Adaptive Humoral Immunity in Atlantic cod (*Gadus morhua*), a Teleost Fish with Evolutionary Losses of MHC II and CD4

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Abbreviations

APC	Antigen-presenting cells
BCR	B-cell receptor
Cas	CRISPR associated protein
CD	Cluster of differentiation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAMPs	Damage-associated molecular pattern molecules
DC	Dendritic cell
DNA	Deoxyribonucleic acid
Ig	Immunoglobulin
ITAMs	Immunoreceptor tyrosine-based activation motifs
MALT	mucosa-associated lymphoid tissue
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
NK cell	Natural Killer cells
PAMPs	Pathogen-associated molecular patterns
pMHC	peptide-MHC complex
PRR	Pattern-recognition receptors
RAG	Recombination activating gene
RNA	Ribonucleic acid
RSS	Recombination signal sequences
TCR	T-cell receptor
TD	T-cell dependent
TI	T-cell independent
TI-1	T-cell independent type 1
TI-2	T-cell independent type 2
TLR	Toll-like receptors
V(D)J	Variable (diversity) and joining-type segments
VLR	Variable lymphocyte receptor

Summary

The main features of our adaptive immune system (AIS) originated about 400 million years ago and are common to all jawed vertebrates. B cells are central to this system, generating a diverse range of specific antibodies for pathogen neutralization. The Major Histocompatibility Complex class I (MHC I) and class II (MHC II) molecules play key roles in the vertebrate's AIS. The MHC II molecules are crucial in antigen presentation to CD4+ T cells, leading to a coordinated immune response and T cell help for antibody production, ensuring a precise reaction against pathogens. Until recently, it was widely assumed that the key features of our AIS, evolved through a series of genetic traits, had been consistently preserved across all jawed vertebrates. However, the AIS of the Atlantic cod (*Gadus morhua*) presents a unique divergence from the traditional vertebrate AIS model. The Atlantic cod has lost the CD4 gene, the MHC II locus and other genes required for the MHC II antigen presentation pathway. Consequently, this results in a distinct paradigm of immune responses in the Atlantic cod compared to other vertebrates.

The evaluation of B cell immune responses, which lead to antibody production, is pivotal for understanding induction of adaptive immunity and mechanisms of defense. Haptens are molecules that can be recognized by surface (B cell receptor) or secreted antibodies but are too small to elicit an antibody response unless conjugated to a carrier molecule. Together, the hapten-carrier conjugate constitutes an antigen and the nature of the carrier determines which pathway of B cell activation that may be engaged, allowing for the investigation of antibody response, specificity and memory. Bacterial vaccination and infection models provide insight into B cell responses to real pathogens, revealing aspects of immune memory and allowing investigation into protective factors needed for host immunity.

The advent of genome-editing technologies like CRISPR/Cas9 has enabled precise manipulation of specific genes and pathways to dissect their roles in immune responses. By integrating classical methods with CRISPR/Cas9, researchers can now achieve an unprecedented level of detail and specificity in probing B cell biology, paving the way for deeper understanding and potential therapeutic advancements in immunology.

The first manuscript delved into the antibody responses of Atlantic cod to various haptenized carriers, which employ different pathways of B cell activation. We found that Atlantic cod produced minimal pre-existing antibodies against 4-Hydroxy-3-nitrophenylacetyl (NP), making it a suitable hapten for further investigation. Upon immunization, the Atlantic cod did not develop an antibody immune response against T cell-dependent (TD) or T cell-independent type 1 (TI-1) carrier antigens. However, a robust and enduring NP-specific antibody response was observed when the fish were immunized with T cell-independent type 2 (TI-2) antigens, lasting up to six months post-immunization. These findings challenge the conventional belief that Atlantic cod have a limited antibody response, highlighting their capacity to generate a potent and long-lasting antibody response, especially when exposed to antigens associated with a TI-2 cellular and molecular mechanism.

In the second manuscript, we challenged the prevailing belief that antibody responses are not important for the observed immunity after vaccination with *Vibrio anguillarum* in Atlantic cod. When exposed to formalin-fixed *V. anguillarum* serotype O2a through bath immunization, the cod produced substantial serum IgM levels that effectively interacted with the bacterial antigens. This resulted in strong immunity against both mucosal and systemic infections by *V. anguillarum* O2a, and partial protection against a different serotype, O2b, with the immunity lasting for over a year. Further investigations revealed that passive vaccination of naïve fish with immune serum or IgM from vaccinated fish conferred protection against a lethal *V. anguillarum* O2a infection,

highlighting the protective role of immune IgM. Notably, the immune serum IgM targeted proteinaceous antigens rather than the LPS O-antigen. This study fundamentally revises our understanding of the Atlantic cod's immune capabilities, proving their ability to mount strong, protective antibody responses against bacterial pathogens even without key immune system components.

In the third manuscript, we established a gene knockout platform for future targeting of immune genes or cells in Atlantic cod. For proof-of-principle studies, we targeted the *slc45a2* gene, linked to pigmentation for inactivation in Atlantic cod embryos. Our evaluation of five CRISPR configurations highlighted the combination of Cas9 mRNA and a single guide RNA as the most effective, yielding a visible albino-like phenotype in 75% of the surviving larvae. Unfortunately, the high mortality rate among injected eggs resulted in overall low efficiency and future research should address this challenge. However, we successfully identified indel mutations in all albino-like phenotype larvae, setting a foundational basis for future research. The advancements made in this study hold promising implications for the broader field of the study of the adaptive immune system, specifically in the exploration of B cells and other immune cell populations in Atlantic cod.

In summary, we demonstrated that Atlantic cod produces potent, long-lasting, specific antibody responses, enriching our comprehension of their antibody capabilities. Further, we revealed their ability to generate specific antibody-based protection against bacterial pathogens, even in the absence of key immune system components. We also established a CRISPR/Cas9-based gene knockout platform in Atlantic cod that paves the way for future genetic and immunological studies, particularly on B and T cells. These findings collectively deepen our understanding of adaptive humoral immunity in Atlantic cod, with potential implications for vaccine development.

INTRODUCTION

1 Background

1.1 The immune systems(s)

The word "immunity" is derived from the Latin *immunis*, which means "to be exempted from public service" or "not to pay one's taxes" (Doherty & Turner, 2007). The term "immunity" is used to describe the biological systems and processes that protect organisms from disease and other harmful substances. The term still has its original liberating meaning, as the immune response often results in an organism being exempt from (protected from) harmful pathogens, especially when the same pathogen is encountered a second time. This is because it has been observed that people who survive an infectious disease usually do not become affected by the same disease again.

Different versions of an immune system can be found throughout different taxa, from bacteria to higher vertebrates, with the sole aim to act as a protective mechanism. The immune system can be classified as either innate or adaptive, as will be remarked later, these concepts are merely for a better comprehension of this topic rather than a factual biological categorization. The innate immune system is characterized by a ready-to-act mechanism while the adaptive immune response implies the development of a memory-induced mechanism after a first encounter with entities recognized as non-self. Bacteria, for instance, carry in their genome what can be classified as an innate immune system such as the restriction-modification system and other systems that can recognize and modify foreign conserved specific sequence motifs, cleaving or degrading unmodified foreign bacteriophage (virus that attacks bacteria) DNA (Vasu & Nagaraja, 2013). Additionally, bacteria have a so-called adaptive immune system known as the CRISPR-Cas system, which can protect them against subsequent viral infections. This system operates in two main stages: the adaptation phase, during which a set of Cas proteins facilitates the incorporation of new and specific spacers derived from bacteriophages, and the interference phase, during which these Cas proteins, with a CRISPR RNA sequence derived from the spacer, identify and break down the infecting bacteriophage's nucleic acids (Bernheim & Sorek, 2020).

Vertebrates possess more intricate and complex innate and adaptive immune systems. The innate immune system is the first line of defense of the body against pathogens, providing immediate, non-specific protection (Zimmerman et al., 2010). It comprises physical barriers, different types of non-immune and immune cells, and chemical and enzymatic defenses. Components of this system trigger inflammation to contain infection immediately. By recognizing conserved molecular structures present in many pathogens through germline encoded pattern-recognition receptors (PRRs), the innate immune system responds in a fast fashion, from seconds to minutes, serving as a crucial sentinel in the overall immune defense as well, while also triggering the more specific adaptive immune system. In contrast, the B and T cells, lymphocytes originated from a common precursor during lymphopoiesis, are the main players of the adaptive immune system, having high diversity and specificity given not through germline-encoded genes but through a genomic rearrangement process known as somatic recombination (Shortman & Wu, 1996). The specific and unique receptors generated through this process in these cells are surface proteins known as B-cell receptors (BCR) and T-cell receptors (TCR), on B and T cells, respectively. BCRs and TCRs recognize epitopes, also known as antigenic determinants. Epitopes are specific portions of an antigen, which is defined as any substance recognized by the adaptive immune system. BCRs and TCRs recognize epitopes through different mechanisms to initiate an

immune response. In general, epitopes can range from small sequences of amino acids to specific groups of non-proteinaceous antigens. Based on their structural attributes, they can be linear, consisting of continuous amino acid sequences reflecting the antigen's primary structure, or conformational, comprising non-sequential amino acid sequences influenced by the protein's tertiary structure (Wu et al., 2016).

