experiment****: Collection, in vitro incubation, MACS and Flow cytometry- analyses (FFC).*

3.8 RNA Isolation

RNeasy Quick start protocol:

Total RNA from total HKL populations , HKL -adherent and HKL- suspension populations and magnetic activated cell sorted populations were isolated using the RNA mini Kit (Qiagen) according to the manufacturing protocol. The cells were frozen with RLT buffer supplemented with 20µl 2M DTT per ml RLT buffer and stored in -20°C until further processing. The use of RLT buffer with DTT during freezing is a common method for preserving RNA integrity. Freezing the cells in RLT buffer with DTT breaks the cell membrane and allows for the lysis of the cells, while DTT helps protect the RNA from degradation by reducing disulfide bonds. 1 volume of ethanol was added which binds to the RNA causing it to form a visible pellet, which can then be collected and further processed for downstream applications. 700ml of the sample was added to a RNeasy Mini spin column placed in a 2 ml collecting tube and centrifuged for 15s at 1000 rpm. Briefly, the cell suspension was transferred to a selective silica membrane, that specifically binds to RNA that is longer than the 200 base-pairs. Waste and RNA with shorter base pairs get collected in a

collecting tube after being centrifuged and discarded. 700 ml RW1 buffer (Qiagen) was added to the RNeast spin column and centrifuged for 15s at 1000xg for washing. After centrifugation 500ml of RPE (Qiagen) was added to the spin column, followed by one 15s 1000xg centrifuging. This step was repeated with and a 2-minute xg centrifuging. 40µl RNase-free water was added to the column and centrifuged for 1 minute. The RNase- free water break the binding between the silica membrane and the RNA in the RNeast spin column. The RNA was collected in a new 1,5 ml collecting tube. The RNA was directly put on ice. 2µl from the samples were quantified by NanoDrop (ND 1000 Spectrophotometer) and then stored at -80°Cuntil further processed. The Nanodrop instrument measures the absorption at the wavelength at 260 and 280 nm to decide the RNA concertation (ng/µl) and the purity of the RNA.

3.9 Preparation of cDNA

DNase treatment

The aim of the DNase treatment is to remove any residual genomic DNA (gDNA) from the RNA sample. A total of 1 ug of isolated RNA (calculated using the formula below) was used.

$$\frac{RNA\ \ concentration\ per\ sample}{1ug} = Volume\ of\ RNA\ needed\ \ for\ 1ug\ in\ 10ul$$

The formula below allows to calculate needed volume of a sample to obtain 1 µg RNA in 10 µL reaction.

$$\frac{RNA\ sample\ conc.}{1\ \mu g} = sample\ vol.^{*}$$

To prepare the RNA samples for reverse transcription, 1 µg of RNA was added to 0,2 ml thin-walled 8 tubes (Thermo Fischer Science) and the volume was adjusted with RNA- free water to 8,5 μl, then a master-mix was prepared by mixing1µl of 10x DNase Buffer and 0,5 µl DNase per sample. 1,5 µL of the master mix was distributed to all samples. The samples were incubated for 30 minutes at 37°C. Following incubation 1µl EDTA, which inhibits the DNase activity and prevents further RNA degradation, was added per sample and incubated

for 10 minutes at 65°C. Samples were immediately used for cDNA synthesis as described below

cDNA synthesis

A total of 20 µL of cDNA sample was prepared, consisting of 9.6 µL of DNase treated RNA (500ng) template and 10.4 µL of master mix. The master mix contained 2 µL of 10x RT buffer, 1.4 µL of MgCl2, 4 µL of 19 mM dNTP, 1 µL of random hexamers, 1 µL of RNase inhibitor, and 1 µL of M-MLV reverse transcriptase (Megascript RT) for each reaction. The Megascript enzymes were added to the mix last and the master mix was kept on ice until use. To ensure the quality of the cDNA synthesis, 3 negative control samples, consisting of 2 samples with RNA and added master mix without Megascript RT, and one sample with all reagents but with no RNA template were included for each 20 test samples. No cDNA synthesis or amplification was expected in the control samples without the Megascript RT enzymes or without RNA.

The samples were incubated under the following conditions: 25°C for 10 minutes, 37°C for 30 minutes, and 95°C for 5 minutes. The incubation was done in a Thermal Cycler (thermos fisher) The cDNA was diluted 1:1,5 before being stored at -20°C until use.

3.10 qPCR

Quantitative PCR (qPCR) was run as 10 µl duplicate reactions on a 7500 Fast Real-Time PCR System according to standard protocol. 2,5 ml Primer mix containing 10uM of forward and reverse primer (2ul forward primer, 2ul reverse primer and 1170ml RNA-free water) and 5ml SYBR GREEN were mixed and added to the wells with a 2,5ml cDNA template. The samples were added in wells in MicroAmpâ Fast Optical 96-Well Reaction Plate. The tray was spun down and placed in the qPCR machine on slow run. To makes sure that there is no contamination or primer dimer in the samples, a melting curve was run to check if the primers worked and if there were any primer dimers. The first tray is a quantification polymerase chain reaction of cDNA samples with primer detection of the “housekeeping gene” elongation factor 1 (EF1) to establish the baseline of the expression. The samples were tested against several genes (Table 1).

Primer list

*Table 1: **Primerlist**. Primer gene, sequence, Genebank nr and melting temperature(TM) in degrees Celsius.*

Gene	Sequence	Genebank nr:	Tm C
EF1a Fw	5′-CCCTCCAGGACGTTTACAAA		66,9
EF1a Rw	5`-CACACGGCCCACAGGTACA		68,3
Mx1/2 Fw	5`-GATGCTGCACCTCAAGTCCTATTA	SY150127131-012	65,9
Mx1/2 Rw	5′-GGATTGGTCAGGATGCCTAAT		64,8
LGA Fw	5`-CACAAAAACCAAGGACGATGAA	SY180315537-011	65,8
LGA Rw	5`-CGGTGCTTTAGTTCAAATGATCTG		65,5
LCA Fw	5`-ATCTCACAGCCAGCCATTC	SY-180315537-013	56,8
LCA Rw	5`-ATCTCACAGCCAGCCATTC		62,9
LHA Fw	5′-CAGCCCTGATCATGTCAAAGA	SY180415611-093	65,2
LHA Rw	5′-AAGATATTCTCTCCTCATCGCA		61,7
LDA Fw	5′-GGCAAGCAACTGAAGAACAC	SY180315537-009	63,7
LDA Rw	5`-TCTCCGCATCACAAAGTTCTC		64,3
LIA-F1	5`-CACCTTTCCCTGAGTGTAGTG		61,2
LIA-R1	5`-CGTAAGGTCCCTTGTGGATAAA		63,7
IFNA1 Fw	5′-CCTTTCCCTGCTGGACCA	SY210315685-091	66,4
IFNA1 Rw	5′-TGTCTGTAAAGGATGTTGGGAAAA		68,0
UBA F1	5`-GACAGTGACACAGCTCAGAAT	SY180315537-025	59,5
UBA R1	5`-CATCAGAGTGCTCTTCCCATAG		62,7
MHCII Fw	5`-GTGGAGCACATCAGCCTCACT		67,4
MHCII Rw	5`-GACGCACCGATGGCTATCTTA		64,9
csfr-1F Fw	5`-CACCAGTAACCCTAACCACTTC	SY180723611-083	62,8
csfr-1R Rw	5`-GACCTGCTTGTCCTGCATTA		62,8
TCRalphaC Fw	AACAATACAGAGGCCACCAC	SY190802154-079	61,6

TCRalphaC Rw	5`-ACCAGTTTGCTTCACATTCTC		62,0
Makro Fw makro Rw	5`-AGGACCTGCTGGTGTTAATG CTGCTCTTTCACCCTTCTCTC	 SY189723611-085	61,6 62,1
SsaMx8a Fw SsaMx8a Rw	5`- GGGGAAACAGCAGCAGGCTCAGAT 5`- GGTCAGCCAGTCTTTGCACCACAAT		74,0 72,3
mIgM	5`-TGAGGAGAACTGTGGGCTACACT	8816715590-30/0	
IRF1 Fw IRF1 Rw	5`-AGGCTAATTTCCGCTGTGTGCA 5`-TTTTGTAGACGCACTGCT	8819781531-30/0	66,8 65,9
IRF9 Fw IRF9 Rw	5`TCTGAAAGCAGTGGGTCAGGATGT 5`ACGTTCTCAGTCCAGAGTGT	NM001173719 NM001173719	

3.11 Statistical analyses

To calculated change in gene expression, the Ct result from the qPCR was used. The Ct value of the sample were normaliced against the reference gene EF1a resulting in Δ All the qPCR samples were run and analysed in duplicates.

ΔCt= Ctsample- Ct reference gene

To account for technical discrepancies, the standard error between the two duplicates was calculated and outlier samples were removed.

$$Relative\ expression: 2^{-\Delta Ct} = 2^{-(Ct\ gene - Ct\ referanse\ gene)}$$

$$Fold\ change: \frac{Relative\ expression\ sample}{Relative\ expression\ media}$$

Fold change was used to compare the gene expression in the stimulated sample to the basal gene expression of the sample.

Gene expression can also be presented as a relative expression calculated using the ΔΔCt method . ΔΔCt expresses (Livak & Schmittgen, 2001). The relative expression compared to the lowest gene expression by the same stimulation.

ΔΔCt=ΔCt sample- ΔCt Reference gene

Relative expression= $2^{\Delta\Delta Ct}$

Both the Fold change and the relative expression was presented after a log transformation in a box plot. Statical significant differences between the treatments were calculated using ordinary one-way ANOVA or t- test after testing against normal distribution. Relative quantitative gene expression analyses were analysed using GraphPad Prism (9th edition). The level of significant was set to $p<0,5$. Outliner: The fold change values and relative expression where tested with Gubbes test to quantify outliner.

4 Result

4.1 In vitro stimulations of primary head kidney leucocytes from adult Atlantic salmon differentially modulates non-classical MHC class I L-lineage gene expression

To further understand which activation signals and signal transduction pathways promote the expression of distinct L-lineage genes a comprehensive transcriptional analysis, mapping the induction of various L-lineage genes in response to different external stimuli, was undertaken. Previous study on L-lineage gene expression showed a stronger induction by virus infection vs. bacterial infection. (Svenning et al., 2019). Virus associated stimuli's was therefore chosen in this experiments. First, basal expression of the non-classical MHC class I L-lineage genes, LDA, LGA, LHA and LIA in primary head kidney leucocytes (HKLs) isolated from adult Atlantic salmon was examined and contrasted with the basal expression of classical MHC class I (UBA) and representative non-classical MHC class I U lineage genes (ULA, UGA and UHA). The relative expression was calculated using the ΔΔCt method against the lowest ΔCt value i.e., all values were compared to the lowest expressed gene, which in this case was LIA. Of note, for basal expression analysis all amplifications, except that for LIA were based on a 1:1,5 dilution of cDNA. Due to the extremely low basal expression of LIA in unstimulated samples LIA amplification was based on undiluted cDNA.

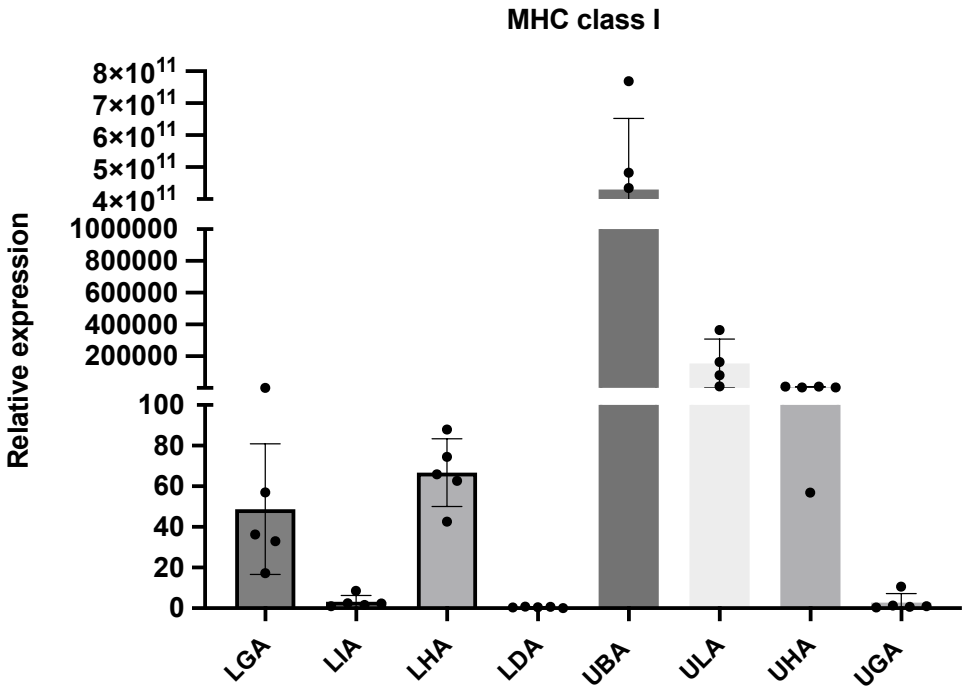


Figure 10: ***Expression of the MHC class I gene in non-stimulated primary HKLs.*** *Relative gene expression of LGA, LHA, LIA, LDA, ULA, UHA and UGA and the classical MHC UBA from adult Atlantic salmon incubated in vitro for 16h culture media (L-15 with 5% FBS and 2% heparin). Relative expression was calculated by first normalising the expression against the reference gene EF1a and then calculating the relative expression relative to the lowest expressed gene (LIA) (Equation 4). Each dot represents an individual fish. Significance was testes using normal one way ANOVA.*

Seen in Figure 10, The basal expression, as expected, of non-classical MHC class I L-lineage genes were markedly lower than for UBA -the classical MHC class I gene. Further, L-lineage gene expression was significantly lower compared to the non-classical U-lineage genes ULA and UHA while the UGA gene has a much low basal expression comparable to that of the L-lineage in HK. There was not a significantly different between the L-lineage genes. However, LIA and LDA expression were lower than that of LGA and LHA. The relative expression was calculated using the ΔΔCt method against the lowest ΔCt value of UGA of the U lineage and the Lowest ΔCt value of LIA.

The basal expression levels of additional genes, subsequently used as control genes to verify successful stimulation and as a mean to evaluate the potential for indirect induction of L-lineage gene expression, were analysed. These included two interferon inducible Mx proteins, (Mx1/2 and Mx8), group 1 type I interferons (IFNa and IFNc) and type II IFNγ as well as interferon

regulatory factor I (IRF1) and interferon regulatory factor 9 (IRF9) which are known to be involved in the IFN signalling pathways.

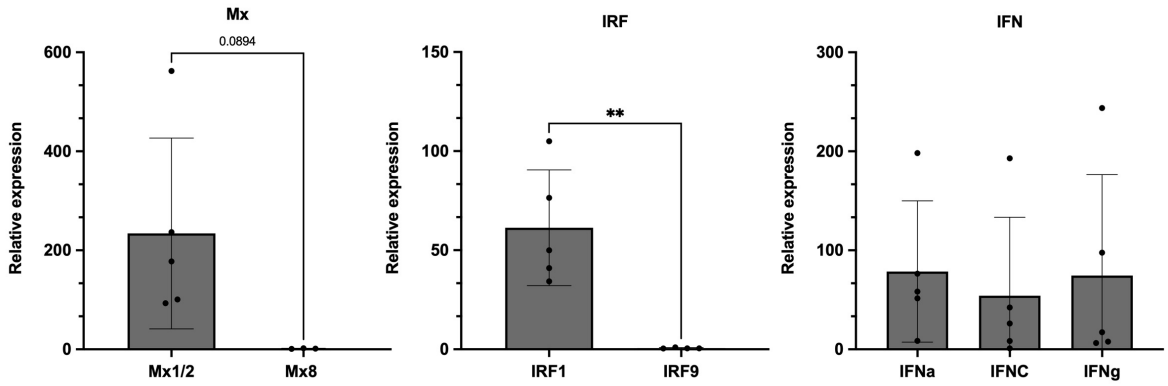


Figure 11: ***Expression of Mx, IRF and IFNs in non-stimulated primary HKL.*** *Relative expression of Mx1/2 and Mx8, IRF1 and IRF9 and the IFNs; IFNa, IFNc and IFNg (=IFNγ) from adult Atlantic salmon incubated in vitro for 16h culture media (L-15 with 5% FBS and 2% heparin). Relative expression was calculated by first normalising the expression against the reference gene EF1a and then calculating the relative expression relative to the lowest expressed gene (Mx8/IFNc/IRF9) (Equation 4). Each dot represents an individual fish. Significance was testes using t-test. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01.*

For Mx proteins the basal expression of Mx1/2 was scattered between the different individuals and overall higher compared to Mx8 showed in Figure 11. There is no significant difference of basal expression of IFNa, IFNc and IFNγ. There was a significantly higher basal expression of IRF1 compared to IRF9.

Next, HKLs from 6 adult Atlantic salmon were isolated and stimulated in vitro for 16h with recombinant (r) IFNa (400Units), rIFNc (400Units), rIFNγ (10ng/ml), Poly(I:C) (10 µg/ml) or R848 (2µg/ml). Gene expressions were analysed by qPCRs using primers for LGA, LHA, LIA, LDA, UBA, IFNa, IFNc, IFNγ, IRF1, IRF9, Mx1/2, Mx8 ULA, UHA and UGA. Fold changes were calculated against the average of the relative expression in cells cultured for the same amount of time in media alone (Equation 3), outlier data points was determined and removed Equation 5.

Gene expression of non-classical MHC L-lineage genes was examined to compare the relative induction response to the IFNs: IFNa, IFNc, IFNγ, Poly (I:C) (a synthetic analog of double-(dsRNA)) and R848 (a synthetic analog of ssRNA) in an attempt to understand the signalling pathways governing L-lineage gene expressions.

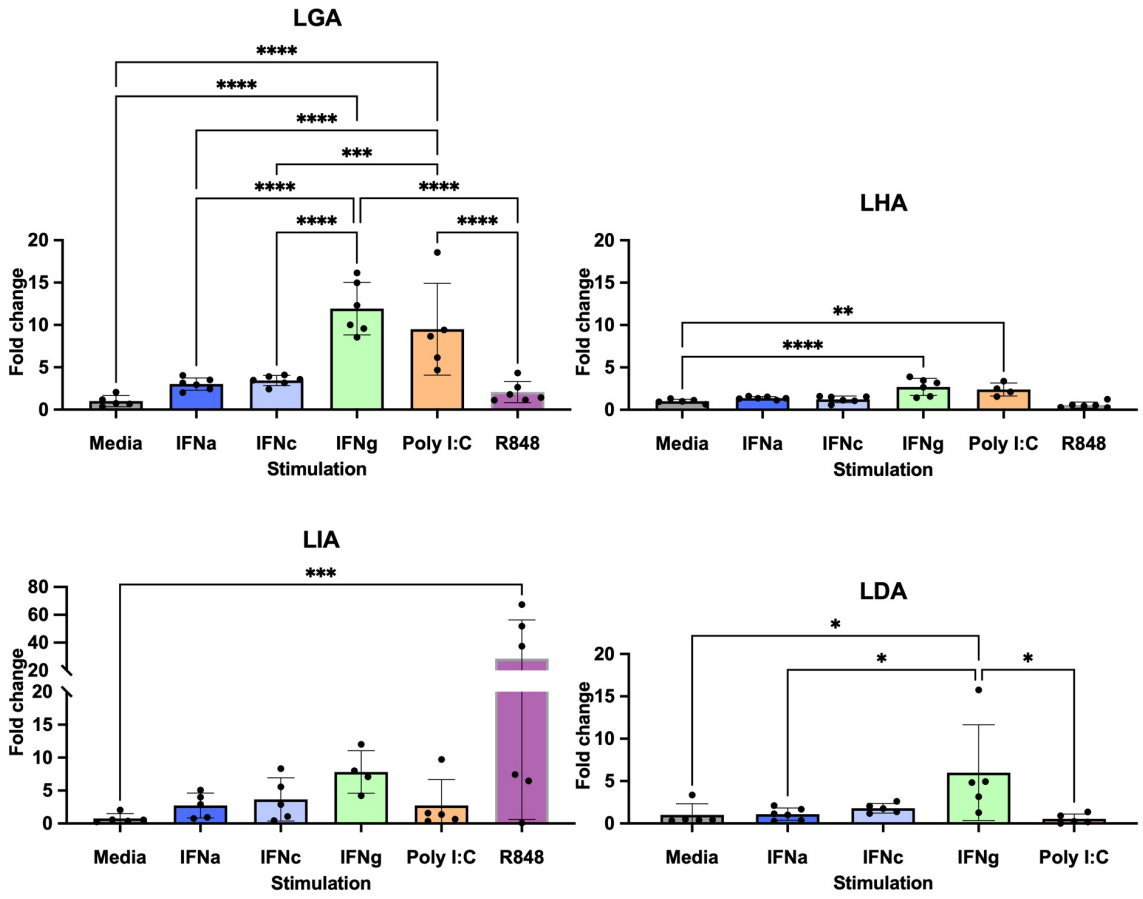


Figure 12: ***Fold change of gene expression of MHC class I, L-Lineage: LHA, LGA, LHA and LDA after in vitro stimulation of primary HKL.*** *The HKLs from adult Atlantic Salmon where stimulated in vitro for 16h with IFNa (blue), IFNc (light blue), IFNg(=IFNγ) (Green), Poly(I:C)(orange) or R848 (purple) in culture media (L-15 with 5% FBS and 2% heparin). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was testes using normal one way ANOVA.. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01, ***p<0,001, ****p< 0,0001.*

LIA gene expression increases with 7-fold change when stimulated with IFNγ compared with a fold change of 5 with IFNc and 3 with IFNa and Poly(I:C) (Figure 12). The gene expression of LIA in response to R848 was significantly higher and varied from individual to individual. Compared to LIA, LGA had a more remarkable fold change in response to stimulation of IFNγ and Poly (I:C) compared to type I IFNs and R848. LGA fold change in stimulation with IFNγ had a fold change 65% higher than the fold change following stimulation with IFNc or IFNa. LHA gene expressions patterns were similarly to LGA, just lower. LDA had no significant induction with any of the stimulants except IFNγ that was significantly higher than media, IFNa

and Poly (I:C). LHA fold changes were significantly induced by both Poly(I:C) and IFNγ compared to basal expression.

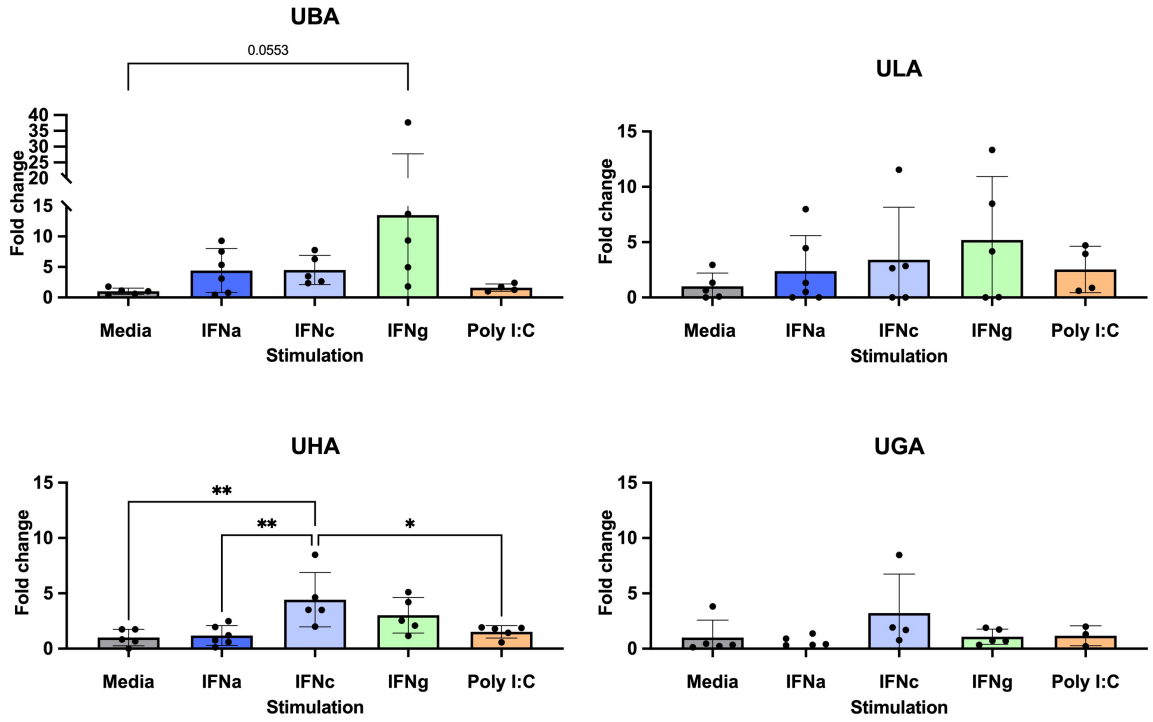


Figure 13: ***Fold change of gene expression of MHC class I, U-Lineage: UBA, ULA, UHA and UGA after in vitro stimulation of primary HKL.*** *The HKLs from adult Atlantic Salmon where stimulated in vitro for 16h with IFNa (blue), IFNc (light blue), IFNg(=IFNγ) (Green), Poly(I:C)(orange) or R848 (purple) in culture media (L-15 with 5% FBS and 2% heparin). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 2) and presented in LOG;2: Significance was testes using normal one way ANOVA.. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01.*

The regarding the U-lineage genes are consistent with previous publications (Grimholt et al., 2020). Shown in Figure 13 UBA was induced in response to IFNγ however, the expression was highly variable from individual to individual. One fish had a fold change around 35 in the stimulation of IFNγ, while other individuals did not have any increase in fold change. ULA was highest expressed in stimuli with IFNγ with a fold change around 5, while UHA and UGA had the highest expression in stimuli with IFNc, fold change around 5. Compared to LGA, there was generally a lower fold increase in response to stimulation with IFNs and Poly(I:C).

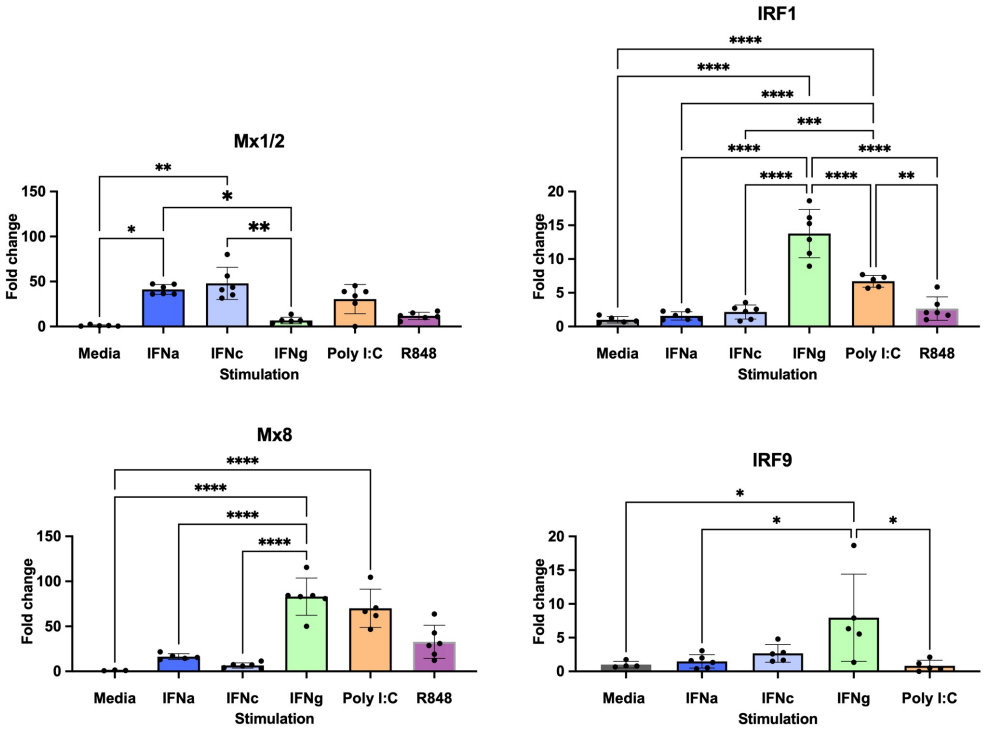


Figure 14: ***Fold change of gene expression of Mx1/2, Mx8, IRF1 and IRF9 after in vitro stimulation of primary HKL.*** *The HKLs from adult Atlantic Salmon where stimulated in vitro for 16h with IFNa (blue), IFNc (light blue), IFNg(=IFNγ) (Green), Poly(I:C)(orange) or R848 (purple) in culture media (L-15 with 5% FBS and 2% heparin). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was testes using normal one way ANOVA. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01, ***p<0,001, ****p< 0,0001.*

Both IRF1 and IRF9 were significantly upregulated in response to IFNγ. With stimulation with Poly(I:C), IRF1 was significantly upregulated (Figure 14). Type I IFNs does not induce significant expression of either IRF1 or IRF9 (Figure 14). Mx1/2 was significantly upregulated by IFNa and IFNc. Mx8 expression shows, on the other hand, a significant upregulation following stimulation with IFNγ and Poly(I:C).

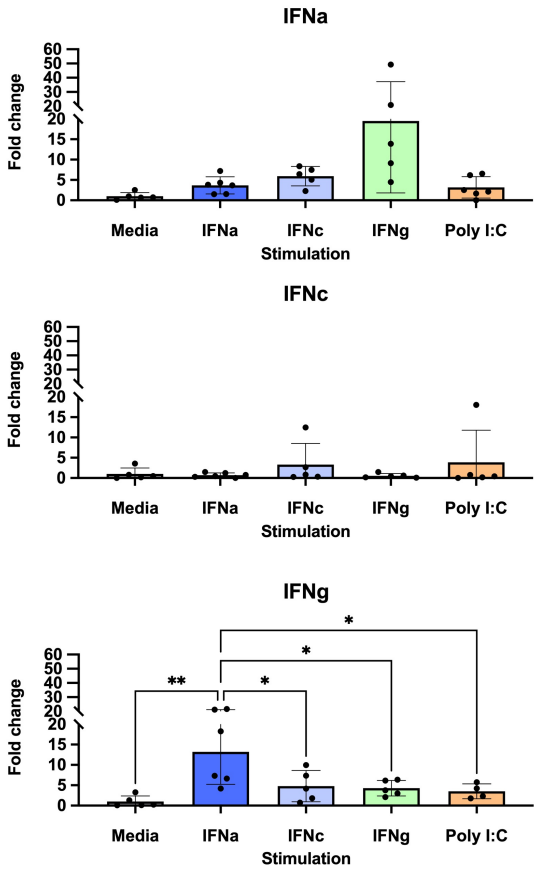


Figure 15: ***Fold change of gene expression of IFNa, IFNc and IFNγ after in vitro stimulation of primary HKL.*** *The HKLs from adult Atlantic Salmon where stimulated in vitro for 16h with IFNa (blue), IFNc (light blue), IFNg(=IFNγ) (Green), Poly(I:C)(orange) or R848 (purple) in culture media (L-15 with 5% FBS and 2% heparin). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was testes using normal one way ANOVA. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01..*

Expression of IFNa shows the tendency of increased expression following stimulation with IFNγ Figure 15. However, the fold change was so scattered between the different individuals that the increased expressions were non-significant. IFNc expression was highest with stimuli with IFNc and PolyI:C. With stimuli of IFNa, the expression of IFNγ was significantly higher. These results suggest that stimuli with IFNs, Poly(I:C), and R848 lead to additional cytokine expression that can indirectly activate the L-lineage genes.

4.2 In vitro co-stimulations of primary head kidney leucocytes from adult Atlantic salmon differentially have an antagonistic effect on non-classical MHC class I L-lineage gene expression

Simultaneously as the in vitro stimulation of primary leucocytes from HK, there were done co-stimulations according to the following combinations IFNa+IFNc, IFNa+ IFNγ, IFNc+ IFNγ, IFNa+ Poly(I:C), IFNc+ PolyI:C, IFNγ +Poly(I:C). The purpose of the stimulation was to investigate if co-stimulation with different IFNs and viral mimics (Poly(I:C)), had an effect on L-linegae gene expression. Co-stimulation with two stimulants can lead to a synergistic effect, antagonistic effect, or no effect at all on gene expression. The effect of gene expression can indicate if the stimulants have the same signalling pathway.

The expression of LGA, LIA, LHA, Mx1/2 and Mx8 genes were analysed using qPCR. Fold change was calculated against the average of the relative expression of media. The outliner of media was removed.