The adaptive immune system has been described to be present in all vertebrates. Jawed and jawless vertebrates diverged around 400 million years ago and although both groups bear an adaptive immune system, unique instances exist in jawless vertebrates such as lampreys and hagfish (Sutoh & Kasahara, 2021). In these animals, lymphocytes do not produce surface immunoglobulins; instead, they generate variable lymphocyte receptors (VLRs). This is a clear example of convergent evolution, as VLRs have been shown to function similarly to TCRs and BCRs in jawed vertebrates. In lampreys, there are three different types of lymphocytes based on different expressed VLRs: VLRA+, VLRB+, and VLRC+ (Hirano et al., 2013). Upon examining the gene-expression patterns of these lymphocyte populations, it is apparent that lymphocytes bearing the VLRB receptor gene expression resemble that of mammalian B cells in many ways. For instance, VLRB+ have been reported to express genes that are similarly expressed in B cells such as paired box 5 (*PAX5*), B lymphocyte-induced maturation protein 1 (*BLIMP1*), B cell lymphoma 6 (*BCL6*), B cell adaptor for phosphoinositide 3-kinase (*BCAP*), *IL8*, *IL17R*, and B cell maturation antigen (*BCMA*) (Das et al., 2019; Guo et al., 2009; Hirano et al., 2013). In contrast, lymphocytes bearing either the VLRA or VLRC have gene expression patterns more closely align with that of mammalian T cells. Despite differences in between the jawed and jawless vertebrates' adaptive immune system, they still have one in common, the use of activation-induced cytidine deaminase for receptor diversification (Sutoh & Kasahara, 2021).

1.2 The immune system of mammals

The immune system in mammals comprises different organs, tissues, and cells bearing hallmark molecules that jointly orchestrate the duty of keeping body's homeostasis. This goal is achieved by keeping body's functions within a normal range that includes keeping away potential pathogens and harmful non-biological entities, as well as protecting itself from anomalous own cells (Evans et al., 2015).

In mammals, the primary, also known as central, lymphoid organs are the thymus and the bone marrow where lymphopoiesis takes place. B and T cells originate in the bone marrow and while B cells mature in this same site, T cells migrate and mature in the thymus (Boehm et al., 2012). Eventually, mature B and T cells will enter circulation and migrate to peripheral, also known as secondary, lymphoid organs which are the spleen, lymph nodes, and mucosa-associated lymphoid tissue (MALT) (e.g., the gut, the nasal and respiratory tract, the urogenital tract, and other mucosa). Importantly, these peripheral lymphoid organs serve as sites to encounter antigens either carried on by dendritic cells (DCs) or in a soluble form transported via afferent lymphatics to be presented to T and B cells (Batista & Harwood, 2009).

Although the division of the immune system into innate and adaptive is largely an artificial categorization rather than a strictly functional biological division (Cooper, 2010). It is useful for the purpose of studying the immune system, timeframe, and its different characteristics according to the cells involved. Thus, it is important to keep in mind that these two systems are highly integrated and continually interact to maintain an organism's homeostasis.

1.2.1 The innate immune system

1.2.1.1 Physical and chemical barriers

Physical and chemical barriers serve as the first line of defense against pathogens and any other external threats. These barriers are crucial in inhibiting the entry of microorganisms and particles that naturally make first contact to the skin, gastrointestinal tract, and other mucosal surfaces. Importantly, gut surfaces comprise a large and thin area that serves as oxygen exchange and nutrients absorption. The need for efficient exchange of nutrients also makes this site more susceptible to pathogen invasion and its thinness an easier portal for pathogens entry and spreading to systemic compartments. The mucosal surfaces, covered by cilia, mucus, and other secretions, play a vital role in protecting against potential external threats (Sperandio et al., 2015). The mucus layer contains a potent mix of antimicrobial peptides and proteins, such as lysozyme and lectins. This layer neutralizes a diverse array of microbes, trapping them along with other particles. These trapped debris are then efficiently expelled during the natural process of mucus turnover. The peristalsis in the intestine is another natural continuous process that effectively prevents pathogen colonization and contributes to excrete them out of the body (Lawley & Walker, 2013). The gastrointestinal tract forms a critical barrier against ingested pathogens. The stomach's acidic environment helps to neutralize many pathogens, while the intestines have a complex microbiota that competes with potential pathogens, limiting their ability to colonize (Smith, 2003).

1.2.1.2 Cellular and molecular components

A natural route of infection implies that pathogens need to overcome the physical and chemical barriers, or these need to be disrupted to facilitate their entry. Thus, the first line of defense encounter after this disruption is led by innate immune cells such as neutrophils, macrophages, DCs, mast cells, basophils, eosinophils, natural killer (NK) cells and innate lymphoid cells (Basset et al., 2003). What is common to all these cells is that they are able to recognize pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs) through PRRs (Tang et al., 2012). These PAMPs are conserved molecular structures found on the surfaces of pathogens, such as bacteria, viruses, fungi, and parasites. Examples of PAMPs include lipopolysaccharides (LPS) found in the outer membrane of Gram-negative bacteria, flagellin found in bacterial flagella, and double-stranded RNA (dsRNA) from viruses. On the other side, DAMPs are molecules released from damaged, stressed, or dying cells within the body (Vénéreau et al., 2015). Examples of DAMPs include high mobility group box 1 (HMGB1) protein, heat shock proteins (HSPs), and ATP. Recognition of PAMPs and DAMPs by PRRs on immune cells leads to activation of innate humoral and cellular immune responses, like production of cytokines or activation of phagocytes, respectively (Wicherska-Pawłowska et al., 2021).

Based on protein domain homology, PRRs in the innate immune system of mammals can be classified into the following five types: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs) (Akira et al., 2006; Creagh & O'Neill, 2006; D. Li & Wu, 2021). These PRRs can be localized on the cell surface (TLRs, CLRs), within the cytoplasm (NLRs, RLRs and ALRs) or in endosomes (TLRs) (Y. Li et al., 2017).

An important characteristic of the immune cells of the innate immune system is that they are able to respond against the menace very fast, in a manner of seconds, as well as be present

during chronic inflammation (Nowarski et al., 2013). These cells have the capacity to exocytose toxic granules and possess high phagocytic activity (Feng et al., 2019; Lacy & Stow, 2011). This latter process is highly important because it first helps to clear the pathogen by taking them up, processing them in cytoplasmic compartments to produce pathogen-derived antigens, and presenting them to T cells. Although different cell types have been described to be phagocytic, the process of presenting antigens is more prominent in macrophages, monocytes, B cells and DCs, commonly named antigens presenting cells (APC), being DCs professional APCs due to their capacity to activate memory T cells as well as naïve T cells (Guermonprez et al., 2002).

1.2.1.3 Humoral components

The humoral components of the innate immune system are molecules found in the extracellular fluid that provide defense against a wide range of pathogens and decaying own cells. They can either act directly against microorganisms, interact with humoral components of the adaptive immune system, or assist the cellular components of the innate immune system. The humoral component of the innate immune system includes the complement system, cytokines, acute phase proteins (APPs), pentraxins, and natural occurring antibodies (Inforzato et al., 2012; Shishido et al., 2012).

The complement system is a group of over 30 proteins that act in cascade to eliminate pathogens. These proteins can directly lyse bacteria, promote inflammation, opsonize pathogens to enhance phagocytosis, and recruit additional immune cells to the site of infection (Reddy et al., 2017). The complement system is activated through three different pathways: the classical pathway, the lectin pathway, and the alternative pathway (Harboe & Mollnes, 2008). Each has a specific role in identifying targets and forming a protease complex necessary for activating C3, a key step to which all three pathways converge. The classical pathway, includes the C1, C4, and C2 protein components that react in this order and triggered by mainly recognizing antibodies within immune complexes; the lectin pathway is similar to the classical pathway but C1 is replaced by a lectin-protease complex containing the mannose-binding lectin (MBL) or ficolin (FCN), and it recognizes microbial sugar chains found in different pathogens like the bacterial peptidoglycan and fungal β-glucans; and the alternative pathway activation relies on complex interaction of complement regulatory molecules such as factor H and properdin. It also has a role in amplifying the C3-activation initiated by the other two pathways (Walport, 2001). The C3b bound to the target forms a protease complex that activates C5, splitting it into C5a and C5b, which then becomes a scaffold for the formation of a membrane-attack complex (MAC) by C6-C9, leading to cytolytic activity. Several leukocytes, including neutrophils and macrophages, recognize the C3b bound to the target and its subsequent cleaved fragment, iC3b, functioning as opsonins. C3a and C5a, analogous peptides released from C3 and C5, respectively, show potent physiological activities that induce leukocyte chemotaxis and degranulation, promoting inflammation and allergic reactions (Sunyer et al., 2005).

Cytokines are a broad category of small proteins that play a pivotal role in cell signaling within the immune system. Produced by several immune cell types, including immune cells like macrophages, B cells, T cells, and mast cells, they function to regulate the balance between humoral and cell-based immune responses, control the maturation, growth, and responsiveness of particular cell populations, and mediate inflammation (Turner et al., 2014). Cytokines can have autocrine, paracrine, or endocrine effects and can either promote or inhibit immune responses,

depending on the context. Their multifaceted roles extend to bridging the innate and adaptive immune systems, influencing tissue repair processes, and contributing to hematopoiesis. Dysregulation of cytokine production or signaling is implicated in numerous diseases, including autoimmune disorders, allergies, and cancer (Yao et al., 2014).