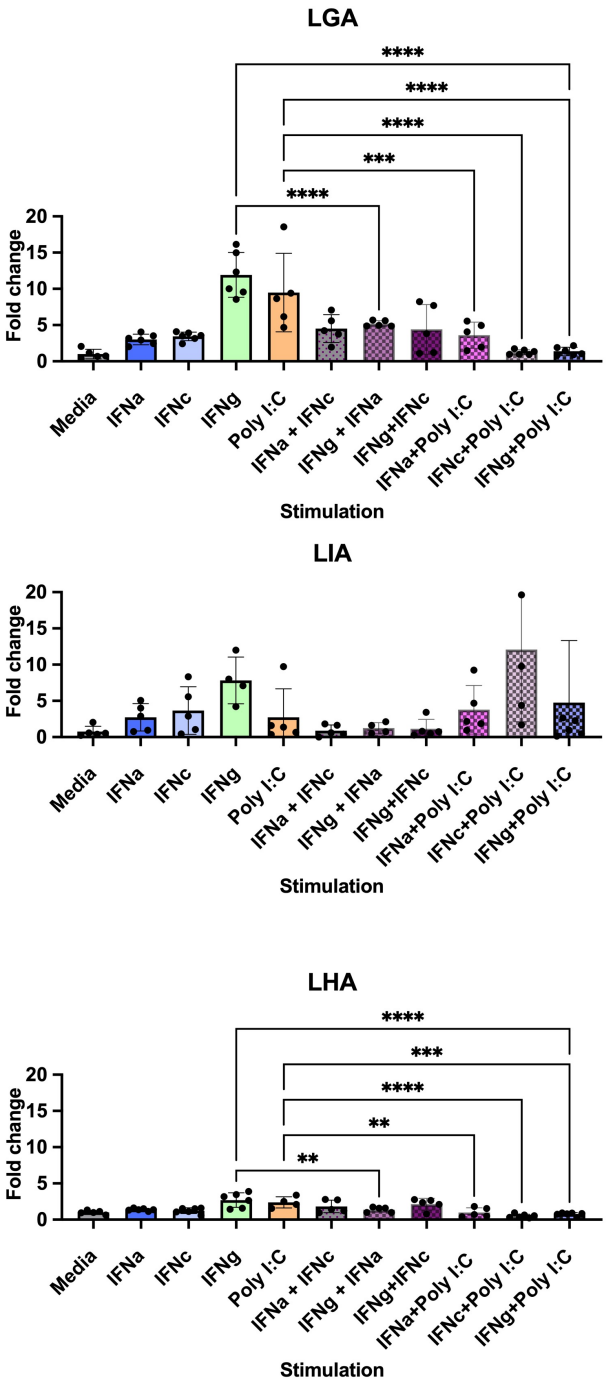


Figure 16: ***Fold change of gene expression of MHC class I, L-Lineage: LHA, LGA and LHA after in vitro co-stimulation of primary HKL.*** *The HKLs from adult Atlantic Salmon where stimulated in vitro for 16h with IFNa (blue), IFNc (light blue), IFNg(=IFNγ) (Green), Poly(I:C)(orange), R848 (purple), IFNa+IFNc(Grey dots) , IFNa+ IFNγ (light purple dots) , IFNc+ IFNγ (purple dots) , IFNa+ Poly(I:C)(pink dots), IFNc+ PolyI:C(gray and purple dots), IFNγ +Poly(I:C) (blue dots) in culture media (L-15 with 5% FBS and 2% heparin). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was testes using normal one way ANOVA. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01, ***p<0,001, ****p< 0,0001.*

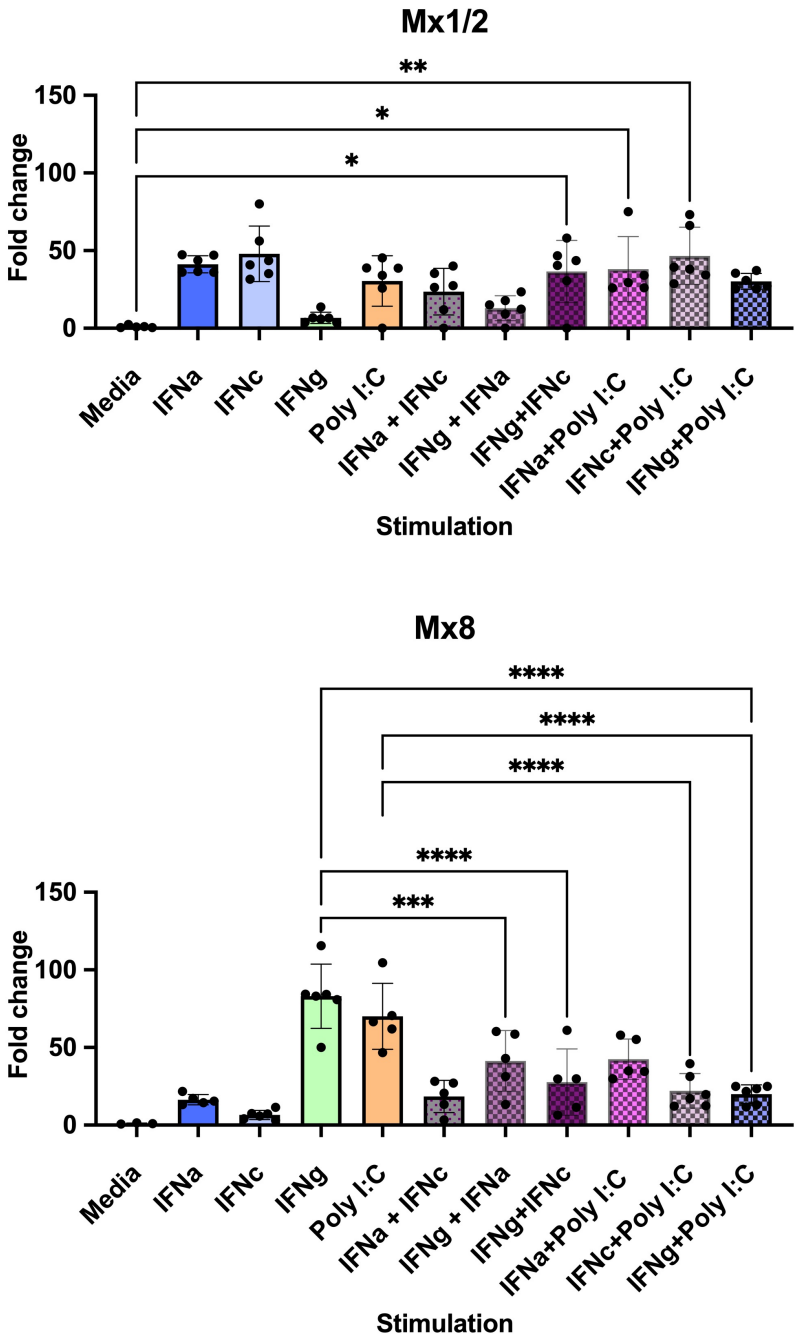


Figure 17: ***Fold change of gene expression of Mx1/2 and Mx8 after in vitro co-stimulation of primary HKL.*** *The HKLs from adult Atlantic Salmon where stimulated in vitro for 16h with IFNa (blue), IFNc (light blue), IFNg(=IFNγ) (Green), Poly(I:C)(orange), R848 (purple), IFNa+IFNc(Grey dots) , IFNa+ IFNγ (light purple dots) , IFNc+ IFNγ (purple dots) , IFNa+ Poly(I:C)(pink dots), IFNc+ PolyI:C(gray and purple dots), IFNγ +Poly(I:C) (blue dots) in culture media (L-15 with 5% FBS and 2% heparin). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was testes using normal one way ANOVA. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01, ***p<0,001, ****p< 0,0001.*

Somewhat surprisingly, there were significantly lower LGA expressions following co-stimulation with IFNγ+IFNa and IFNγ+ Poly (I:C) compared with IFNγ alone, indicating that concurrent stimulation and activation of multiple signalling pathways have an antagonistic effect on LGA expression (Figure 16). Co-stimulation with IFNc and IFNγ also has an antagonistic effect however not significantly different from IFNγ alone. LHA gene induction has the same overall pattern as LGA. LIA expression following co-stimulation with IFNγ is universally lower compared to single stimulation with IFNγ indicating an antagonistic effect. Co-stimulation with type I IFN also has an antagonistic effect. In contrast, the expression of LIA following stimulation with IFNc+ Poly (I:C) appear to have a synergistic effect however, there is large individual variation.

The co-stimulation of IFNa and IFNc had an antagonistic effect on the fold change of Mx1/2 (Figure 17). Co stimulation with Poly(I:C) has not influenced gene expression. Mx8 expression was significantly antagonistic when IFNγ was in a co-stimulation vs when IFNγ was stimulant alone.

4.3 In vitro stimulations of primary head kidney leucocytes from adult Atlantic salmon with JAK I inhibitor modulates non-classical MHC class I L-lineage gene expression

To understand if the JAK/STAT signalling pathways are directly involved in the expression on L-lineage InSoulutionTM JAK Inhibitor I (Calbiochem) was used to inhibit the activities of Janus protein tyrosine kinases (JAKs) In mammals the JAK/STAT pathway has been shown to be inhibited by Calbiochem which is a highly potent ATP- competitive inhibitor primary targeting JAK1 (Thompson et al., 2002). To examine if the JAK/ STAT signaling pathways was involved in L-lineage gene regulations, focusing on LIA and LGA, the JAK Inhibitor I was used in combination with the stimulants IFN type I and II, Poly(I:C) and R848.

Since, to my knowledge, there was no previous publicized experiment where the JAK inhibitor I (Calbiochem) have been used to inhibit JAKs in fish, two different experiments were carried out to evaluate the impact of the inhibitor on the basal expression of L-lineage genes as well as on the transcriptional induction in response to external stimuli. One with the timepoints 12h and 24h and one with two different concentrations of the inhibitor: 15nM and 150nM.

4.3.1 Stimualtion with JAK I Inhibitor for the timepoints 12h vs 24h

HKL were isolated and stimulated with the stimulants IFNa, IFNc, IFNγ and Poly(I:C) with 15nM JAK inhibitor 1 for two timepoints 12h and 24h. The expression of LIA, LGA and Mx1/2 was analysed using qPCR.

Here the inhibitor JAK inhibitor I (Calbiochem) was added in two different concentrations, and the timepoint for the RNA isolation was 12h. The RNA was isolated and qPCR was run. The resulting foldchange from to separated trails were combined. Mx1/2 was included as a control.

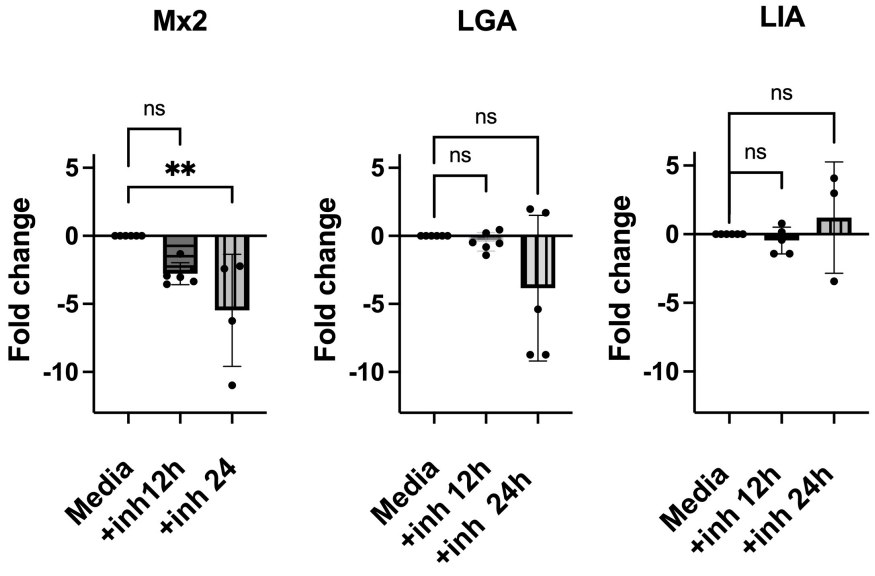


Figure 18: ***Fold change of gene expression of Mx1/2, LGA and LIA after in vitro non-stimulated primary HKL.*** *The HKLs from adult Atlantic Salmon incubated in vitro for 12h (grey) and 24h (light grey) in culture media (L-15 with 5% FBS and 2% heparin) with the inhibitor JAK1 Inhibitor (Calbiochem). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was testes using t-test. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01.*

Figure 18 shows that there was no significant difference between the expression of Mx1/2, Mx8, LGA and LIA with either of the two concentrations of inhibitor. The fold change between the 15nm inhibitor and 150nm inhibitor were not significant. The rest of the experiments were continued with the concentration of 15nm inhibitor and a incubation time of 12 hours. Figure 33 in the appendix shows no significant difference in gene expression for LIA and LGA at the two timepoints at 12h and 24h.

4.3.2 Stimualtion with JAK I Inhibitor at the consecrations 15nM vs 150nM

In Primary cell inhibition trail 2 and 3 the HKL was collection, isolation and stimulation done identically as in inhibition trail 1. Timepoint for the RNA isolation was 16h. The inhibitor InSoulutionTM JAK inhibitor I was added in two different concentrations. The gene expression were analysed using qPCR. The resulting foldchange from the separated trails 2 and 3 were merged.

Gene expression was investigated for the L-lineage LGA and LIA. Control genes were Mx1/2 that literature shows respons to Type I IFN, and Mx8, which has been shown to respond to type II IFNs (Robertsen et al., 2019).

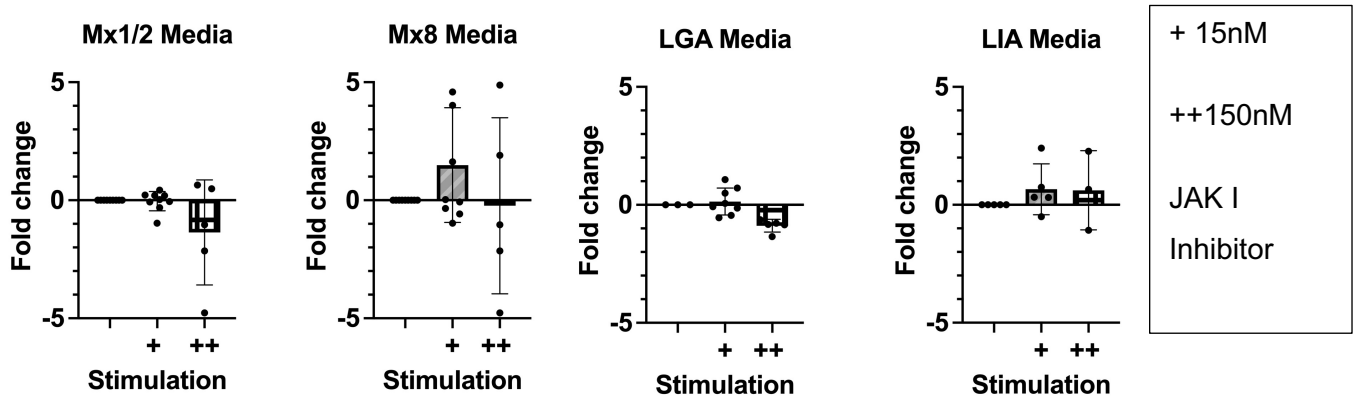


Figure 19: ***Fold change of gene expression of Mx1/2, Mx8, LGA and LIA after in vitro non-stimulated primary HKL.*** *The HKLs from adult Atlantic Salmon incubated in vitro for 16h in culture media (L-15 with 5% FBS and 2% heparin) with the inhibitor JAK1 Inhibitor (Calbiochem) with the consecrations 15nM(grey) and 150nM(white). Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was tested using T-test.*

Figure 19 shows that there was no significant difference between the expression of Mx1/2, Mx8, LGA and LIA with either of the two concentrations of inhibitor. The fold change between the 15nm inhibitor and 150nm inhibitor were low. The rest of the results were continued with the concentration of 15nm inhibitor.

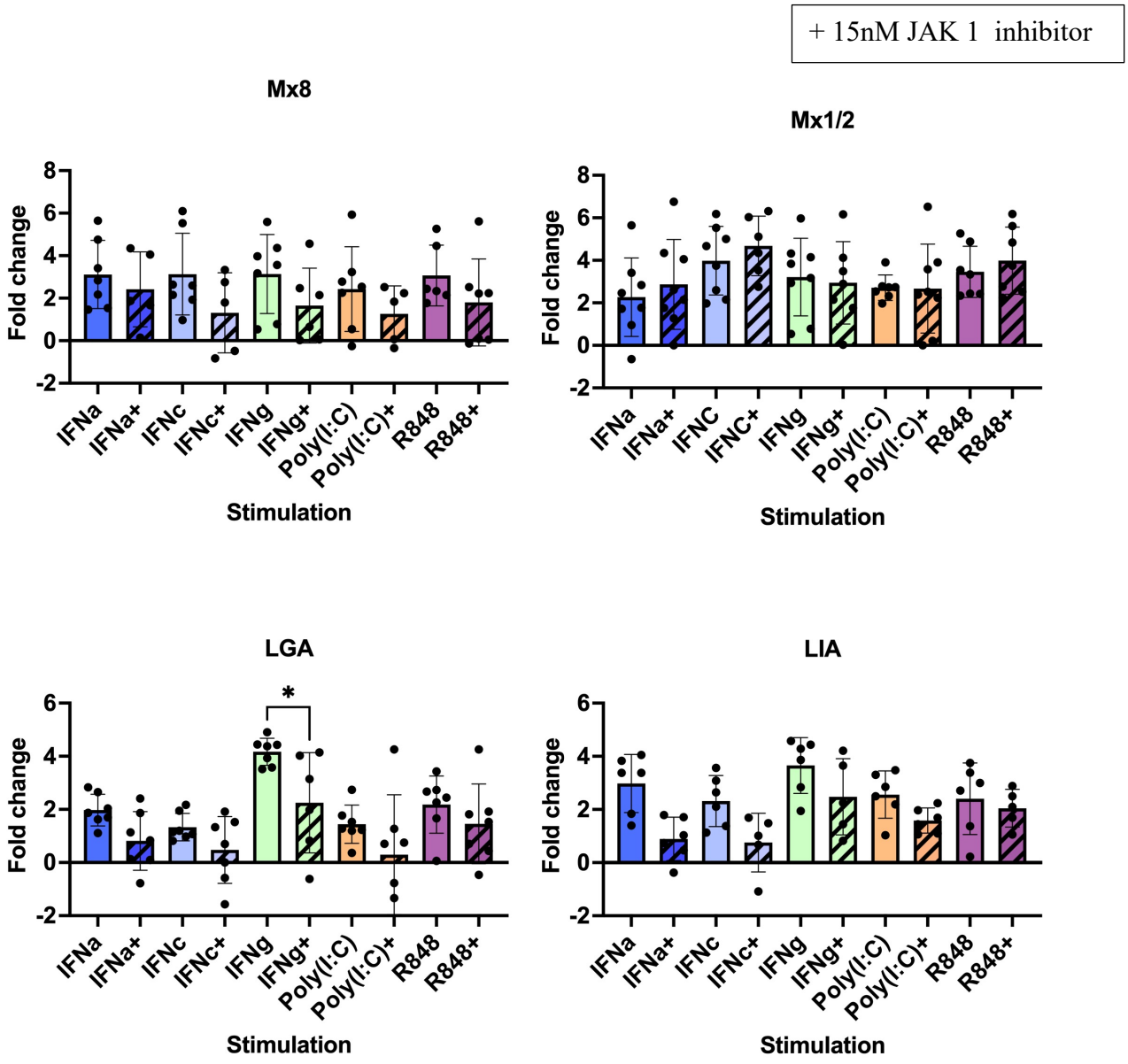
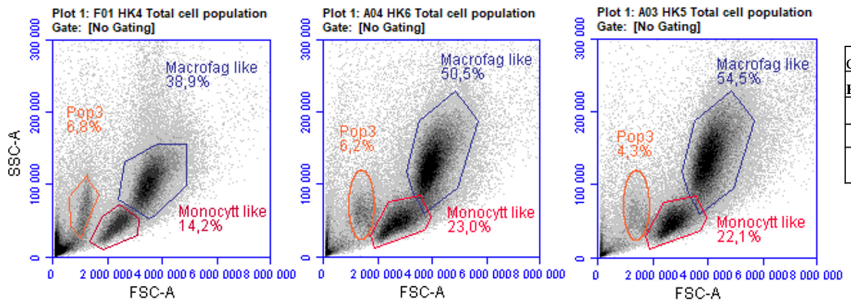


Figure 20: ***Fold change of gene expression of Mx1/2, Mx8, LGA and LIA after in vitro stimulated primary HKL.*** *The HKLs from adult Atlantic Salmon incubated in vitro for 16h in culture media (L-15 with 5% FBS and 2% heparin) stimulated with IFNa (blue), IFNc (light blue), IFNg(=IFNγ) (Green), Poly(I:C)(orange), R848 (purple) with the inhibitor JAK1 Inhibitor (Calbiochem) with and without the consecrations 15nM. Each dot represents an individual fish. The fold change was calculated against the average of the relative expression of media against the reference gene EF1a (Equation 3) and presented in LOG;2: Significance was testes using normal one way ANOVA. Asterisk indicates the degree of significance between the different genes. *p<0,05.*

Following a 12-hour incubation with 15nM JAK I inhibitor the gene expression of Mx8, LGA and LIA show an overall trend of decreasing in fold change for all the stimulations used (Figure 20). LIA expression following stimulation with IFNa decreased with 67% when the inhibitor was included. IFNγ and Poly(I:C) stimulated HKL had a 31% and 38% decrease in induction respectively. For LGA fold change on IFNγ stimulated cells where significantly lower (46%) in the presence of inhibitor IFNa stimulated HKL had a decrease of 58% in induction with the inhibitor. Mx 8 shows similar decrees as LGA and LIA. Mx1/2 on the other hand showed a slight increase in gene expression with inhibitor for all the stimulations except IFNγ and R848 where no change was observed. The change is not significantly due to the shadderd individuals, but is indicating that JAK/STAT is involved in the induction of LGA in stimuli with Poly(I:C).

4.5 Optimization of Magnetically activated cell sorting as a method to enrich IgM+ B cells from total HKLs

As B cells express classical MHC class I and class II molecules and are central in antigen-presenting and activation of the adaptive immune response, we chose to focus our further investigation into the transcriptional regulation of L-lineage genes on B cells. Studies on specific populations of fish immune cells remain challenging due to the limited availability of antibodies to differentially expressed surface markers and to date, there are no antibodies specific to any of the L-lineage genes available. Therefore, the specific cell type must be isolated to examine if that cell type expresses L-lineage genes. The head kidney is composed of several different cell types and what cell types potentially express L-lineage is currently unknown. The composition of different cell populations also differ somewhat from individual to individual. Primary head kidney leucocytes from three different adult Atlantic salmon were analysed using Flow cytometry. Fish 5 and 6 were from the same trial, while fish nr 4 was from a separate trial. Based on the scatter profiles, two different cell populations, large internally complex granulocyte/macrophage-like cells and a smaller, less internally complex population of monocyte/lymphocyte-like cells were identified. The blue marks macrophage-like cells based on side-scatter and forward-scatter. The red area is monocyte-like cells, while the yellow area, marked with “pop3” was debri . The cell populations differ from an individual, as shown in table B.



Cell count	100000		
HK	**Macrofag like**	**Monocytt like**	**Pop3**
4	38,90 %	14,0 %	6,80 %
5	54,00 %	22,07 %	4,31 %
6	50,50 %	22,95 %	6,27 %

Figure 21: **Flow cytometry dot plot of HKL population from three adult salmon**. A) The represent HKL based on cell size (FSC) and complexity (SSC). The different cell poppulation are gated. Macrofag-like cells are gated in blur, Monocytt-like gated in red and Pop3 is gated in yellow. B) Tables show the share of the different gated cells.

Based on size and complexity, the share of macrophage- like cells was the highest for all the individuals, and monocyte-like share was between 14%- 23% (Figure 21). The “Pop3” was based on their cell size probably debride cells.

To isolate B-cells, Magnetic activating cell sorting (MACS) was used. Before proceeding to further experiments, a characterization of the purity of the MACS sorted cells were undertaken. Total HKL were incubated overnight, 16h, in culture media with 5% FBS before subjected to magnetic separation. The HKL were stained with anti-IgM followed by magnetic beads and run through a magnetic column. The “flow-through” were the cells that did not bind to the anti - IgM, and magnetic beads and thus did not attach to the magnetic column. The HKL and the flow through cells were collected and stained with anti- IgM and analyzed using Flow cytometry. The flow cytometry result from HKL was compared to flow through to examine how many B- lymphocytes were not sorted out in MACS.

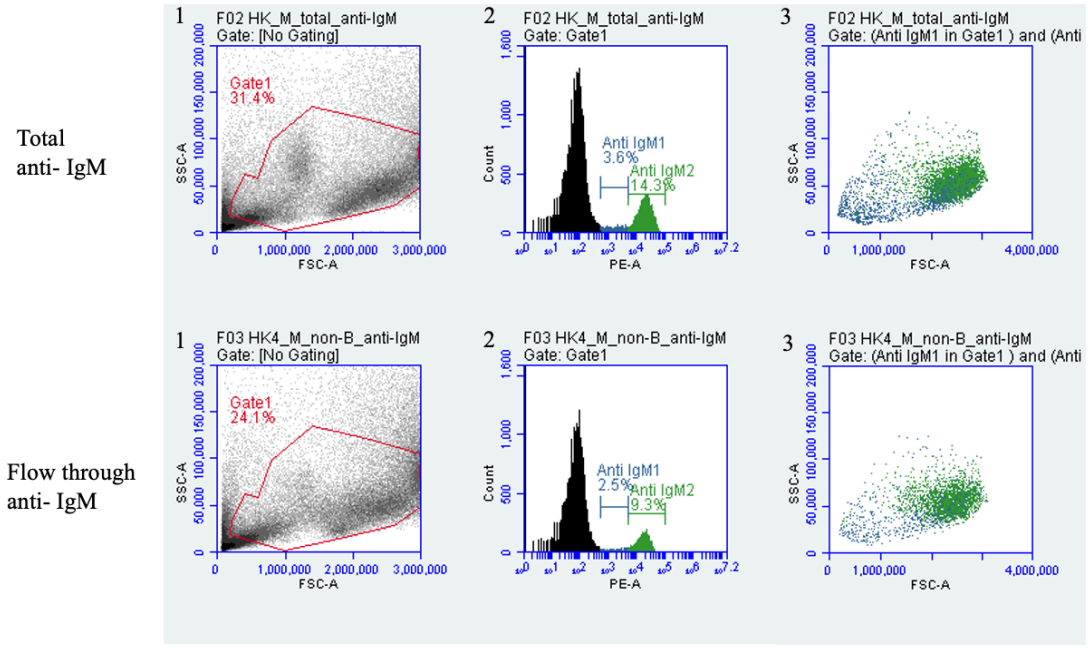


Figure 22: ***Flow cytometry reveals two anti-IgM stained populations****. 1) The plot represent HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of HKL cells and the flow through from MACS. 2) Shows light absorbance of two gated anti- IgM-stained populations. 3) The Plot represent forward (FSC-A) and side scatter (SSC-A) of the anti-IgM stained cells. Blue cells Anti- IgM1, green in Anti-IgM2.*

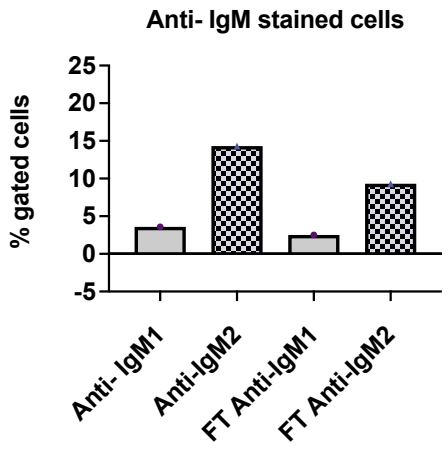


Figure 23: ***Comparing the % counted Anti-IgM1 and Anti-IgM2 stained HKL in suspension sorted (Anti-IgM1, Anti-IgM2) and flow through from MACS (FT Anti-IgM 1, FT Anti-IgM2) from Adult salmon.***

As shown by flow cytometry (Figure 22) the forward (FSC-A) and side scatter (SSC-A) show two cell populations binding to anti-IgM. The Anti-IgM2 gate was likely lymphocytes based on their size and complexity. However, the cells in anti-IgM1 gate are likely showing positive staining due to Anti- IgM binding to Fc- receptor bound IgM and represents macrophages and other cells rather than B-cells. In a comparison of the Flow through and total cell suspension, there were around 30% fewer IgM-stained cells in the Flow through compared to the entire cell suspension. That means that 30% of all the IgM expression cells were sorted out as B-cells (Figure 23).

4.5.1 Prior to MACS minimize the number of IgM surface positive macrophage-like cells in the culture

To exclude selection of macrophage contamination within the IgM^+ MACS purified cells total isolated HKL were separated before the MACS into suspension- and adhesion cells in an effort to get a cleaner B-cell population after MACS. Different immune cells have different surface proteins and will have different abilities to bind to plastic. Lymphocytes will not attach to the plastic, but macrophages and dendritic cells typically will (Kumagai et al., 1979). Thus, HKL were incubated in plastic bottles in culture media with 1% FBS for 72h and divided into two groups: suspension and adhesion cells. The groups were stained with Live/ dead Fixable Viability to define a gate for living cells.

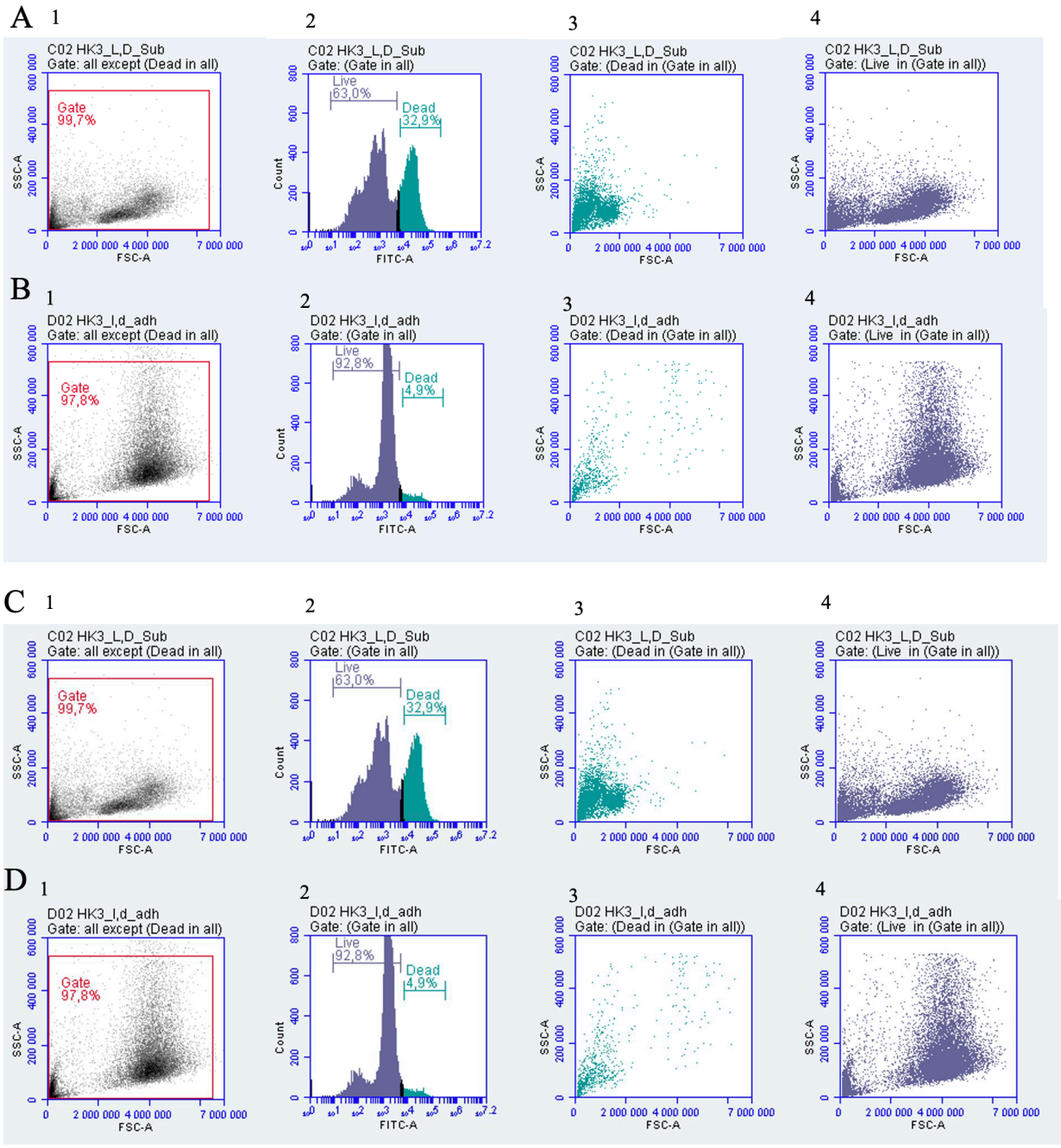


Figure 24: **Flow cytometry result of Live/ dead staining of A,C: Suspension cells (HK3, HK1) B, D: Adherent cells (HK3,HK1) from Adult Atlantic salmon** 1) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells (A) and Adherent cells (B). 2) Shows light absorbance of live (purple) and dead cell-stained (blue) cells (FITC-A). The plot represents forward (FSC-A) and side scatter (SSC-A) of (3), Dead cells and (4) live cells.

As expected, dead cells were collected in the suspension, so the share of dead cells was higher in suspension compared to adherent cells (Figure 24). The live/ dead staining was used to make gates of living cells for the rest of the experiments. The side and forward scatter showed two different cell populations in the suspension group vs the adherent group. The adherent cell population had bigger cells with higher complexity. This was likely monomacrophage-like cells, dendritic cells, and/ or granulocytes. The HK cells in the suspension group were smaller and less complex. These likely include B and T lymphocytes. The gate "Living" was adjusted to this result. The two populations were stained with Anti- IgM and analysed to see the percent of anti-IgM-stained cells.

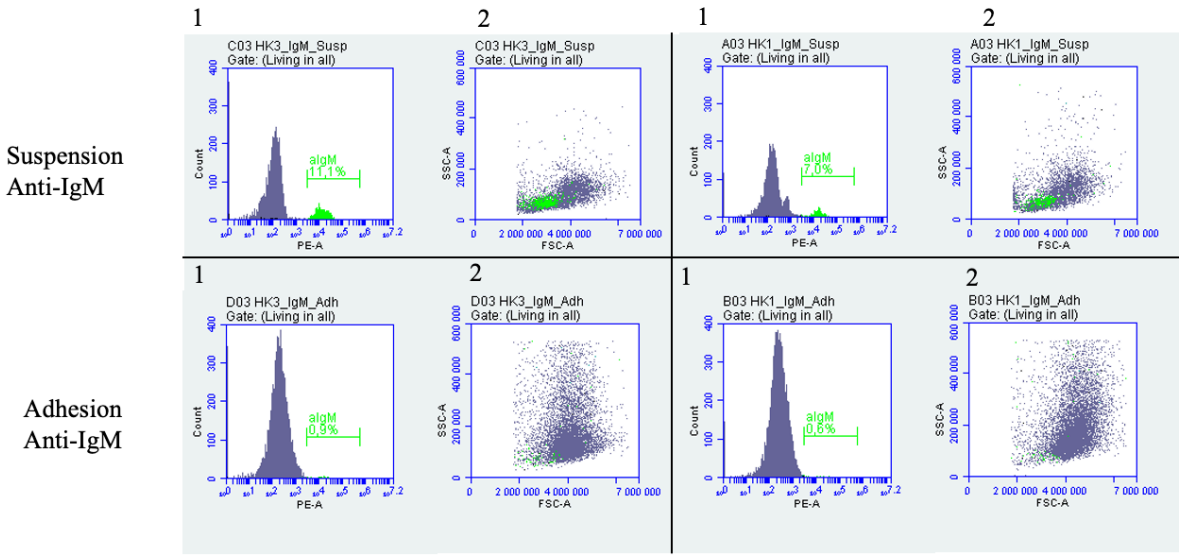


Figure 25: **Flow cytometry result of anti-trout IgM staining of suspension and adhesion sortet HKL from two Adult salmon HKL.** 1) Shows light absorbance of anti-throut IgM stained, live cells (PE-A). 2) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells and Adherent cells. Anti-IgM stained cells are marked in green.