Acute phase proteins (APPs) are produced mainly by the liver in response to inflammation or tissue injury, APPs have different functions that aid in the immune response (Jain et al., 2011). Some, like C-reactive protein (CRP) and mannose-binding lectin (MBL), can bind to pathogens and promote their destruction by phagocytes (Vasta et al., 1999). Others, like fibrinogen and serum amyloid A, participate in the inflammatory response.

Defensins and cathelicidins are small antimicrobial peptides present in different cells of the innate immune system, including neutrophils, macrophages, and epithelial cells (Auvynet & Rosenstein, 2009). They act by disrupting the membranes of bacteria, fungi, and some viruses, resulting in their death. Collectins and ficolins are a group of soluble PRRs that recognize and bind to specific carbohydrate patterns found on the surfaces of microorganisms, leading to opsonization or activation of the complement system (Holmskov et al., 2003).

Natural antibodies, immunoglobulins mostly of the IgM class, are produced mainly by a type of B cells named B-1 B cells without the need of prior antigen exposure. They can be polyreactive, meaning that they bind a broad range of epitopes present on pathogens or foreign molecules and initiate an immune response (Hoffman et al., 2016).

1.2.2 The adaptive immune system

In mammals, as stated earlier, the hallmark of the adaptive immune system is that after an encounter with a an antigen it can develop a specific immune response that eventually will develop into an immune memory against future encounters with the same antigen (Moser & Leo, 2010). This second immune response is specific, faster, and more robust than the first encounter and is driven by T and B cells, and the subsequent production of specific antibodies (Fig. 1) (Kurosaki et al., 2015).

The adaptive immune response relies on complex cellular interactions and molecular mechanisms with particular characteristics that makes it very different from the innate system counterpart. These cellular and molecular components allow the adaptive immune system to have the ability to recognize specific antigens (specificity), the ability to recognize a vast array of antigens (diversity), the ability to respond more effectively upon subsequent exposures (memory), and the ability to wind down the immune response after the pathogen is eliminated (self-limitation) (Müller et al., 2018).

A central process of this immune response is the antigen recognition by B and T cells. In T cells this process has a pivotal role in dictating crucial functions not only to themselves but also to B cells. This process is mediated by specialized molecules known as the Major Histocompatibility Complex (MHC) (Pishesha et al., 2022). These molecules have the sole function of binding peptides and present them to T cells. These molecules have a groove on their extracellular surface that has the ability to pocket and bind peptides forming a peptide-MHC (pMHC) complex. MHC molecules are highly polymorphic and heterozygous, features that increase the range of peptides that can be bound (Reche & Reinherz, 2003). As will be described later, T cell receptors recognize

features of a complex formed by the peptide antigen and the MHC molecule and stabilized by other molecules.

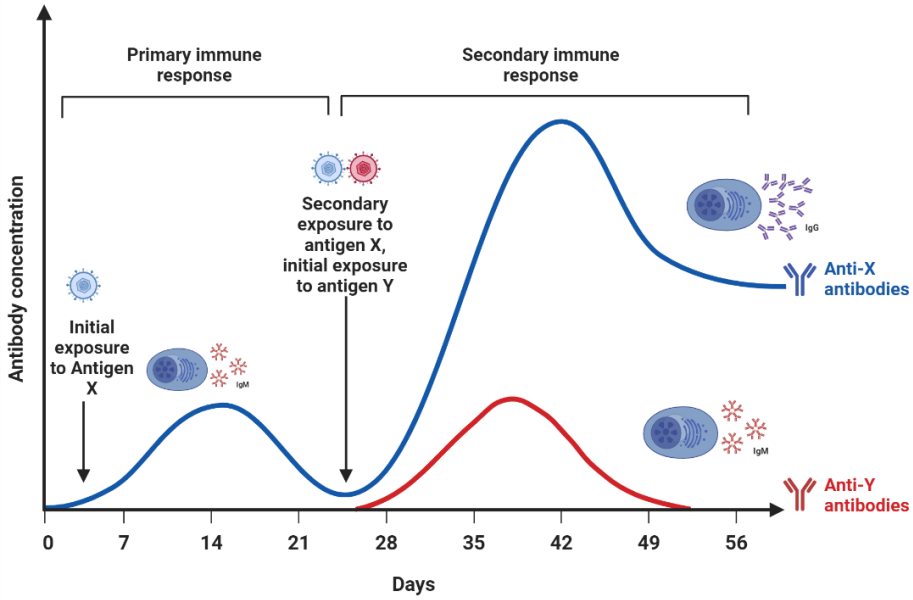

Figure 1. Schematics of the kinetics of primary and secondary immune responses. The blue curve represents the response to Antigen X. At the initial exposure to Antigen X, a primary immune response is triggered, producing IgM antibodies. Upon secondary exposure to Antigen X, the immune system recalls the antigen, leading to a heightened secondary response dominated by isotype-switched and higher-affinity IgG antibodies. The red curve indicates the response to Antigen Y, with its first encounter resulting in IgM antibody production. The primary immune response is characterized by IgM production. In contrast, the secondary immune response denotes a faster and more robust reaction upon re-encounter with an antigen, primarily involving IgG antibodies. Figure made in https://app.biorender.com.

B and T cells differ from any other type of cells in that their ultimate function of recognizing an antigen is not germline encoded. Instead, they are the outcome of the recombination of their own DNA to produce a high diversity of unique receptors to recognize specific antigens (Magadán, 2020). These receptors are known as T-cell receptor (TCR) on the surface of T cells and B cell receptor (BCR) on B cells. This molecular mechanism named somatic recombination, genes from different clusters encoding different segments of the BCR and TCR are rearranged in a manner that has been described historically as stochastically, producing a unique receptor that will bind specifically to a unique epitope on an antigen (Rees, 2020). This results in immense diversity, allowing recognition of a myriad of epitopes. Furthermore, the resultant BCR or TCR is unique per cell (Calis & Rosenberg, 2014). This is a fundamental prerequisite for adaptive specificity, selection, and memory.

T cells closely collaborate with APCs, particularly with professional APCs, the DCs. This collaboration is pivotal to the orchestration of the adaptive immune response. DCs present the antigen-derived peptides on their surfaces via MHC II molecules to activate naïve T (Fig. 2) (Germain, 1994).

Activated CD4+ T cells interact with B cells bearing pMHCII and activate them. This contact induces expression of key molecules and secretion of cytokines from CD4+ T cells (Muntasell et al., 2007). These molecules and cytokines further stimulate B cells to produce

pathogen-specific antibodies. T cells, B cells, and APCs function in a harmonized sequence to detect, combat, and maintain memory against specific pathogens, fortifying a comprehensive and persistent immune protection. B cells can specifically re-engage and stimulate CD4 effector T cells.

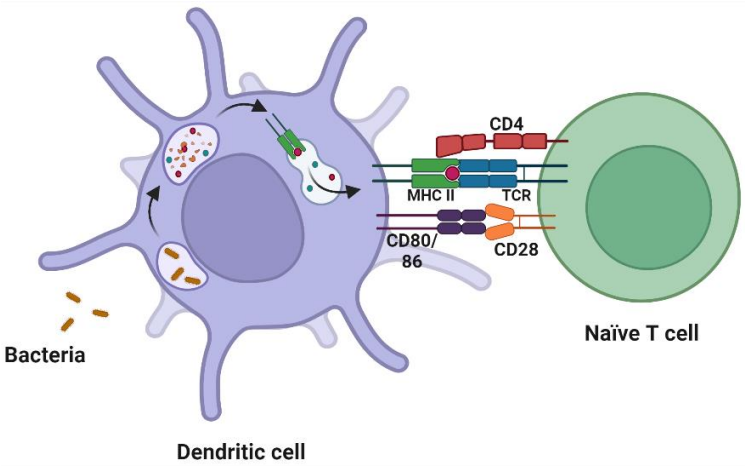

Figure 2. Antigen presentation to T cells and their activation. Extracellular antigens are taken up, processed, and presented on MHC-II molecules by antigen-presenting cells such as dendritic cells to T cells. The peptide-MHC-II complex is then recognized by naïve CD4+ T cells through their T cell receptors (TCRs) and CD4 molecules stabilize this interaction by interacting directly with the MHC-II molecule. Figure made in https://app.biorender.com.

1.2.2.1 T cells

Immunity mediated by T cells represents a critical branch of the adaptive immune system, direct antigen recognition and stimulation signals will produce different subsets with different effector functions. T cells can be categorized either as αβ or γδ T cells. The two main classes of αβ T cell subsets express either a cell-surface protein called CD4 or CD8, also known as co-receptors, and are classified accordingly (Attaf et al., 2015). Thus, CD4+ T cells are known as helper T cells, while CD8+ T cells are also known as cytotoxic T cells (Andersen et al., 2006). These αβ T cells are highly involved in the adaptive immune system, thus they will be the focus henceforth.