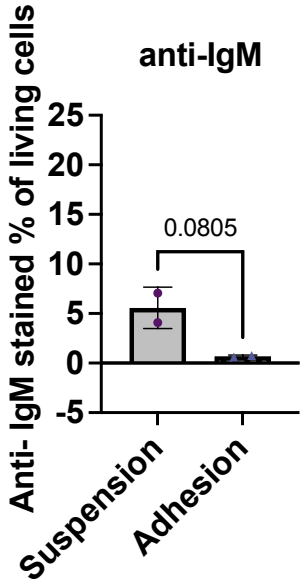


Figure 26: ***Comparing the % counter, live, Anti- Trout IgM stained cells sorted in suspension and Adhesion.*** *Marked are the significant level between the two groups. Significance was tested using t-test.*

The adherent cells are not stained at all of the anti-IgM (Figure 25). The share of anti-IgM was higher in suspension cells compared to Adhesion (Figure 26) .

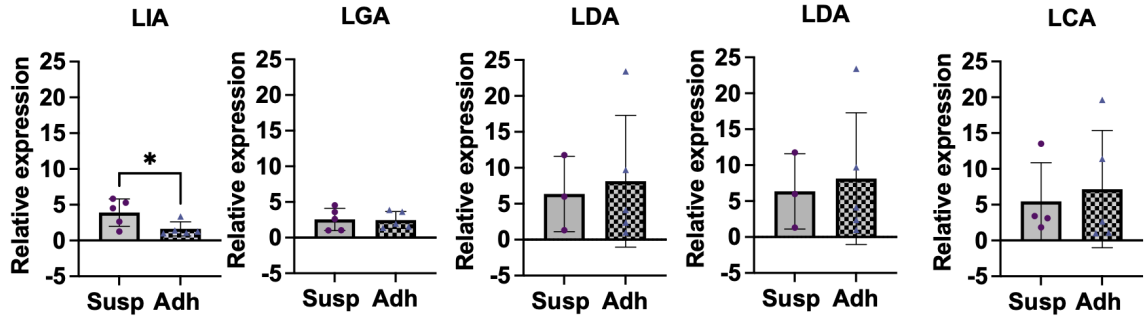


Figure 27: ***Relative gene expression of L-lineage in the sortet suspension (Susp) and Adhesion (Adh) of five fish incubated for 72h. MACS trial 1.*** *Relative expression of LIA , LGA, LDA, LDA and LCA are calculated using the ΔΔCt method (Equation 4: ΔΔCt-method).*

Figure 27 shows that ncMHC L-lineages are similarly expressed in suspension and adherent.

4.6 Purity and viability of MACS sorted IgM+ Bcells from HK of MACS

Next, HKL were isolated and incubated in culture media with 1% FBS in plastic bottles and separated into adherent and suspension groups. Unlike the previous trial, the cells were incubated for 48h instead of 72h to allow for minimal interference from Fc receptorbound IgM on macrophages, but shorter to increase B-cells viability. Primary B-cells in vitro survive for a short period of time.

Suspension-divided fraction of HKL cells were sorted using MACS. Post sorting flow cytometry with anti-IgM staining was run to assess the number of believed B-cells in the sorted solutions. The purity of the sorted B- cells samples were examined by comparing %anti- IgM in the suspension with flow-through from MACS.

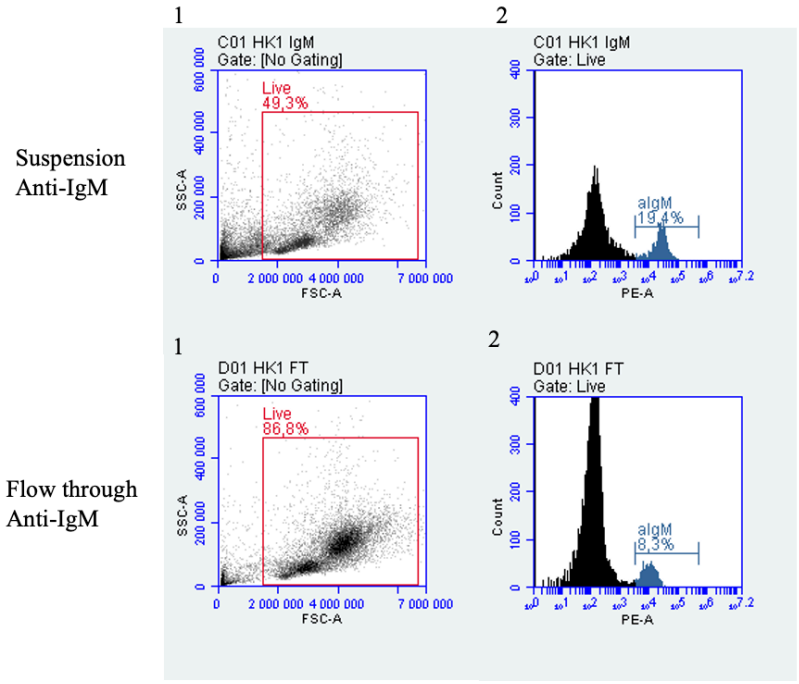


Figure 28 **Flow cytometry result of anti-trout IgM staining of suspension and adhesion sorted HKL from Adult salmon HKL nr 1 uncubated for 48h.** 1) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells and flow through after MACS. Within the red gate there are living cells 2) Shows light absorbance of anti-trout IgM stained, live cells (PE-A). Anti IgM cells are marked in blue.

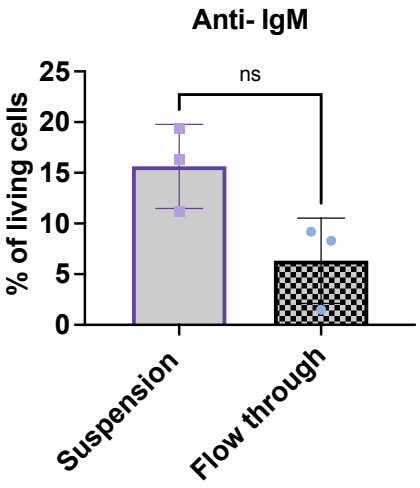


Figure 29: ***Comparing the % counter, live, Anti- Trout IgM stained HKL cells sorted in suspension and flow through from MACS from adult grown salmon.*** *Each dot represents an individual fish. Significant level was calculated using t-test.*

Figure 29 shows flow cytometry result with anti- IgM staining and in the appendix show the flow cytometry result in Flow cytometry Comparing the % Anti-IgM positive stained cells in the flow through from MACS to the total suspension population (Figure 28, and Figure 34 and Figure 35). The number of anti-IgM-stained cells was reduced from suspension to flow through with 67%. That means that 67% of the B-cells were collected. The flow cytometry charts show a large difference between fish 1 and 2 and 3 when it comes to % of anti-IgM.

4.7 L- lineage gene expression in Adherent, non-adherent and IgM sorted cells

As flow cytometry analysis clearly shows different cell populations in adherent HK cells and suspension HK cells. Thus, basal gene expression of L-lineage genes was examined to see if there was a difference in expression between the two groups. *csf-1r*, known as markers of monocyte-macrophage lineages in mammals and were used as a control marker for macrophages (Jenberie et al., 2018). TCR alpha was used as a control gene for T-cells. UBA and MHC II were included since UBA is expressed on all cells and MHC II are expressed on dendritic cells, monocytes and B-cell. IgM gene expression was done to see if the MACS was successful.

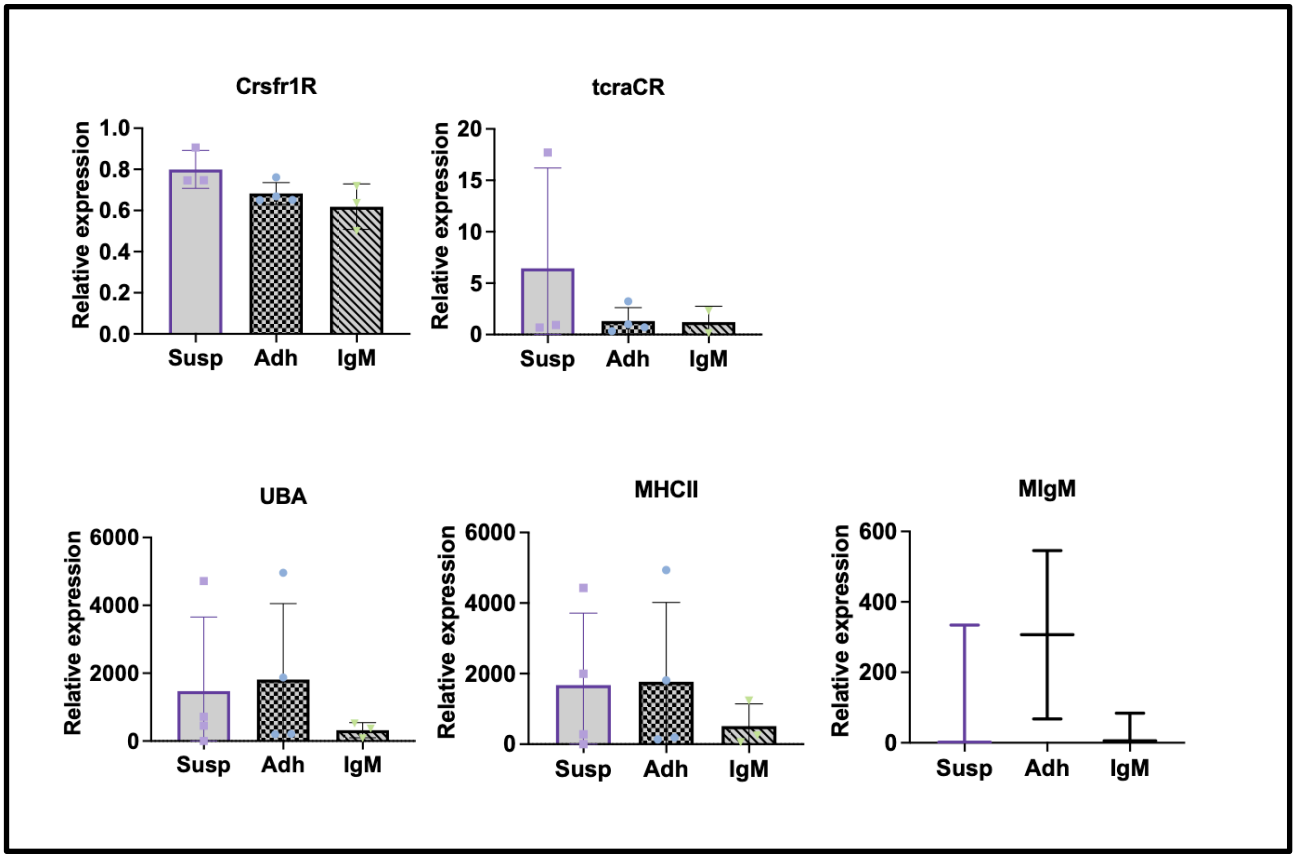


Figure 30: ***Relative gene expression of Crsfr1R, tcraCR, UBA, MHC II and MIgM in the sorted suspension (Susp) and Adhesion (Adh) of five fish incubated for 48h.MACS trial 2*** *Relative expression of LIA , LGA, LDA, LDA and LCA are calculated using Equation 4: ΔΔCt-method. Each dot represents an individual fish. A significant level was calculated using One way ANOVA.*

There was an expression of UBA, MHC II and mIgM in all the different groups (Figure 30). There was the highest relative expression of MHC II in the adherent cells. MHC II was found on Dendritic cells, +, +. UBA, MHC class I are not highly expressed on IgM-positive cells compared to the adherent and suspension cells. The MHC II and UBA gene expression establish what we previously know. The IgM gene detection on the other hand where relative low in the B-cell batch and higher in the Adhesion batch. But the expression were quite different between the different fish.

LIA, LGA, LHA and LDA gene expression after the MACS sorting was examined.

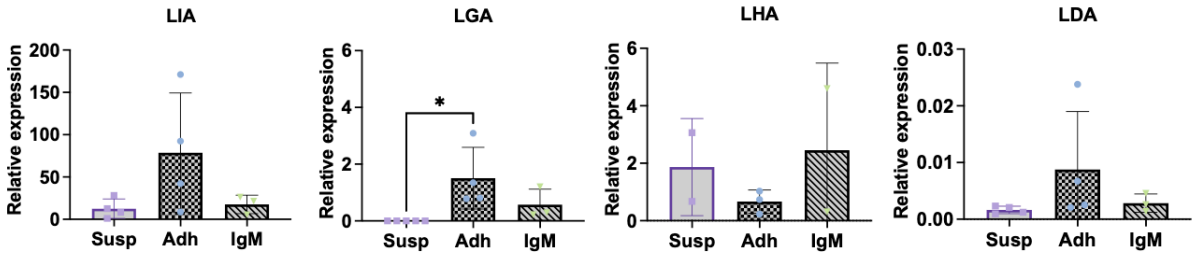


Figure 31: **Relative gene expression of L-lineage in the sorted suspension (Susp) and Adhesion (Adh) of five fish incubated for 48h MACS trial 3.** Relative expression of LIA , LGA, LHA and LDA are calculated using the ΔΔCt method (ref. appendix). Each dot represents an individual fish. A significant level was calculated using one way ANOVA. Asterisk indicates the degree of significance between the different genes. *p<0,05. **p<0,01.

Similarly, a third trial was done identically to trial 2, which also yielded a decrease of ant-IgM stained cells from suspension to flow through was 65%, which was the same as in trail 2, viewed in Figure 37 Figure 38 and Figure 39 in the appendix. qPCR was run against the genes LIA, LGA and LDA of Suspension, Adherent and B-cell-sorted cells to see if there were different expressions of the L-lineage in the different sorted groups.

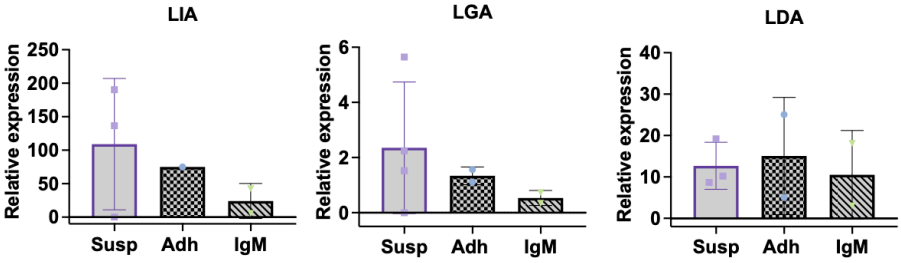


Figure 32: **Relative gene expression of L-lineage in the sorted suspension (Susp) and Adhesion (Adh) of five fish incubated for 48h. MACS trial 3** Relative expression of LIA , LGA and LDA are calculated using the ΔΔCt method (ref. appendix). Each dot represents an individual fish. A significant level was calculated.

From adhesion MACS trail 1, there was a significantly higher expressed LIA in suspension than in adhesion (Figure 28). The relative expression of LIA was, on the other hand, higher in adhesion rather than in suspension in MACS trial 2 Figure 31 and trial 3(Figure 32). For LGA, LDA, LHA and LCA there was no significant difference in the relative expression when

comparing the different populations. Based on the expression data from the control genes, it is not possible to infer much with regard to the cell type-specific basal expression of L-linegae genes suffice it to say that MACS sorted IgM-positive cells express LIA, LGA, LHA and LDA as do both adherent and suspension cell fraction derived from HKLs.

5 Discussion

5.1 Unique gene expression of different L-lineages in response to various stimulants

LGA, LIA, LHA and LDA all have unique expression patterns in response to in vitro stimulation with typical viral associated stimuli, such as IFNs , Poly (I:C) and R848, in primary HKL in adult Atlantic salmon. Gene expression of LGA and LIA are both strongly induced by IFNγ, and moderately induced by type I IFNs. LGA was highly expressed in cells stimulatied with Poly(I:C), while LIA had a stronger response to R848 stimulation. LDA shows a significant induction by IFNγ but differs from the other examined L-lineage subtypes by not being induced by type I IFNs, Poly(I:C) or R848. This may indicate that the different L-linages have different tasks in anti-microbial defences.

LHA and LDA were not induced by type I IFNs in these experiments. However, the LDA and LHA gene expression may be induced later than the investigated 12h timepoint and have a role later in the immune response. There may be a mechanism that regulates the timing of induction by IFN of the other L-lineage genes. Evidence supporting this is a previous study that shows the different L-lineage subtypes are expressed at different timepoints post-stimulation (Svenning et al., 2019). Different induction by stimulants at different time points indicates different immunological tasks.

In Svennings study, 2019, LIA expression was higher than LGA in stimulation with Poly(I:C) at the time point 24h in primary isolated HKL (Svenning et al., 2019). In this thesis, the LGA were higher expressed than LIA by the timepoint 12h. Comparably this study was done at different time points and in a different life stages of Atlantic salmon. Svenning`s study was done on parr, while this study was done on adult Atlantic salmon (1,5-2kg). During smoltification, the immune system changes, and an assumption can be that the expression pattern of the L-lineage may change as well. The functional role of the various L-lineage molecules may change during the life of Atlantic salmon and reflect their life in fresh water and salt water.

5.2 Oppositely regulations of the L-linage by multiple stimuli

Not only do the L-lineages genes differ in transcriptional response and kinetics, but also the transcription of these genes was found to be oppositely regulated by the addition of multiple stimuli. Combine stimulation with IFNa, IFNc, IFNγ, Poly (I:C) and R848 show a significantly antagonistic effect on LGA, LHA and Mx8 gene expression. The only synergistic effect was found on LIA in co-stimulated with IFNc and Poly(I:C) however, this was not significant. Interestingly the same co-stimulations didn't have an antagonistic effect on Mx1/2 gene expression. This excludes the suspicion that the observed antagonistic effect could be due to a chemical and or sterical interaction occurring among the different stimuli (recombinant proteins and synthetical analogues to ds and ssRNA) previously to or during the stimulation. The Mx1/2 expression was not synergistic but not antagonistic ether. This may indicate that Mx1/2 gene expression is regulated via another signalling pathway compared to LIA, LGA and Mx8.

Type I IFN and type II IFN may induce gene expression through an overlapping signalling pathway and get overloaded, resulting in an antagonistic effect. During a viral infection, the ssRNA and dsRNA are detected by intracellular TLR and cytokines, and IFNs Type I and type II will increase. Thus there will be both IFNa, IFNc, IFNγ and virus RNA present at the same time. It is, therefore, somehow surprising that the combination of this, mimicking a virus infection more accurately than a single stimulus leads to an antagonistic effect in LIA, LGA and Mx8. When Atlantic salmon were infected with SAV3 virus (Svenning et al., 2019), the LIA and LGA expression were upregulated. The negative effect may have a purpose to downregulate different L-lineages. It would be interesting to co-stimulate over a longer period and induce the different stimulates at different timings.

Type I IFNs induce gene transcription through ISRE elements. ISRE elements are identified in LIA, LGA, LHA and LHA (Svenning et al., 2019). There is no identified GAS element in LGA (Grimholt et al., 2020; Svenning et al., 2019). GAS, the main promotor element for IFNγ induction, are only found in LDA and LCA, and a GAS-like element has been identified in LIA (unpublished data, personal communication Dr. Eva-Stina Edholm). These experiments show that there is a strong induction of LGA, LIA, LHA and LDA gene expression in response to IFNγ. This indicates that IFNγ may induces gene transcription by another promoter element that GAS, perhaps through GAS-like elements. Another

explanation can be that IFNγ induces an indirect gene expression of the L-lineage genes. IFNγ induces secretion of IFN type I, that leads to the induction of transcription through type I IFNs receptors.

5.3 The potential role of L-lineage genes in the Immunity of Atlantic Salmon

Compared to ncMHC U- lineage and classical MHC class I UBA, L- lineages are expressed at very low levels in non-stimulated primary HKL in adult salmon. The ncMHC U-lineage are like the L- lineage non-polymorph, but their sequences are more like the classical MHC class I gene I (Grimholt et al., 2015). In response to IFNγ stimulation, the U-linage; ULA, UGA and UHA had a lower increase in fold change than LIA, LGA and LDA. This can indicate that compared to the U-linage, the L -lineage gene expression is more inducible.

An unpublished result from the fish immunology and vaccinology research group at UiT (personal communication Dr. Maryam Iman) showed that LGA and LIA complexes are detected in the membrane fraction of Atlantic salmon cell linages. This indicates that LGA and LIA are expressed on the cell surface. The detection of the L-lineage complex and the increased gene expression by anti-microbial stimulants strongly indicate that the L-lineage has a role in the immune system. Some cell types may have the gene expression of LIA and LGA, but not necessarily the ability to present the ncMHC on the cell surface.

The L-lineage gene, LIA is perhaps the most highly conserved L-lineagesubfamily and has orthologs conserved in all Salmonidae species examined to date, including whitefish (Coregnus cluepaformis) and grayling (Thymallus thymallus) as well as Northern pike (*Esox Lucius*) (Grimholt & Lukacs, 2021). This evolutionary conservation implies that LIA is essential and might bind to a conserved ligand. Other L-lineage genes like LGA are more variable in different salmonids; its found in some but not all species examined and is often identified as a pseudogene (unpublished data, personal communication Eva-Stina Edholm). This indicates more species' induvial evolutionary devolvement and might indicate binding to a more diverse, possibly species-specific receptor. The different evolution conservations of LIA and LGA support the hypothesis that the different subtypes of the L-lineage have different tasks in the immune system. The limited polymorphism and conserved antigen

presentation pattern of the ncMHC make them attractive vaccine targets that could be recognised in genetically diverse populations.

Due to the hydrophobic residues in the binding groove of L-lineagegenes, a hypothesis is that the L-lineage presents lipids antigen to conserved T cells and NK cells, similar to ncMHC CD1 (Grimholt et al., 2015). Lipid-loading on the CD1 ncMHC are tightly regulated by various cytokines (Prigozy et al., 1997). The classical MHC class I need a peptide to be stabilised before it can merge to the cell surface (Abbas et al., 2016). The L-lineage MHC may be loaded with well-conserved lipids from microbes due to a similar loading pathway like the CD1 ncMHC and classical MHC class I.

Another hypothesis is that the subtypes of L-lineage may present a lipid structure, within or produced by the cell itself in response to microbial infection. When presenting lipids from the cell itself, there is no evolutionary arms race, and the gene may stay conserved. Different cytokines, IFNs and PAMPs may induce intracellular release of lipids, that gets loaded on the different L-lineage ncMHC. The gene expression of the subtypes of L-lineage may be induced by the same stimuli.

The L-lineage ncMHC may not present lipids at all. During infection, expression of the L-lineage subtypes is induced by signalling and expressed on the cell surface, where they bind to other immune cells and stimulate a more general immune response.

5.4 Inhibitor of JAK1 in the JAK/ STAT pathway

To examine if both IFNs and TLR ligands (ds and ssRNA) directly induced L-linages gene expression a JAK1 inhibitor was used to block signalling via the JAK/STAT pathway. The result shows that the JAK1 inhibition (Calbiochem) had a down-regulation effect on gene expression of LIA and LGA with all the stimulants. The inhibitor's effect on IFNs was stronger compared to the inhibitor's effect on viral RNA mimics stimulants. The inhibitor may not affect the signalling pathway of LIA and LGA gene induction through the TLRs directly. R848 and Poly (I:C) induce IFNs secretion, which again induces gene expression of L-linage.

The reduction of expression of LGA, LIA and Mx8 may be caused by inhibition of the secondary IFNs stimulation.

Collectively, these data indicate that LIA and LGA are differentially induced in response to multiple different external stimuli, utilizing both type I IFN and type II IFNγ induced JAK/STAT dependent signaling. It is interesting that both the JAK1 inhibition and the co-stimulation result in a different pattern for the Mx1/2 expression, but a more similar for Mx8, LIA and LGA gene expression. Mx1/2 was, during these experiments, intestinally chosen as a control gene expedited to be induced by IFN type I. Mx8 was chosen as a control gene and was expected to be induced by IFNγ. This is again indicated that different signalling pathways for induction of the different genes.

The JAK I Inhibitor (Calbiochem) inhibits JAK 1 in mammals. The inhibitor has not been used in experiments with fish before, and the efficiency with which this inhibitor functions on various JAKs in Atlantic salmon is still unknown. The downregulation of LGA, LIA and Mx8 with the JAK inhibitor indicates that the JAK I inhibitor (Calbiochem) inhibits a JAK1 gene and that it is important in the induction of these LIA and LGA. This again means that the JAK/STAT signalling pathway is likely involved in regulating the expression of LIA and LGA. There are multiple JAK1 and JAK2 genes in Atlantic salmon because of the whole genome duplication in salmonids (Grimholt et al., 2020). There are expressions of three different JAK I by stimulation with IFNγ. (Grimholt et al., 2020). Induction of Mx1/2 was not inhibited by the JAK I inhibitor (Calbiochem). Mx1/2 induction may involve another JAK1 gene than the JAK1 involved in the LGA, LIA and Mx8 signalling pathway. The basal expression of Mx1/2 is significantly inhibited with a concentration of 150nm with the inhibitor. It is described on the inhibitor that it may, with high concentrations, affect other kinases.

5.5 Cell-specific gene expression of the different ncMHC, L-lineage

L- lineage gens expression varies and may include specialised roles for combating specific pathogens. The parallel peripheral antigen-presenting cells in mammals selectively express

distinct CD1 genes in response to certain activating stimuli, such as TLR antagonists and certain cytokines. Structural L-lineage is found to be able to bind hydrophobic structures. A hypothesis is that L-lineage has similar abilities to mammal CD1 (Grimholt et al., 2015). There are identified different ncMHC CD1 expressed on different cell types (Dougan et al., 2007). The expression of the other L-lineage genes is identified in various organs in Atlantic salmon (Svenning et al., 2019), but what cells express the specific L-lineage unknown. The different signalling induction of the different L-linages also strengthens the hypothesis that the different subtypes of the L-lineage gene are expression on different cells.

There are a few possible ways to sort Atlantic salmon cells due to the lack of antibodies. The availability of a cross-reactive anti-trout IgM made it possible to do isolate B cells and investigate the L-lineage gene expression. B-cells are critical antigen-presenting cells and express both the MHC class I and class II. In mammals, B-cells are found expressing the isoform CD1c of nonclassical MHC CD1(Reinink & Van Rhijn, 2016). B- cells are highly found expressed in the HK of Atlantic salmon and trout.

5.6 MACS method optimalisation

The aim of doing the MACS was to get a clean B-cell population so that gene expression of L-lineage could be examined. The purity of the MACS was analysed using flow cytometry with anti-trout -IgM staining, comparing the sorted cells with the flow through from the magnetic column.

In the first trial, the HKL were incubated overnight (16h) culture media with 5% FBS and the result from the Flow cytometry showed two Anti- IgM staining populations. This was probably due to macrophage Fc receptors that bind to Atlantic salmon IgM. Trout anti-IgM binds to the FcR-bound IgM. With a 72h incubation of in-culture media with 1% FBS and sorted out adherent cells, the flow cytometry shows only one anti-IgM-stained cell population. Doing the magnetic beats staining sorting (MACS) only on the suspension group increases the share of B -cells after MACS.

There was a decrease of 66% in anti-IgM between the suspension and the Flow through. This reveals a purity of 66% sorted B-cells. The qPCR shows that there was a low relative

expression of Crsfr and TCR alpha in the sorted B-cell. This means that there are some macrophages and T-cells that have been included in the B- cell batch. To get a higher purity of B-cells adjustment need to be made. Cleaning of the magnetic column with sorting buffer should have been done 1-2 times more. The magnetic column should maybe have a bigger magnetic surface and the radio between cells and antibodies should be optimised.

The gene expression of the control genes Crsfr1R and TCR alpha shows that the separation based on adherent/ non- adherent was not clean. Crsfr1R are a marker of macrophages expressed only in the adherent group. The flow cytometry shows that the number of dead cells in suspension is high. This is because macrophages-like cells that debrie during the incubation time will be collected in the rest. The RNA from macrophages will therefore be detected in suspension. The suspension was run through MACS, and the Crsfr1R were detected in the B-cell sorted group.

There was also a lower share of live anti-IgM-stained cells in trial 2 compared to trial 1, even if they were done identically. Reasons for this can be a less successful anti-IgM staining before flow cytometry, or just less viable B-cells due to incubation and isolation reasons.

5.7 L-lineage expression in B-cells?

From the experiments done in this thesis, there can't be concluded if B cell express the different L-linages because of the not optimal B-cell purity after MACS. qPCR shows gene expression of L-lineage in suspension, adherent and B-cells sorted samples. But if LIA, LGA, LHA and LDA are more expressed in adherent or suspension wearies between the trials. The relative expression of LIA in suspension varies a lot from individual to induvial. The reason for this can be that some fish are battling an infection. There was an ongoing POX virus epidemic at the Havbruksstasjonen during the period of the trials. The POX epidemic should not have affected the sampled fish, but there is always a possibility. Even if there cannot be concluded that B -cells express L-linage, this can be a hint that it does.

The previous stimulations trials show that the basal expression of the L-lineage was relatively low, which makes identifying the specific cell types that express the L-lineage challenging. This trail with stimulation primary HKL shows that L-linage, especially LIA and LGA fold

change, increases with stimulation with IFNγ. For future experiments, stimulation of the cells before sorting can make detection easier.

5.8 Future perspectives

The biological function of the ncMHC L-linages in Atlantic Salmon immunity are only starting to be tapped into, and there is a need for more studies to fully understand the role of these molecules. In this thesis, signalling induction of gene expression of the different L-lineage and establish the hypnotises that the subtypes of L-lineage ncMHC are differently induced by various stimuli. Further, the timing of the protein expression on the L-lineage cell membrane should be investigated. And what the L- lineage subtypes ncMHC binding grove present

The MACS method must also be optimised so the resulting sorted B-cell population are cleaner. Other methods with higher resolution, like Prime flow, should be used to investigate the gene expression of L-lineage in B-cells.

A method used to sort cells is Fluorescence-activated Cell sorting (FACS). FACS is a specialised type of flow cytometry that sort cells, one by one, into different containers based on specific light scattering and fluorescent characteristic of each cell. This method could be used to collect a clean B-cell population. However, the FACS method is expensive, and when doing many experiments, it may be more effective with a less advanced MACS sorting.

Another method that can be optimised and used is PrimeFlow. In fish, monoclonal and polyclonal antibodies that recognise specific cell types are scarce, making it challenging to identify and study particular populations of cells. An alternative is the PrimeFlow RNA Assay. This assay makes it possible to analyse cells one- by one by targeting the gene expression with a gene-specific primary probe par. The cells get permeabilised and incubated with a gene-specific probe that gets an Oregon nucleotide pair that will hybridise to a specific mRNA sequence. A preamplifier is added and binds to the oligo pair. The amplifier attaches to the preamplifier, and the binding is added to a label probe. The labelling probes are

fluorescently and will be detected using a flow cytometer. There can be two signals from one cell or no signal at all. But the Prime Flow are not yet optimized for fish. So, the MACS method should be optimized to control the prime flow. Designing probes to low expression targets is difficult, so even though the prime flow was planned for my master experiments, there were no optimal probes designed towards L- lineage genes and our attempts to optimize the experiments failed.

6 Conclusion

In this thesis, in vitro stimulation with IFNa, IFNc, IFNγ and Poly (I: C) has shown increased gene expression of the non-classical L-lineage genes LIA, LGA, LHA and LDA. By inhibition of the JAK1, the gene induction by the IFNs and Poly(I: C) decreased for LIA, LGA and the control gene Mx8, indicating the JAK/STAT signalling pathways are essential in gene expression.

B-cells sorting was improved due to the suspension/ adhesion sorting prior to the MACS. Investigations in L-lineage gene expression-specific cells did not show clear answers due to the not optimal B-cell sorting. The L-lineage was expressed in all the different sorted groups. Therefore, what cells have L-lineage gene expression and L-lineage protein expression should be investigated further, including other methods.

7 References

Abbas, A. K., Lichtman, A. H., & Pillai, S. (2016). *Basic Immunology: functions and disorders of the immune system* (5th Edition ed.). Elsevier.