CD4+ helper T cells (Th cells) are central in activating B cells and stimulating these and other immune cells by secreting specific cytokines that guide different immune processes. For example, activating B cells for antibody production and macrophages for improved phagocytosis and can also exhibit cytotoxic effects on virus-infected cells (McKinstry et al., 2010). Different Th cell subsets have been historically classified based on their main secreted cytokine(s). Cytokines play a crucial role in T cell activation and in modulating the immune functions of other cells. Specific cytokines stimulating CD4+ T cells induce them to differentiate into distinct subsets, each with unique roles and attributes. The best characterized of these subsets are Th1, Th2, and Th17 cells (X. Zhu & Zhu, 2020). Th1 cells release Interferon-gamma (IFN-γ), which enhances macrophage activity, while Th2 cells produce cytokines like IL-4, IL-5, and IL-13, promoting B cell and eosinophil functions (Cope et al., 2011; J. Zhu, 2015). These two subsets, known for promoting cell-mediated and humoral responses respectively are part of a broader CD4+ T cell lineage landscape (Kim & Cantor, 2014). Th17 cells are often associated with autoimmune diseases, and follicular helper T cells assist B cells in their antibody production (Cosmi et al.,

2011). Over the past few decades, more subsets like Th22, Th9, follicular helper cells (Tfh), and various T regulatory cell (Tregs) subpopulations have been identified (Schmitt & Ueno, 2015). Tregs produce anti-inflammatory cytokines, including IL-10 and TGF-β, modulating or suppressing several immune cells, maintaining immune homeostasis, and preventing excessive inflammation and autoimmune diseases (Yamasaki et al., 2013). Within these subsets, significant heterogeneity and plasticity in cytokine production patterns are observed, particularly *in vivo*. This diversity in cytokines secretion underscores the extensive functional potential of T cells and the intricate balance required for optimal protective immunity.

CD8+ T cells have the ability to target and eliminate cells infected by intracellular pathogens, such as viruses, and transformed cells, like those found in tumors (Thakur et al., 2019). They possess a unique TCR on their surface that enables them to recognize specific peptides presented by MHC I molecules on the surface of target cells. Once a CD8+ T cell recognizes this pMHCI complex, it becomes activated and can directly kill the infected or transformed cell through the release of perforins and granzymes (Duan & Thomas, 2016). Additionally, CD8+ T cells produce several cytokines, like IFN-γ, which can further shape the immune response. Their cytotoxic function, in conjunction with CD4+ helper T cells and B cells, makes them indispensable for adaptive immunity and ensuring the body's defense against a myriad of pathogens and tumorigenic threats.

Memory T cells are a specialized subset of T cells that play a pivotal role in the adaptive immune response and beyond. After an initial encounter with a foreign pathogen, most of the activated T cells (known as effector T cells) die off following their defensive role. However, a small fraction persists and transforms into memory T cells (Ahmed & Gray, 1996). These cells have the remarkable ability to "remember" previously encountered pathogens. This means that if the same pathogen tries to invade the body again, memory T cells can recognize it swiftly and mount a faster and more robust immune response than the first time. Memory T cells can be categorized either as central memory and effector memory T cells, each with unique functions and localizations in the body (J. T. Chang et al., 2014). Central memory T cells mainly reside in lymphoid tissues, like the lymph nodes and spleen. They have a higher proliferative potential, when re-exposed to an antigen, they can expand rapidly and give rise to a vast army of effector T cells to combat the invader or anormal cells (Roberts et al., 2005). In contrast, effector memory T cells are often found in peripheral tissues and provide immediate protection against pathogens trying to re-establish an infection (Belz et al., 2020). These cells have a quicker response time than central memory T cells but do not proliferate as extensively. Both these types of memory T cells collectively ensure that the body is ready and equipped to handle repeat encounters with pathogens, providing both immediate and sustained defense mechanisms. Their long lifespan and rapid response capabilities are the foundation for the effectiveness of vaccines, which prime the immune system by generating memory T cells (Knight & Wilson, 2021).

The TCR is a heterodimer, composed of two distinct protein chains: the α and β chains, making up the αβ T cells. Another lineage, often regarded as “unconventional”, is the γδ T cells, which are characterized by a TCR constituted of γ and δ chains (Morath & Schamel, 2020). These chains have transmembrane domains and short intracellular tails that lack intrinsic signaling capability. Instead, the TCR associates with a set of signaling molecules called CD3, which consists of γ, δ , ε, and ζ chains (Fig. 3) (Birnbaum et al., 2014). These CD3 chains contain

immunoreceptor tyrosine-based activation motifs (ITAMs) which, upon antigen recognition by the TCR, become phosphorylated, triggering a cascade of intracellular signaling events leading to T cell activation. The αβ T cells, being the more abundant type of T cells, are primarily found in mammalian lymphoid organs and blood. The γδ T cells are found on mucosal sites mainly. As it was mentioned earlier, CD4+ and CD8+ T cells need to recognize peptides presented on MHC molecules, while γδ T cells are not restricted to these MHC molecules to recognize antigenic peptides (Y. Chen et al., 2022).

Each of the αβ chains has a variable (V) and a constant (C) region. The variable regions of both chains come together to form the antigen-binding site of the TCR, enabling the recognition of a specific pMHC complex (Bhati et al., 2014). The diversity in the variable regions, resulting from gene rearrangement processes during T cell development, allows for the vast array of TCRs to recognize a vast number of antigens. These genes are recombined in a similar fashion as it takes place in the V(D)J recombination in B cells (see B cells section).

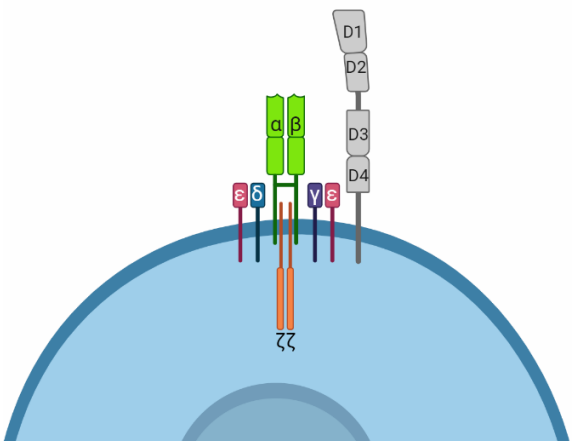

Figure 3. Schematics of the T Cell Receptor (TCR) complex and CD4 co-receptor within a T cell's membrane. α and β represent the αβ heterodimer chains of the TCR, which are vital for antigen recognition and subsequent T cell activation. The ε, γ, and δ chains make up the CD3 complex. These chains, in conjunction with the TCR, play a pivotal role in signal transduction following antigen recognition. D1, D2, D3, and D4 represent the four extracellular domains of the CD4 co-receptor. Figure made in https://app.biorender.com.

Structurally, CD8 exists mainly as a heterodimer composed of two distinct chains: CD8α and CD8β (Y. Li & Mariuzza, 2013). Each of these chains contains an extracellular Ig-like domain, a transmembrane segment, and a short cytoplasmic tail. This interaction not only stabilizes the association between the T cell and an antigen-presenting cell but also aids in signal transduction, initiating a cytoplasmic cascade leading to T cell activation. As it will be described later, the CD8αβ co-receptor plays a major role in CD8+ T cell activation by increasing antigen sensitivity and by stabilizing the TCR-pMHCI interaction at the cell surface (Holler & Kranz, 2003). There is also a CD8αα homodimer variant, which is not CD8+ T cell specific, is less common and can be found on NK cells, γδ T cells, and intestinal intra-epithelial lymphocytes, and also expressed on some dendritic cell subsets (Leishman et al., 2001; Moebius et al., 1991; Vremec et al., 2000). The CD8αβ form is found on approximately 90% of cytotoxic T cells (Norment & Littman, 1988). The functional role of the CD8αα homodimer has not been fully determined, although a regulatory

role has been proposed in the case of intestinal intra-epithelial lymphocytes (Geng & Raghavan, 2019).

The molecular structure of CD4 is composed of four extracellular immunoglobulin-like domains (D1-D4), a hydrophobic transmembrane segment, and a short intracellular tail (Y. Li & Mariuzza, 2013). The D1 domain, situated furthest from the cell surface, is responsible for binding to the MHC II expressed on antigen-presenting cells. Through this interaction it crucially stabilizes the interaction between the TCR-pMHCII (Glassman et al., 2018). This interaction enhances the specificity and affinity of the TCR for its cognate antigen presented by MHC class II. The intracellular tail of the CD4 molecule contains a specialized sequence of amino acids that can interact with the protein Lck, a tyrosine kinase. This kinase is pivotal for initiating intracellular signaling cascades upon T cell receptor engagement, leading to T cell activation. The CD4 co-receptor not only strengthens the interaction between the T cell and antigen-presenting cells but also ensures the appropriate signaling pathways activated within the T cell.