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish & shellfish immunology*, *90*, 210-214. https://doi.org/10.1016/j.fsi.2019.04.066

Adams, E. J., & Luoma, A. M. (2013). The adaptable major histocompatibility complex (MHC) fold: structure and function of nonclassical and MHC class I–like molecules. *Annual review of immunology*, *31*, 529-561. https://doi.org/10.1146/annurev-immunol-032712-095912

Barraza, F., Montero, R., Wong-Benito, V., Valenzuela, H., Godoy-Guzmán, C., Guzmán, F., Köllner, B., Wang, T., Secombes, C. J., & Maisey, K. (2020). Revisiting the teleost thymus: current knowledge and future perspectives. *Biology*, *10*(1), 8. https://doi.org/10.3390/biology10010008

Bjørgen, H., & Koppang, E. O. (2022). *Anatomy of teleost fish immune structures and organs* (1st Edition ed.). Springer, Cham. https://doi.org/10.1007/978-3-030-85420-1_1

Chen, Z., Omori, Y., Koren, S., Shirokiya, T., Kuroda, T., Miyamoto, A., Wada, H., Fujiyama, A., Toyoda, A., & Zhang, S. (2019). De novo assembly of the goldfish (Carassius auratus) genome and the evolution of genes after whole-genome duplication. *Science Advances*, *5*(6), eaav0547. https://doi.org/10.1126/sciadv.aav0547

Decker, T., Lew, D. J., Mirkovitch, J., & Darnell Jr, J. E. (1991). Cytoplasmic activation of GAF, an IFN‐gamma‐regulated DNA‐binding factor. *The EMBO journal*, *10*(4), 927-932. https://doi.org/10.1002/j.1460-2075.1991.tb08026.x

Dougan, S., Kaser, A., & Blumberg, R. (2007). CD1 expression on antigen-presenting cells. *T Cell Activation by CD1 and Lipid Antigens*, 113-141. https://doi.org/10.1007/978-3-540-69511-0_5

Fiskeridirektoratet. (2022, 25.05). *Økt salg av oppdrettsfisk i 2021*. Fiskeridirektoratet. https://www.fiskeridir.no/Akvakultur/Nyheter/2022/okt-salg-av-oppdrettsfisk-i-2021

Flajnik, M. F. (2018). A cold-blooded view of adaptive immunity. *Nature Reviews Immunology*, *18*(7), 438-453. https://doi.org/10.1038/s41577-018-0003-9

Flajnik, M. F., & Kasahara, M. (2010). Origin and evolution of the adaptive immune system: genetic events and selective pressures. *Nature Reviews Genetics*, *11*(1), 47-59. https://doi.org/10.1038/nrg2703

Forskrift av bruk av dyr i forsøk (2015). https://lovdata.no/pro/#document/SF/forskrift/2015-06-18-761?searchResultContext=1530&rowNumber=21&totalHits=211

Grimholt, U. (2016). MHC and evolution in teleosts. *Biology*, *5*(1), 6. https://doi.org/10.3390/biology5010006

Grimholt, U., Fosse, J. H., & Sundaram, A. Y. (2020). Selective stimulation of duplicated Atlantic salmon MHC pathway genes by interferon-gamma. *Frontiers in immunology*, *11*, 571650-571650. https://doi.org/10.3389/fimmu.2020.571650

Grimholt, U., Tsukamoto, K., Azuma, T., Leong, J., Koop, B. F., & Dijkstra, J. M. (2015). A comprehensive analysis of teleost MHC class I sequences. *BMC evolutionary biology*, *15*(1), 32. https://doi.org/10.1186/s12862-015-0309-1

Grimholt, U., Tsukamoto, K., Hashimoto, K., & Dijkstra, J. M. (2019). Discovery of a novel MHC class I lineage in teleost fish which shows unprecedented levels of ectodomain deterioration while possessing an impressive cytoplasmic tail motif. *Cells*, *8*(9), 1056. https://doi.org/10.3390/cells8091056

Haller, O., Staeheli, P., Schwemmle, M., & Kochs, G. (2015). Mx GTPases: dynamin-like antiviral machines of innate immunity. *Trends in microbiology*, *23*(3), 154-163. https://doi.org/10.1016/j.tim.2014.12.003

Hedfors, I. A., Bakke, H., Skjødt, K., & Grimholt, U. (2012). Antibodies recognizing both IgM isotypes in Atlantic salmon. *Fish & shellfish immunology*, *33*(5), 1199-1206. https://doi.org/10.1016/j.fsi.2012.09.009

Jenberie, S., Thim, H. L., Sunyer, J. O., Skjødt, K., Jensen, I., & Jørgensen, J. B. (2018). Profiling Atlantic salmon B cell populations: CpG-mediated TLR-ligation enhances IgM secretion and modulates immune gene expression. *Scientific reports*, *8*(1), 3565. https://doi.org/10.1038/s41598-018-21895-9

Jørgensen, S. M., Lyng-Syvertsen, B., Lukacs, M., Grimholt, U., & Gjøen, T. (2006). Expression of MHC class I pathway genes in response to infectious salmon anaemia virus in Atlantic salmon (Salmo salar L.) cells. *Fish & shellfish immunology*, *21*(5), 548-560. https://doi.org/10.1016/j.fsi.2006.03.004

Kasinrerk, W., Baumruker, T., Majdic, O., Knapp, W., & Stockinger, H. (1993). CD1 molecule expression on human monocytes induced by granulocyte-macrophage

colony-stimulating factor. *Journal of immunology (Baltimore, Md.: 1950)*, *150*(2), 579-584. https://doi.org/10.4049/jimmunol.150.2.579

Kumagai, K., Itoh, K., Hinuma, S., & Tada, M. (1979). Pretreatment of plastic Petri dishes with fetal calf serum. A simple method for macrophage isolation. *Journal of immunological methods*, *29*(1), 17-25. https://doi.org/10.1016/0022-1759(79)90121-2

Lien, S., Koop, B. F., Sandve, S. R., Miller, J. R., Kent, M. P., Nome, T., Hvidsten, T. R., Leong, J. S., Minkley, D. R., & Zimin, A. (2016). The Atlantic salmon genome provides insights into rediploidization. *Nature*, *533*(7602), 200-205. https://doi.org/10.1038/nature17164

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408. https://doi.org/10.1006/meth.2001.1262

Lutfalla, G., Crollius, H. R., Stange-Thomann, N., Jaillon, O., Mogensen, K., & Monneron, D. (2003). Comparative genomic analysis reveals independent expansion of a lineage-specific gene family in vertebrates: the class II cytokine receptors and their ligands in mammals and fish. *BMC genomics*, *4*(1), 29. https://doi.org/10.1186/1471-2164-4-29

Maccari, G., Robinson, J., Ballingall, K., Guethlein, L. A., Grimholt, U., Kaufman, J., Ho, C.-S., De Groot, N. G., Flicek, P., & Bontrop, R. E. (2017). IPD-MHC 2.0: an improved inter-species database for the study of the major histocompatibility complex. *Nucleic acids research*, *45*(D1), D860-D864. https://doi.org/10.1093/nar/gkw1050

Malik, M. S., Teige, L. H., Braaen, S., Olsen, A. B., Nordberg, M., Amundsen, M. M., Dhamotharan, K., Svenning, S., Edholm, E. S., & Takano, T. (2021). Piscine orthoreovirus (PRV)-3, but not PRV-2, cross-protects against PRV-1 and heart and skeletal muscle inflammation in Atlantic salmon. *Vaccines*, *9*(3), 230. https://doi.org/10.3390/vaccines9030230

Matsuo, A., Oshiumi, H., Tsujita, T., Mitani, H., Kasai, H., Yoshimizu, M., Matsumoto, M., & Seya, T. (2008). Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. *The Journal of Immunology*, *181*(5), 3474-3485. https://doi.org/10.4049/jimmunol.181.5.3474

Moody, D. B. (2006). TLR gateways to CD1 function. *Nature immunology*, *7*(8), 811-817. https://doi.org/ 10.1038/ni1368

Nærings- og Fiskeriderpartementet (2021). *Havbruksstraegien- Et hav av muligheter* https://www.regjeringen.no/no/dokumenter/havbruksstrategien-et-hav-av-muligheter/id2864482/

Ogg, G., Cerundolo, V., & McMichael, A. J. (2019). Capturing the antigen landscape: HLA-E, CD1 and MR1. *Current Opinion in Immunology*, *59*, 121-129. https://doi.org/10.1016/j.coi.2019.07.006

Pestka, S., Langer, J. A., Zoon, K. C., & Samuel, C. E. (1987). Interferons and their actions. *Annual review of biochemistry*, *56*, 727-777.

Platanias, L. C. (2005). Mechanisms of type-I-and type-II-interferon-mediated signalling. *Nature Reviews Immunology*, *5*(5), 375-386. https://doi.org/10.1038/nri1604

Porcelli, S., Morita, C. T., & Brenner, M. B. (1992). CDlb restricts the response of human CD4− 8− T lymphocytes to a microbial antigen. *Nature*, *360*(6404), 593-597. https://doi.org/10.1038/360593a0

Press, C. M., & Evensen, Ø. (1999). The morphology of the immune system in teleost fishes. *Fish & shellfish immunology*, *9*(4), 309-318. https://doi.org/10.1006/fsim.1998.0181

Prigozy, T. I., Sieling, P. A., Clemens, D., Stewart, P. L., Behar, S. M., Porcelli, S. A., Brenner, M. B., Modlin, R. L., & Kronenberg, M. (1997). The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. *Immunity*, *6*(2), 187-197. https://doi.org/10.1016/S1074-7613(00)80425-2

Robertsen, B. (2006). The interferon system of teleost fish. *Fish & shellfish immunology*, *20*(2), 172-191. https://doi.org/10.1016/j.fsi.2005.01.010

Robertsen, B. (2008). Expression of interferon and interferon-induced genes in salmonids in response to virus infection, interferon-inducing compounds and vaccination. *Fish & shellfish immunology*, *25*(4), 351-357. https://doi.org/10.1016/j.fsi.2008.02.004

Robertsen, B. (2018). The role of type I interferons in innate and adaptive immunity against viruses in Atlantic salmon. *Developmental & Comparative Immunology*, *80*, 41-52. https://doi.org/10.1016/j.dci.2017.02.005

Robertsen, B., Bergan, V., Røkenes, T., Larsen, R., & Albuquerque, A. (2003). Atlantic salmon interferon genes: cloning, sequence analysis, expression, and biological activity. *Journal of Interferon & Cytokine Research*, *23*(10), 601-612. https://doi.org/10.1089/107999003322485107

Robertsen, B., Greiner-Tollersrud, L., & Jørgensen, L. G. (2019). Analysis of the Atlantic salmon genome reveals a cluster of Mx genes that respond more strongly to IFN gamma than to type I IFN. *Developmental & Comparative Immunology*, *90*, 80-89. https://doi.org/10.1016/j.dci.2018.09.004

Sahoo, B. R. (2020). Structure of fish Toll-like receptors (TLR) and NOD-like receptors (NLR). *International journal of biological macromolecules*, *161*, 1602-1617. https://doi.org/10.1016/j.ijbiomac.2020.07.293

Sanchez, D. J., Gumperz, J. E., & Ganem, D. (2005). Regulation of CD1d expression and function by a herpesvirus infection. *The Journal of clinical investigation*, *115*(5), 1369-1378. https://doi.org/10.1172/JCI200524041

Seif, F., Khoshmirsafa, M., Aazami, H., Mohsenzadegan, M., Sedighi, G., & Bahar, M. (2017). The role of JAK-STAT signaling pathway and its regulators in the fate of T helper cells. *Cell communication and signaling*, *15*, 1-13. https://doi.org/10.1186/s12964-017-0177-y

Shum, B. P., Guethlein, L., Flodin, L. R., Adkison, M. A., Hedrick, R. P., Nehring, R. B., Stet, R. J., Secombes, C., & Parham, P. (2001). Modes of salmonid MHC class I and II evolution differ from the primate paradigm. *The Journal of Immunology*, *166*(5), 3297-3308. https://doi.org/10.4049/jimmunol.166.5.3297

Sieling, P. A., Jullien, D., Dahlem, M., Tedder, T. F., Rea, T. H., Modlin, R. L., & Porcelli, S. A. (1999). CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity. *The Journal of Immunology*, *162*(3), 1851-1858. https://doi.org/10.4049/jimmunol.162.3.1851

Smed-Sörensen, A., Moll, M., Cheng, T.-Y., Loré, K., Norlin, A.-C., Perbeck, L., Moody, D. B., Spetz, A.-L., & Sandberg, J. K. (2008). IgG regulates the CD1 expression profile and lipid antigen-presenting function in human dendritic cells via FcγRIIa. *Blood, The Journal of the American Society of Hematology*, *111*(10), 5037-5046. https://doi.org/10.1182/blood-2007-07-099549

Smith, N. C., Rise, M. L., & Christian, S. L. (2019). A comparison of the innate and adaptive immune systems in cartilaginous fish, ray-finned fish, and lobe-finned fish. *Frontiers in immunology*, *10*, 2292. https://doi.org/10.3389/fimmu.2019.02292

Solbakken, M. H. (2016). *Evolutionary and functional insight into the teleost immune system—lessons learned from Atlantic cod and other teleosts* [PhD, University of Oslo]. https://www.duo.uio.no/handle/10852/77174

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., & Schreiber, R. D. (1998). How cells respond to interferons. *Annual review of biochemistry*, *67*(1), 227-264. https://doi.org/10.1146/annurev.biochem.67.1.227

Svenning, S., Gondek-Wyrozemska, A. T., van der Wal, Y. A., Robertsen, B., Jensen, I., Jørgensen, J. B., & Edholm, E.-S. (2019). Microbial danger signals control

transcriptional induction of distinct MHC class IL lineage genes in Atlantic Salmon. *Frontiers in immunology*, 2425. https://doi.org/10.3389/fimmu.2019.02425

Svingerud, T., Holand, J. K., & Robertsen, B. (2013). Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell culture. *Virus research*, *177*(2), 163-170. https://doi.org/10.1016/j.virusres.2013.08.004

Svingerud, T., Solstad, T., Sun, B., Nyrud, M. L. J., Kileng, Ø., Greiner-Tollersrud, L., & Robertsen, B. (2012). Atlantic Salmon Type I IFN subtypes show differences in antiviral activity and cell-dependent expression: evidence for high IFNb/IFNc–producing cells in fish lymphoid tissues. *The Journal of Immunology*, *189*(12), 5912-5923. https://doi.org/10.4049/jimmunol.1201188

Thompson, J. E., Cubbon, R. M., Cummings, R. T., Wicker, L. S., Frankshun, R., Cunningham, B. R., Cameron, P. M., Meinke, P. T., Liverton, N., & Weng, Y. (2002). Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor. *Bioorganic & medicinal chemistry letters*, *12*(8), 1219-1223. https://doi.org/10.1016/S0960-894X(02)00106-3

United Nations. (2022). *The Sustainable Development Goals Report*. https://unstats.un.org/sdgs/report/2022/The-Sustainable-Development-Goals-Report-2022.pdf

Van Den Elsen, P. J. (2011). Expression regulation of major histocompatibility complex class I and class II encoding genes. *Frontiers in immunology*, *2*, 48. https://doi.org/10.3389/fimmu.2011.00048

Verhelst, J., Hulpiau, P., & Saelens, X. (2013). Mx proteins: antiviral gatekeepers that restrain the uninvited. *Microbiology and Molecular Biology Reviews*, *77*(4), 551-566. https://doi.org/10.1128/MMBR.00024-13

Veterinærinstituttet. (2023). *Fiskehelserapporten 2022* (5a/2023). https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2023/fiskehelserapporten-2022

Yamaguchi, T., & Dijkstra, J. M. (2019). Major histocompatibility complex (MHC) genes and disease resistance in fish. *Cells*, *8*(4), 378. https://doi.org/ 10.3390/cells8040378

Yamaguchi, T., Takizawa, F., Furihata, M., Soto-Lampe, V., Dijkstra, J. M., & Fischer, U. (2019). Teleost cytotoxic T cells. *Fish & shellfish immunology*, *95*, 422-439. https://doi.org/10.1016/j.fsi.2019.10.041

Ziegler, F., Jafarzadeh, S., Skontorp Hognes, E., & Winther, U. (2022). Greenhouse gas emissions of Norwegian seafoods: From comprehensive to simplified assessment. *Journal of Industrial Ecology*, *26*(6), 1908-1919. https://doi.org/10.1111/jiec.13150

Zwollo, P. (2018). The humoral immune system of anadromous fish. *Developmental & Comparative Immunology*, *80*, 24-33. https://doi.org/10.1016/j.dci.2016.12.008

Zwollo, P., Haines, A., Rosato, P., & Gumulak-Smith, J. (2008). Molecular and cellular analysis of B-cell populations in the rainbow trout using Pax5 and immunoglobulin markers. *Developmental & Comparative Immunology*, *32*(12), 1482-1496. https://doi.org/10.1016/j.dci.2008.06.008

8 Appendix 1

8.1 Figures

Gene expression was investigated for the L-lineage LGA and LIA. Control genes were MX1/2 that literature shows respond to Type I IFN and Mx8 that has been shown to respond to type II IFNs (Robertsen et al., 2019).

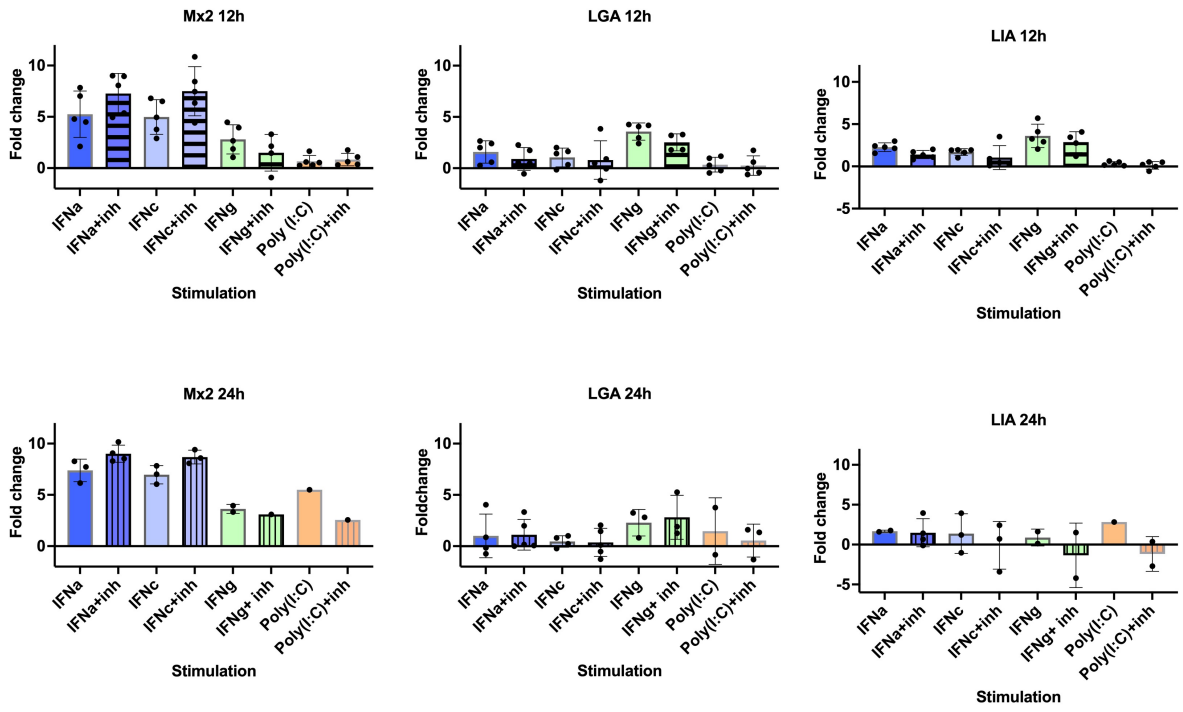


Figure 33: **Gene expression of Mx1/2, LGA and LIA stimulated primary HKL with inhibitor** JAK1 Inhibitor (Calbiochem) **at the timepoint 12h and 24h**. Fold change in the expression of Mx1/2, LGA and LIA in adult Atlantic salmon incubated in vitro with the stimulations . Each dot represents an individual fish. The fold change was calculated against the relative expression of media for the individual fish against the reference gene EF1a (ref, Appendix) and presentet as LOG: 2. The result was normally distributed and tested with normal one-way ANOVA.