The MHC molecules are transmembrane glycoproteins encoded in the large locus known by the same name. This system is divided into two main types of MHC molecules: class I (MHC-I) and class II (MHC-II) (Pishesha et al., 2022). Different T cell subsets defined by specific molecules bared on their surface, recognize specifically these molecules, establishing axes with significant distinctions between them. MHC-I molecules are present on the surface of all nucleated cells in the body and are made up of a heavy (α) chain, which is a transmembrane glycoprotein with three extracellular domains (α1, α2, and α3), and the β2-microglobulin, a non-transmembrane protein, that are non-covalently bound (Fig. 4). The peptide-binding groove is formed by the polymorphic α1 and α2 domains (Thibodeau et al., 2019). The α3 domain, being more conserved, interacts with the CD8 molecule on T cells. While β2-microglobulin helps in stabilizing the MHC-I structure, it does not directly participate in peptide binding. For instance, if a cell is infected with a virus or is cancerous, the MHC-I molecules will present peptides of the viral or cancer-associated proteins on the cell surface as a pMHC-I complex to CD8+ T cells, initiating an immune response. Conversely, MHC-II molecules are found on specialized immune cells known as antigen-presenting cells (APCs) including macrophages, dendritic cells, and B cells (Villadangos, 2001). Dendritic cells (DC) are often called professional APCs because they have the special ability to activate both memory and new naive T cells (Bell et al., 1999). Structurally, MHC-II comprises two chains, α and β chains, both contributing to the peptide-binding groove (Fig. 4). When APCs take up a pathogen or antigen, they break it down and display the resulting peptides on the groove of these MHC-II molecules. These pMHCII complexes are then recognized specifically by CD4+ T cells. When B cells display these pMHCII complexes, they become activated, proliferate, and differentiate into B cells and plasma cells to produce specific antibodies later in the immune response.

TCR recognizes specifically antigenic peptides presented on the groove of the MHC molecules. As stated above, CD8+ T cells identify peptides present on MHC I, while CD4+ T cells recognize them on MHC II molecules. Through these interactions, T cells coordinate the immune response by identifying antigens and getting activated and acquiring effector functions as well as stimulating other immune cells such as B cells. The CD4 and CD8 co-receptors recognize and stabilize the interaction between the TCR and pMHC complexes by direct contact to MHC molecules. This first cell-cell contact through pMHC-TCR triggers a cytosolic cascade serving as

the first and main activation signal. Other cell-cell secondary or co-stimulatory signals are also required to induce T cells survival and proliferation such as CD28 on T cells contacting B7.1 and B7.2 on APCs. APCs can release different cytokines (e.g., IFN-γ, IL-4, IL-6, IL-12, IL-23), introducing a third signaling pathway. Depending on the secreted cytokines, CD4+ T cells take diverse differentiation routes, leading to the different effector T cell subsets, each executing unique responses (Schmitt & Ueno, 2015).

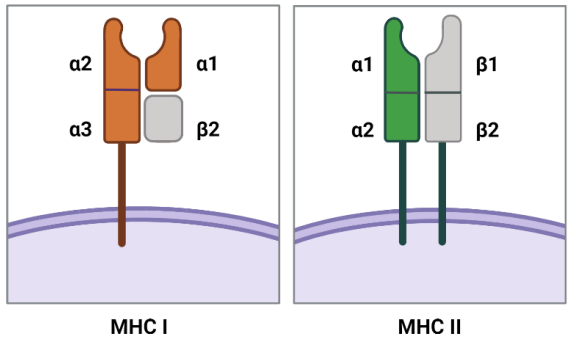

Figure 4. Structural representation of the MHC-I and MHC-II. MHC I molecules are composed of a heavy α-chain and a β2-microglobulin light chain. The α-chain contains three domains: α1, α2, and α3, with α1 and α2 forming the antigen-binding groove, and α3 anchored to the cell membrane. The β2-microglobulin is non-covalently associated with the α3 domain. In contrast, MHC II molecules consist of an α-chain and a β-chain, both spanning the membrane. The antigen-binding groove is constituted by the α1 and β1 domains, while α2 and β2 domains lie closer to the cell membrane. Figure made in https://app.biorender.com.

During T cell maturation in the thymus, they undergo two critical processes known as positive and negative selection, both of which are essential for ensuring immune tolerance and preventing autoimmunity (Klein et al., 2014). Positive selection ensures that T cells can recognize and bind to self MHC molecules. T cells that cannot bind to self MHC molecules with adequate affinity are eliminated, as they would be non-functional in the periphery. Following this, negative selection takes place, where T cells with high affinity for self-peptides presented by MHC molecules are deleted. This process ensures that T cells that might recognize and react against the body's own proteins (autoantigens) are removed from the repertoire, thereby preventing potential autoimmunity. Together, positive and negative selection processes shape the T cell repertoire to be both functional (able to recognize antigens in the context of self MHC) and safe (not reactive against self-antigens) (Lo & Allen, 2013). Any breakdown or inefficiency in these processes can lead to a lack of immune tolerance and the emergence of autoimmune disorders, where the immune system mistakenly attacks the body's own tissues.

1.2.2.2 B cells

The BCR is a membrane-bound immunoglobulin (Ig) that acts as the antigen receptor on B cells. Structurally, the BCR is composed of two main chains: the heavy chain (H) and the light chain (L), linked by disulfide bonds (Fig. 5) (Melchers, 2005). Each BCR molecule contains two identical heavy chains and two identical light chains. The H and L chains both have distinct variable (V) and constant (C) regions. The V regions, which include the heavy chain variable region (VH) and the light chain variable region (VL), are responsible for antigen binding (Chailyan et al., 2011). The VH region gains its unique structure from a molecular mechanism that recombines the V-D-J genes during B cell development. Meanwhile, the VL region is made up of only the recombination of the V-J genes. Together, the VH and VL domains create a unique antigen-binding site, giving the BCR its specificity (Chailyan et al., 2011). The constant (C) regions comprise the heavy chain constant regions (CH) and the light chain constant region (CL). The CH has different C domains, thus differences in the C regions used to make up an antibody determine the classes or isotype, such as IgM, IgD, IgG, IgA, or IgE (Fig. 11) (Ollila & Vihinen, 2005). Consequently, this variation dictates the effector functions of the BCR or antibody, including interactions with complement proteins or Fc receptors on other immune cells (James, 2022). On the other hand, the CL region of the light chain is less diverse and does not govern effector function in the same manner. The BCR has a transmembrane region to anchor it to the B cell membrane. As part of it, an intracellular tail can communicate with other intracellular proteins. However, this tail lacks standalone signaling capacity. The BCR is associated with Igα (CD79a) and Igβ (CD79b) chains, non-polypeptide components of the BCR complex . These chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their intracellular domains. When the BCR identifies and binds an antigen, the intracellular tails interact with CD79a and CD79b, then these ITAM motifs undergo phosphorylation, triggering intracellular signaling pathways that activate the B cell (Chu & Arber, 2001).

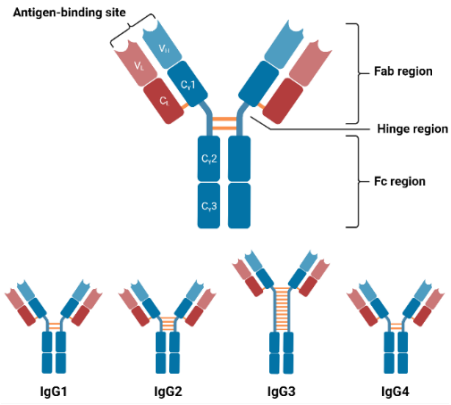

Figure 5. Schematic of the structure of the IgG antibody. Antibodies have a Y-shaped comprising four polypeptide chains: two heavy chains (γ chains) and two light chains (κ or λ chains). Through disulfide bonds, the heavy chains are interconnected, while the light chains are linked to the heavy chains via disulfide bonds and non-covalent interactions. The antibody's two Fab regions (fragment antigen binding) house the antigen-binding sites, whereas its Fc region (fragment crystallizable) engages with other cells and molecules within the immune system. Figure made in https://app.biorender.com.

The high diversity of BCRs and consequently specificity for an antigen is accomplished through a genomic mechanism known as somatic recombination, which arranges different V(D)J genes segments (Fig. 6), to produce a complete V-region exon (Fig. 7). This process involves the recombination of variable (V), diversity (D) (only for heavy chain) and joining (J) gene segments within the immunoglobulin (Ig) loci that encodes the BCR (Fig. 6). Two stages are involved in this process: heavy chain rearrangement and light chain rearrangement (Chi et al., 2020).

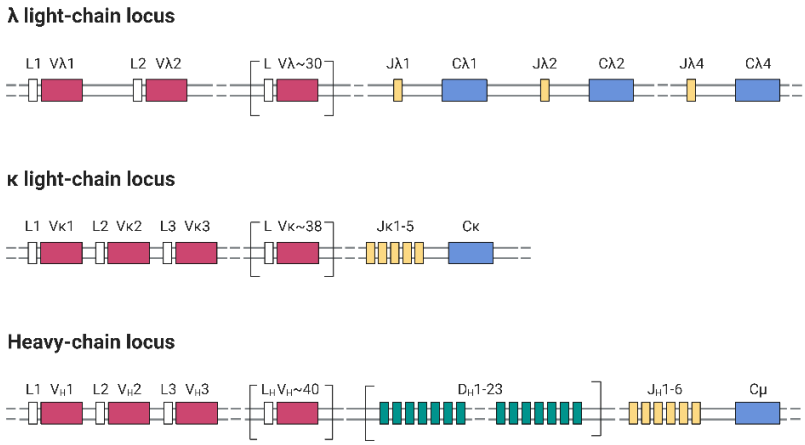

Figure 6. Schematic representation of the genomic arrangement of the Ig light (L) and heavy (H) chains. The κ L-chain locus, consists of numerous variable (V), joining (J), and constant (C) gene segments, which recombine to form a functional V-J-C gene arrangement. Similarly, the λ L-chain locus, also consists of V, J, and C segments, though in fewer numbers compared to the κ locus. The H chain locus is more complex, containing multiple V, diversity (D), J, and C gene segments. The recombination of these segments, specifically the V-D-J recombination, generates a functional gene that encodes the heavy chain of the antibody. This genomic arrangement, governed by the process of V(D)J recombination, is crucial for generating the diversity of antibodies necessary for the adaptive immune system to recognize a vast array of antigens, while the constant regions ensure the structural and functional integrity of the resulting antibodies. Figure retrieved from https://app.biorender.com.

In heavy chain rearrangement, the IgH locus, which encodes VH and CH, consists of multiple V, D, and J segments along with the different C segments that define the isotype (Chi et al., 2020). VDJ and VJ recombination are fundamental processes in the maturation of B cells and their diversity to recognize a vast array of antigens. For the heavy chain formation, each variable (V), diversity (D), and joining (J) gene segment within the immunoglobulin heavy chain locus is flanked by recombination signal sequences (RSS) (Feeney et al., 2000). These sequences are recognized by enzymes encoded by the recombination-activating genes (RAG-1 and RAG-2) (Schatz & Ji, 2011). Upon recognition, the RAG enzymes cleave the DNA at specified points adjacent to the V, D, and J segments. Initially, one D and one J segments are joined together, followed by the joining of one V segment to the DJ complex, thereby forming a contiguous VDJ exon which encodes the variable region of the heavy chain of the BCR.

The light chain rearrangement, on the other hand, involves either the kappa (κ) or lambda (λ) light chains, each encoded by separate Ig locus (Igκ and Igλ) with multiple V and J segments (Schatz & Ji, 2011). The light chain undergoes a similar but slightly simpler recombination process

known as VJ recombination. This process also starts with the recognition of RSS flanking the variable (V) and joining (J) gene segments, facilitated by the action of RAG enzymes. One V and one J segments are then joined to form a contiguous VJ exon, which encodes the variable region of the light chain of the BCR.

When these V, (D), and J segments are recombined, the junctions between them can undergo further modifications. This is achieved through random insertions and deletions that occur at the junctions between V-(D)-J segments during recombination and plays a critical role in achieving the vast diversity of BCRs. In both the heavy and light chains, the diversity of the V region is augmented by nucleotide additions and deletions. The introduced nucleotides are categorized as P-nucleotides and N-nucleotides (Braams et al., 2023). P-nucleotides are designated as such due to their palindromic (P) sequences at the gene segment ends, whereas N-nucleotides get their name because they are non-template-encoded (N) (Jackson et al., 2013). These insertions and deletions add another layer of randomness to the recombination process, further increasing the BCR diversity.

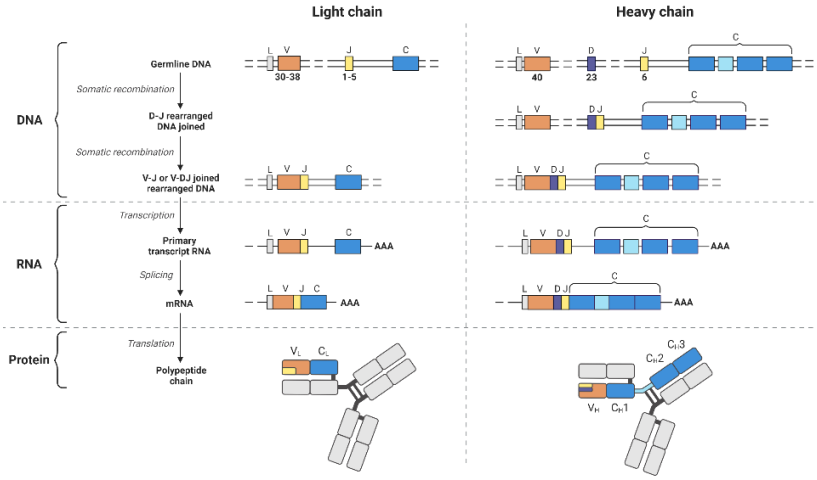

Figure 7. Schematic representation of the somatic recombination mechanism of the immunoglobulin (Ig) light (L) and heavy chains (H) in B cells. The L chain locus displays multiple V (variable) segments, several J (joining) segments, and constant (C) segments. In contrast, the H chain's germline DNA presents multiple V, D (diversity), and J segments followed by C segments. During the first phase of somatic recombination for the H chain, one D segment joins one J segment, forming a DJ-rearranged DNA segment. Meanwhile, the L chain does not undergo this step due to the absence of D segments. In the subsequent recombination step, the L chain pairs one V segment with one J segment, resulting in a VJ-rearranged DNA segment. For the H chain, one V segment is combined with the prearranged DJ segment, creating a V-DJ-rearranged DNA segment. This rearranged DNA for both chains is then transcribed into a primary RNA transcript. After intervening sequences are removed via splicing, a mature mRNA forms, which includes V, (D for heavy chain), J, and C regions. Translation of this mRNA in the L chain produces a polypeptide chain encompassing the VL and CL domains. For the H chain, translation yields a polypeptide featuring the VH domain and C domains. Ultimately, the L and H chains assemble to constitute a full immunoglobulin molecule, with the V regions of both chains crafting the antigen-binding sites and the constant regions dictating the immunoglobulin isotype and consequently its effector functions. Figure retrieved from https://app.biorender.com.

The resulting recombined V(D)J exons remain separated from their respective constant regions by J segments and J-C intron. The recombined V(D)J are then transcribed to produce a transcript that includes the C regions. Further splicing and removal of introns constitute the complete BCR. This mechanism of recombination and assembly ensures each B cell displays a

unique BCR, pivotal for recognizing specific epitopes and thereby sustaining the body's immune defense against a myriad of pathogens (Chi et al., 2020).

After successful rearrangement of both heavy and light chains, the B cell expresses its unique BCR on its surface, composed of two H and two L chains featuring a variable region for the antigen-binding site specific to a particular antigen. Through BCR rearrangement, the immune system can theoretically achieve a very high diversity, calculated to be in the order of at least 10^{18} (Elhanati et al., 2015). This diversity enables B cells to recognize and respond to a wide myriad of antigens. This complex process is tightly regulated to ensure proper B cell development and prevent the emergence of self-reactive B cells that could harm the body's own tissues.

The V(D)J rearrangement can produce BCRs that recognize self-antigens, leading to potential autoimmunity. To counteract this, B cells have a mechanism called receptor editing. If a B cell's receptor binds strongly to self-antigens, it can re-initiate V(D)J recombination to modify its receptor, thereby changing its specificity (Townsend et al., 2010). This process allows the B cell another chance to generate a receptor that does not react with self-antigens. If receptor editing fails to produce a non-self-reactive BCR, the cell undergoes apoptosis (programmed cell death). It is worth noting that while T cells have a robust negative selection process in the thymus to eliminate self-reactive cells, receptor editing in B cells is less complete, making the peripheral tolerance mechanisms, like anergy, crucial for preventing B cell-mediated autoimmunity (Meffre & O'Connor, 2019).

Mammalian B cells can be broadly categorized into two main populations: B-1 and B-2 B cells. Each of them has unique characteristics that play key roles in adaptive immunity. B-1 B cells are mostly generated during fetal and neonatal development and are found primarily in the peritoneal and pleural compartments in mammals (Hardy, 2006). They have a more restricted diversity of BCRs producing polyreactive low-affinity natural antibodies, providing a broad, first-line defense against pathogens without specific antigen stimulation. These cells have the ability to self-renew in the periphery. On the other hand, B-2 B cells, generated in the bone marrow after birth, reside mainly in the spleen, lymph nodes, and other secondary lymphoid tissues (Montecino-Rodriguez & Dorshkind, 2023). They exhibit a highly diverse range of BCRs, producing memory B cells and plasma cells that secrete high-affinity antibodies in response to specific pathogens. Furthermore, unlike B-1 cells, B-2 cells require T cell help for full activation.

B cell activation can be achieved either with cognate cell-cell help from CD4+ T cells or without it. The intrinsic nature of the antigen (e.g., proteinaceous, non-proteinaceous) bound by a specific BCR along with non-BCR mediated signals determines whether or not B cells will be assisted by T cells to be activated. The B cell activation is categorized based on this T cell assistance either as T cell-dependent (TD) or T cell-independent (TI) (Allman et al., 2019). This TI categorization can be further divided into two subcategories based on the different molecular mechanisms associated with their activation: T cell-independent type 1 (TI-1) and T cell independent type 2 (TI-2) (Mond et al., 1995). Hence, the antigens that activate each of these pathways are named after them.