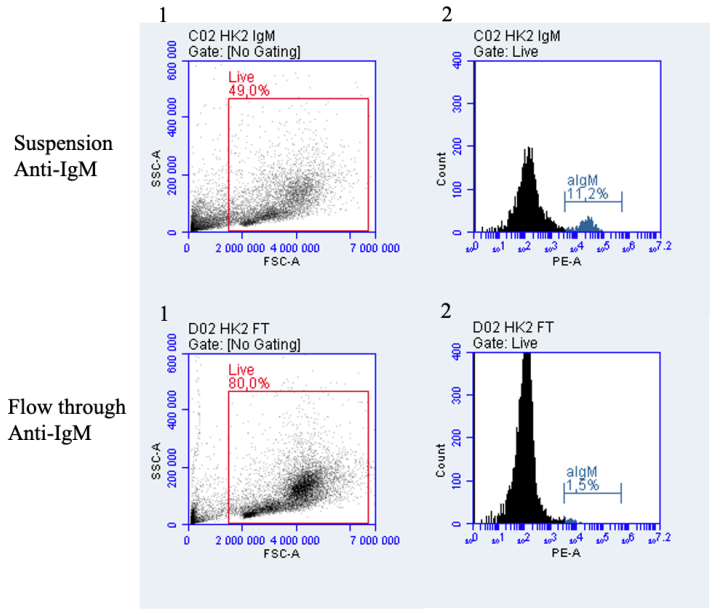


Figure 34:**Flow cytometry result of anti-trout IgM staining of suspension and adhesion sortet HKL from Adult salmon HKL nr2 .** 1) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells and flow through after MACS. Within the red gate there are living cells 2) Shows light absorbance of anti-trout IgM stained, live cells (PE-A)

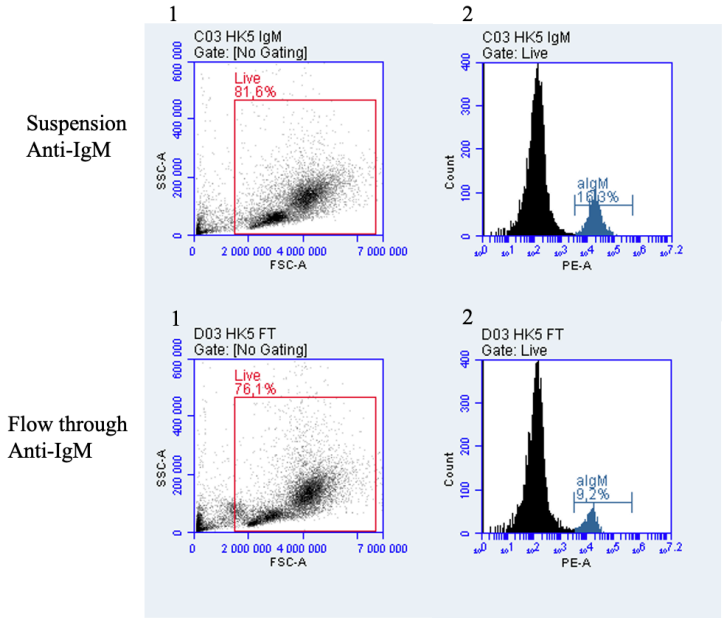


Figure 35: **Flow cytometry result of anti-trout IgM staining of suspension and adhesion sortet HKL from Adult salmon HKL nr3.** 1) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells and flow through after MACS. Within the red gate there are living cells 2) Shows light absorbance of anti-trout IgM stained, live cells (PE-A)

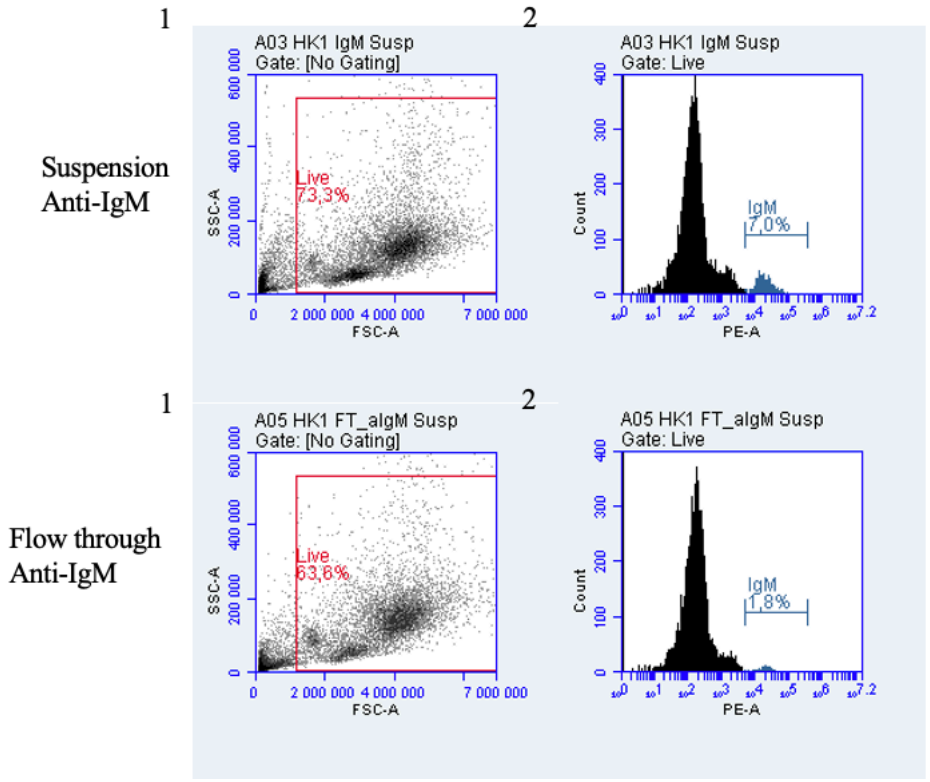


Figure 36: **Flow cytometry result of anti-trout IgM staining of suspension and adhesion sortet HKL from one of three Adult salmon HKL from 2. Trail MACS.** 1) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells and flow through after MACS. Within the red gate there are living cells 2) Shows light absorbance of anti-trout IgM stained, live cells (PE-A)

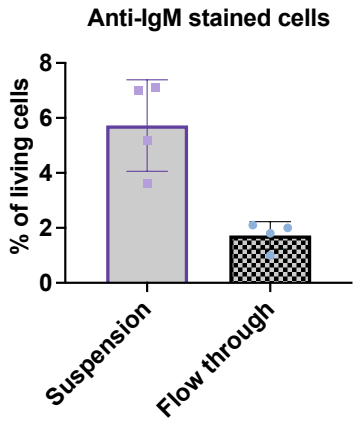


Figure 37: ***Comparing the % counter, live, Anti- Trout IgM stained HKL cells sorted in suspension and flow through from MACS from adoult grown salmon.*** *Each dot represents an individual fish. Significant level was calculated.*

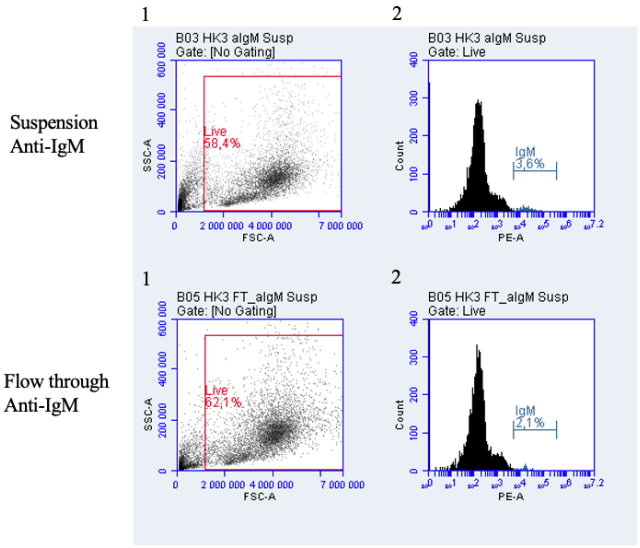


Figure 38: **Flow cytometry result of anti-trout IgM staining of suspension and adhesion sortet HKL from Adult salmon HKL nr3. Trail 2** 1) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells and flow through after MACS. Within the red gate there are living cells 2) Shows light absorbance of anti-trout IgM stained, live cells (PE-A)

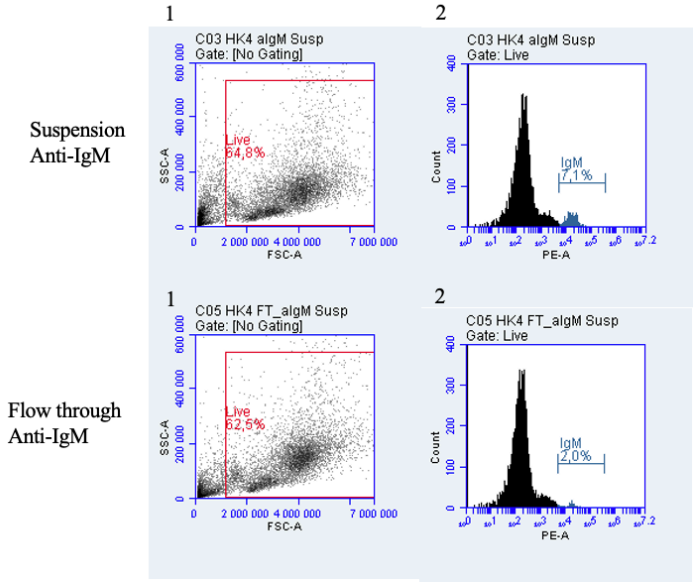


Figure 39: **Flow cytometry result of anti-trout IgM staining of suspension and adhesion sortet HKL from Adult salmon HKL nr3. Trail 2** 1) The Plot represents HKL size and complexity in forward (FSC-A) and side scatter (SSC-A) of suspension cells and flow through after MACS. Within the red gate there are living cells 2) Shows light absorbance of anti-trout IgM stained, live cells (PE-A)

8.2 Formulas

Equation 1 DCt*:*

$\Delta \mathrm{Ct} = \mathrm{Ct\ sample} - \mathrm{Ct\ relative\ "houskeeping"\ gene}$

Relative expression:

Equation 2: Relative expression

$Relative\ expression: 2^{-\Delta \mathrm{Ct}} = 2^{-(Ct\ gene - Ct\ referanse\ gene)}$

Fold change

Equation 3: Fold change

$$Fold\ change: \frac{Relative\ expression\ sample}{Relative\ expression\ media}$$

DDCt method for gene relative expression (Livak & Schmittgen, 2001):

Equation 4: ΔΔCt-method

ΔΔCt=DCt sample- ΔCt Referance gene

Relative expression= $2^{-\Delta\Delta Ct}$

Gubbes test to quantify outliner:

Equation 5: Gubbes test to quantify outliners

$$Z = \frac{Mean - value}{SD}$$

Based on a normal distribution and a test statistic (Z) that is calculated from the most extreme data pint. The test statistic corresponds to a p-value that represent the likelihood of seeing that outliner assuming the underlying data is gaussian.

8.3 Reagents

Table 2: Reagents: Name, Produced and Catalog no.

	Reagents		**Producer**	**Catalog no.**	**Notes**
Cell collection and isolation	Countess™ Cell Counting Chamber Slides		Invitrogen™	C10228	Used with Countess™ 3 Automated Cell Counter; Catalog number: AMQAX2000
	Heparin 5000 IE/ml		LEO (LEO Pharma Norge AS)	464327	Heparinnatrium 5000 IE/a.e./ml
	Leibovitz's L-15 Medium		Gibco™	11415-049	
	NaCl 1,5M				
	Penicillin-Streptomycin (10,000 U/mL)	P/S	Gibco™	15140122	
	Percoll TM stock		Cytiva™	17-0891-01	
	RNAlater™ Stabilization Solution		Invitrogen™	AM7021	
	HyPure Molecular Biology Grade Water (Nuclease free, Deonized,		Cytiva™	SH30538.02	

	Distilled, 0,1 um Serile Filtered)				
	RNeasy Mini Kit		Qiagen, Hilden, Germany	74106	
	Fetal Bovine Serum (FBS)	FBS	Biowest, South America	S1810	
	Trypan Blue Stain 0,4%		Thermo Fisher Scientific Inc.	T10282	
	Trypsin- EDTA solution		Sigma-Aldrich C	T4049-100ML	
DNase treatment	DNAse buffer x10 Reaction Buffer with MgCL2		Thermo Fisher Scientific Inc.	01095433	
	Dnase, Rnase-free		Thermo Fisher Scientific Inc.,	01107503	
	EDTA 50 mM		Thermo Fisher Scientific Inc.	01090869	
cDNA syntheses	10mM dNTP Mix (w dTTP)		TaqMan® Reverse Transcription Reagents	N8080234	
	10x RT buffer				
	MgCl2 25mM				
	Random Hexamers,				
	RNAse inhibitor				
	MultiScribe™ Reverse Transcriptase		Invitrogen™	4311235	

qPCR	MicropAmp Fast 96-well Reaction Plate (0,1 ml)		Applied biosystems by life technologies	4346907	
	SYBR TM Select Master Mix SGreen Slow		Applied biostems, Thermo Fisher Scientific Inc.		
MACS and flow cystometry	Anti-Mouse IgG1 MicroBeads		Miltenyi Biotec	130-047-102	
	Bovine Serum Albumin heat shock fration, pH, >98%	BSA	Sigma-Aldrich R	A9647-100G	
	Dulbecco`s Phosphate Buffer waline w /o Magnesium, W/o Calcium Sterile Filter	PBS	Biowest	S1810-100	MS01D5
	EDTA 2mEDTA		Thermo Scientific		
	Fixable Viability Dye eFluor^TM 780		Invitrogen	L34977	
	MS Columns		Miltenyi Biotec	130-042-201	

8.4 Instruments

Table 3: Instruments used and producer

Instruments	**Producer**
Contess™ FL Automated Cell Counter	Introgen™ (Katlognr: 311986)
NanoDrop 1000 Spectrophotometer	Thermo Scientific™
7500 Fast Real-Time PCR	Applied Biosystem
Flow Cytometer	AB Accuri TM C6 Plus

8.5 Protocols and settings

8.5.1 Cell isolation

TABLE: overview of components used in making transport media, culture media and Percoll ingredients.

Solution	**Components**	**Volume**
Transport media	L-15 FBS Heparin P/S	 820ml 162ml 5ml
Culture Media	L-15 P/S FBS	 5ml 5% for suspension cultures
L-15 media	L-15 FBS	41ml 820ml

	Heparin	163ml
90% Percoll	Percoll stock 1,5M NaCl	90ml 10ml (3ml 5M NaCl + 7ml dH_2O
54% Percoll	90% Percoll L-15 media	59ml 41ml
25% Percoll	90% Percoll PBS 1X Heparin	28ml 72ml 288ml

8.5.2 DNase treatment

TABLE: DNase treatment

Reagents	**Volume µl**
1ug RNA + RNA free water	8,5
Master mix: 10DNAse buffer DNase	1,5 1,0 0,5
Total volume	10

8.5.3 cDNA syntheses

TABLE: Preparation for cDNA syntheses per sample:

Reagents	**Volume µl per reaction**
Template:	9,6

RNA+ RNA free water	
Mastermix: 10x RT buffer $MgCl_2$ 19mM dNTP Random Hexamers, RNAse inhibitor Megacript RT	 2,0 1,4 4,0 1,0 1,0 1,0
Total volume:	20

8.5.4 qPCR

TABLE: Reactions set up qPCR per sample

Reagents per	**Volume** ml
SYBR Green Slow	5,00
Forward primer	0,05
Reverse primer	0,05
H_20	3,90
sample	1,00
Total volume	10,00