The TD immune responses are pivotal to the adaptive humoral immune response. In this immune response, the typical antigen is a proteinaceous antigen (Allman et al., 2019). Upon specifically binding antigens trough the BCR, it initiates a cytosolic cascade and also mediates the

efficient uptake of antigens through receptor-mediated endocytosis, followed by proteolysis in cytosolic compartments. Antigenic peptides generated by this process will be bound to MHC II molecules that are eventually trafficked to the B cell surface and presented to CD4+ T cells (Adler et al., 2017). Activated CD4+ T cell through their TCR recognize the pMHC-II complex on B cells serving as the first signal for B cell activation. This interaction facilitates the second crucial co-stimulatory signal that ensues between CD40L on the T cell and CD40 on the B cell (Adler et al., 2017). The T cell then releases cytokines, further stimulating the B cell, serving as the third co-stimulatory signal (Fig. 8). These signals induce B cell proliferation in germinal centers. Germinal centers are specialized structures within secondary lymphoid organs, triggered by antigen exposure, where B cells undergo maturation. Structurally, germinal centers have two zones: the dark zone for B cell proliferation and somatic hypermutation, and the light zone for B cell selection based on BCR affinity, crucial events for effective secondary immune responses against infections (Lau & Brink, 2020). Affinity maturation is a central process that enhances the binding affinity of BCR to antigens over time. B cells undergo somatic hypermutation and those ones with higher affinity BCRs are favored during clonal selection, receiving survival signals from follicular helper T (Tfh) cells. Concurrently, B cells may undergo class switch recombination, altering the antibody isotype (e.g., from IgM to IgG), enhancing antibody functionality (Stavnezer et al., 2008). Post-selection, high-affinity B cells differentiate into memory B cells for long-term immunity or plasma cells for high-affinity antibody production (Eisen, 2014). Some may exit germinal centers to other lymphoid organs, broadening the immune response. Through these coordinated steps, affinity maturation ensures a more effective and lasting immune response.

Upon engagement of the TCR or BCR to MHC molecules, Src family kinases are activated, leading to a cascade that involves either ZAP-70 (for T cells) or Syk (for B cells) (Geahlen, 2009; W. Zhang et al., 1998). This cascade activates several downstream pathways, including the MAPK, PI3K-Akt, and NF-κB pathways. Another crucial event is the surge in intracellular calcium levels, which activates calcineurin, leading to the activation and nuclear translocation of the transcription factor NFAT. NFAT, in conjunction with other transcription factors like AP-1 and NF-κB, drives the expression of genes vital for T and B cell responses(Ruland & Mak, 2003).

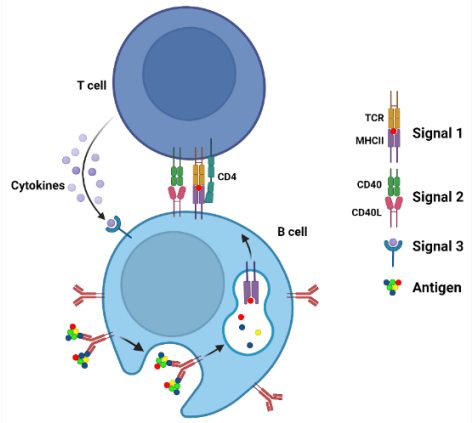

Figure 8. T-cell dependent antibody production. Antigens are taken up, processed, and presented on MHC-II molecules by B cells. These peptide-MHC-II complexes are then recognized by helper T cells through their T cell receptors (TCRs). The CD4 molecules stabilize this interaction by interacting directly with the MHC-II molecule. This interaction activates the B cells. Figure made in https://app.biorender.com.

The TI-1 immune responses activate B cells without cell-cell engagement with CD4+ T cells, typically in response to antigens with other innate immunostimulatory properties, such as LPS (Mond et al., 1995). TI-1 antigens can activate B cells by specifically engaging the BCR (Fig. 10). Some TI-1 antigens function as polyclonal B cell activators or mitogens, they can stimulate B cells irrespective of their antigenic specificity. In TI-1 responses, BCRs usually recognize LPS from bacteria, but can also recognize this and other PAMPs through TLRs on the same B cell, triggering a second signal (Fig. 9). These signals trigger an intracellular cascade that leads to activation and proliferation of the B cells. This proliferation leads to the production of memory and plasma cells that can secrete specific immunoglobulins. As an example, LPS from Gram negative bacteria can bind the TLR4, a PRR, and initiate downstream signaling pathways, including the NF-κB pathway, which promotes B cell activation, proliferation, and eventually antibody production (Pone et al., 2012). Concurrently, the engaging of PAMPs to other PRRs leads to the production and release of different cytokines, which can further stimulate B cells. As B cells become activated, they upregulate several co-stimulatory molecules. Although T cells are not directly involved in a cell-cell manner during TI-1 responses to stimulate B cells, it has been suggested that other immune cells can stimulate B cells with co-stimulatory molecules, providing additional humoral stimulatory activating signals (Peter & Warnatz, 2005). The outcome of TI-1 responses is typically a rapid production of polyclonal IgM antibodies. These responses exhibit limited class-switch recombination or affinity maturation, resulting in antibodies of relatively lower affinity compared to those from TD responses. In high concentrations, certain TI-1 antigens can trigger the proliferation and antibody secretion in up to a third of all B cells. However, in lower concentrations, only those B cells with specific BCRs for the epitope will be activated and proliferate.

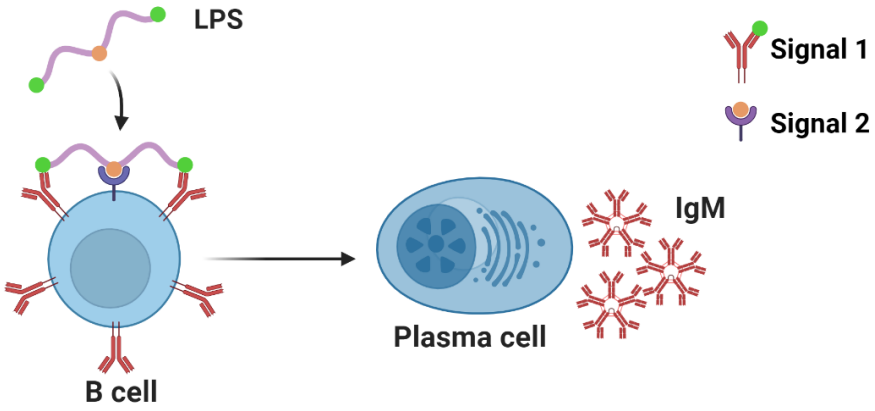

Figure 9. T-cell independent type 1 (TI-1) antibody response. Antigens such as lipopolysaccharides (LPS) are recognized by B-cell receptors (signal 1) and Toll-like receptors (signal 2) simultaneously. These two signals are essential for triggering the expansion of B cells and the subsequent production of antibodies. Figure made in https://app.biorender.com.

The TI-2 antigens are typically repetitive, highly organized structures, and multivalent antigens such as bacterial polysaccharides, that can cross-link multiple BCRs (Mond et al., 1995). Their repetitive nature allows for the efficient and robust stimulation of B cells. However, the

antibodies produced in response to TI-2 antigens have been described as typically of a limited isotype, mainly IgM (Fig. 4). Upon binding to the TI-2 antigens, the BCR undergoes a conformational change, activating the associated Src-family kinases (Yamanashi et al., 1991). These events collectively activate several transcription factors, including NF-κB, promoting B cell activation, proliferation, and differentiation. The activated B cells then initiate the production and release of IgM. This direct response of B cells to these antigens enables a fast reaction to many pathogens. In addition to BCR signaling, co-stimulatory signals can enhance the TI-2 response. For instance, engagement of TLRs on B cells by PAMPs can synergize with BCR signaling, amplifying the activation (Barr et al., 2006). Moreover, DCs can secrete cytokines that modulate the immune environment, influencing B cell responses indirectly. For instance, the secretion of BAFF by dendritic cells can promote B cell survival and differentiation (Fig. 10) (Jeurissen et al., 2004). These factors provide survival and maturation signals to B cells. Some TI-2 antigens are known to induce the formation of long-lasting PCs and memory B cells in both mice and humans, and due to these characteristics they are components in numerous widely used vaccines (Obukhanych & Nussenzweig, 2006).

However, defining specific characteristics of a TI antigen that consistently apply to all TI antigens has been challenging (Mond et al., 1995). Certainly, the unique element that unequivocally characterize all is that they cannot bind to MHC class II restriction elements, which would be needed for presentation to T helper cells (Harding et al., 1991).

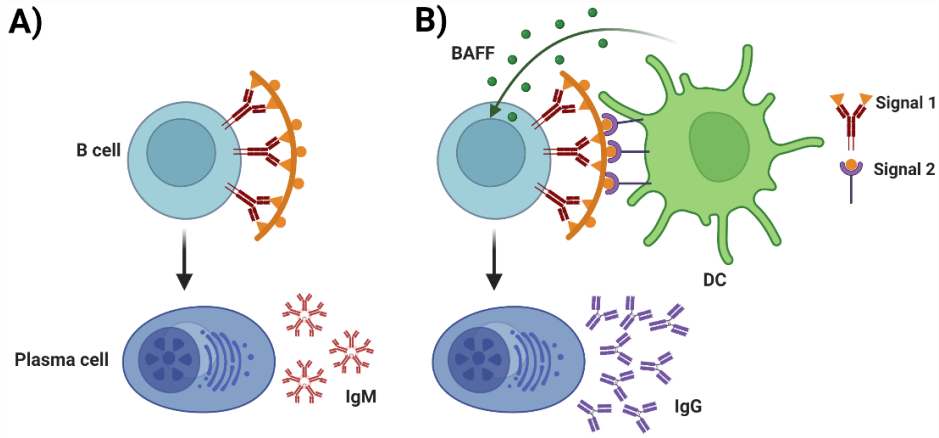

Figure 10. T cell independent type 2 (TI-2) antibody response. B-cell receptors (BCRs) recognize antigens, typically polysaccharides. These antigens contain repetitive epitopes that can be recognized by multiple BCRs. This BCRs cross-linking triggers B cell activation and their expansion, resulting in antigen-specific antibody production. Figure made in https://app.biorender.com.

Upon B cell activation, especially when assisted by helper T cells in TD responses, the activated B cell begins to proliferate rapidly in the germinal centers. Here, some activated B cells can undergo class switch recombination, changing their immunoglobulin isotype. Following these maturation processes, some of the activated B cells differentiate into plasma cells (PCs). These PCs, equipped with an extensive rough endoplasmic reticulum and a large Golgi apparatus,

produce, and secrete antibodies into the bloodstream and lymph. In humans, IgA and IgM PCs have shown to express functional BCR on their cell surface and can therefore respond to antigenic stimulation (Pinto et al., 2013). While many PCs are short-lived, some become long-lived in the bone marrow, producing antibodies for extended periods. Wilmore et al. (2021) have shown that long-lived PCs express surface IgA in the lamina propria of the small intestine and the bone marrow sharing transcriptional similarities compared to those expressing IgG and IgM in the bone marrow.

Immunoglobulins, commonly known as antibodies, are glycoproteins produced by B cells and are essentially the secreted form of the B-cell receptor. There are five different classes of immunoglobulins, distinguished by C regions that have unique structures and properties. These are: IgG, IgA, IgM, IgE, and IgD (Fig. 11) (Keyt et al., 2020). The distinctive functional properties of the different classes and subclasses of antibodies are conferred by the CH domains. Each has distinct structural attributes, functions, and distributions in the body. They elicit their effects through several mechanisms. Firstly, they can directly neutralize pathogenic entities (L. L. Lu et al., 2018). By binding to viruses or bacterial toxins, antibodies prevent these agents from entering host cells or exerting their toxic effects. Secondly, through a process called opsonization, antibodies coat the surface of pathogens, marking them for uptake and destruction by phagocytic cells, such as macrophages and neutrophils. The Fc region of the antibody binds to Fc receptors on these phagocytic cells, facilitating phagocytosis (Achkar et al., 2015). Additionally, the classical pathway of the complement system is activated when the complement protein C1q binds to antibodies that are attached to the surface of pathogens, leading to a cascade of events that can lyse bacteria and cells, opsonize pathogens, and recruit inflammatory cells. Another mechanism is the antibody-dependent cellular cytotoxicity (ADCC) wherein certain immune cells, like natural killer (NK) cells, recognize and bind to the Fc region of antibodies bound to target cells, triggering the release of cytotoxic molecules that destroy the target cell (Von Holle & Moody, 2019). Antibodies can also cause agglutination by crosslinking and clumping together pathogens or their products, which prevents the spread of infectious agents and makes them easier targets for phagocytosis (Hoces et al., 2020). Moreover, in the context of allergies, IgE antibodies can bind to allergens and crosslink Fc receptors on mast cells and basophils, triggering the release of inflammatory mediators like histamine (Shamji et al., 2021). Lastly, by binding to antigens, antibodies can facilitate their uptake by antigen-presenting cells, enhancing the presentation of the antigenic peptides on MHC molecules and promoting T cell responses (Lanzavecchia, 1996).

IgG is by far the most abundant immunoglobulin in serum and has several subclasses (IgG1, 2, 3, and 4 in humans) (Fig. 5) (Vidarsson et al., 2014). It makes up approximately 75% of the total immunoglobulins in human serum. It is particularly essential for long-term immunity, secondary immune responses, and maternal immunity passed to fetuses via the placenta (Vidarsson et al., 2014). Its effector functions include opsonization, neutralizing pathogens, activating the complement system, and mediating ADCC (de Taeye et al., 2020). Functionally, IgG can enter tissues easily and is found both in the blood and extracellular fluid, allowing it to recognize and combat invading pathogens effectively.

The IgA, on the other hand, mainly exists in mucosal areas such as the gut, respiratory tract, and urogenital tract, as well as in secretions like saliva and breast milk. There are two main forms: serum IgA, mainly present in its monomer form; and secretory IgA, as a dimeric form (de

Sousa-Pereira & Woof, 2019). The latter, which is more abundant, provides a crucial first line of defense on body surfaces by neutralizing pathogens and preventing their colonization. Beyond mucosal defense, IgA also plays roles in immune regulation and tolerance, particularly in the gut.

The IgM is typically the first antibody produced during a primary immune response (Boes, 2000). It is present in the blood and, to a lesser extent, in lymphatic fluid. Structurally, it often exists as a pentamer, allowing it to effectively agglutinate pathogens and activate the complement system. Its size prevents it from easily entering extravascular spaces, confining its function primarily to the bloodstream. It acts as an early responder, rapidly neutralizing pathogens during the initial stages of an infection.

IgE is a key player in mediating Type I hypersensitivity reactions by binding to Fc receptors on immune cells, triggering the release of inflammatory mediators upon allergen exposure. Besides allergic reactions, IgE plays a crucial role in defending against parasitic infections. It employs potent inflammatory and expulsive forces against local antigen provocation, albeit with a risk of damaging host tissues (Sutton et al., 2019).

IgD's functions are still to be elicit. It is found mainly co-expressed with IgM on the surface of the majority of mature B cells prior to antigenic stimulation. IgD acts as an antigen receptor, playing a crucial part in the initial stages of immune response. It has been described that it has several unique roles in enhancing mucosal immunity by arming basophils and other innate immune cells with secreted IgD. IgD's evolutionary conservation from ancient vertebrates to humans underscores its immunological significance, with a non-canonical class-switching mechanism from IgM to IgD in the human upper respiratory mucosa contributing to the generation of IgD-secreting B cells, further emphasizing its importance in mucosal immunity (K. Chen & Cerutti, 2011).

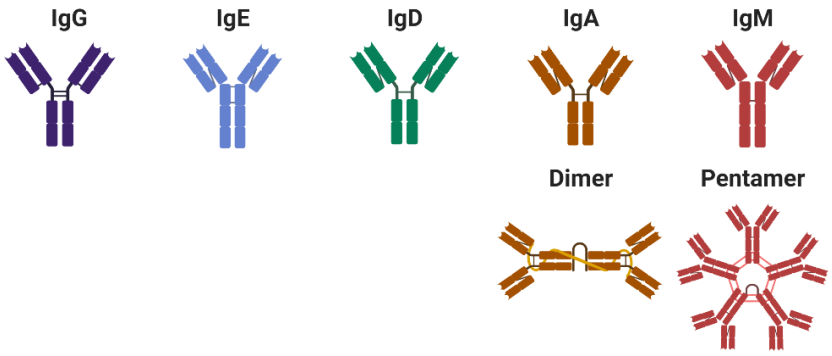

Figure 11. Schematic representation of immunoglobulin (Ig) isotypes. IgG highlighting immunoglobulin Y-shaped monomeric structure commonly involved in opsonization, virus neutralization, and complement activation. IgE, plays a fundamental role in allergic reactions and parasite defense. IgD functions mainly as a B-cell receptor. IgA is represented in two forms: a monomer and its distinctive dimeric form, connected by a J-chain, which is the primary immunoglobulin found in mucosal secretions. IgM usually exists as a pentamer, which means it is composed of five monomer units connected by a J chain. This structure allows it to effectively bind multiple identical pathogens or antigens. Its large size and pentameric form make it effective in agglutinating pathogens and activating the complement system. Figure made in https://app.biorender.com.

1.3 The immune system of teleosts

With a rich diversity of over 30,000 extant ray-finned fish species, teleosts comprise approximately 98% of them with a vast evolutionary distance between clades (Ravi & Venkatesh, 2018). These species have colonized and adapted to different environments, each presenting a unique array of challenges and conditions. The habitats they occupy span from the freshwater realms to the saline expanse of saltwater bodies and go across a climate spectrum from cold to tropical zones. Beyond this, they have acquired survival strategies to cope with a variety of water conditions such as different pH levels, oxygen concentrations, and temperature gradients. Some species succeed in the cold, oxygen-abundant waters of polar domains, while others are skilled at enduring the warm, oxygen-scarce waters of stagnant tropical ponds. Additionally, there are those that have ventured into highly specialized habitats such as the abyssal deep-sea vents, where they face extreme pressure and temperature, or the challenging desert pools, which need somehow rapid life cycle adaptations. These multifaceted environmental adaptations have likely influenced the evolution of a diverse array of immune system setups among different clades and species of fish, generating a spectrum of genomic and functional immunological traits. The divergence may be even more accentuated when comparing the immune system of fish with those of mammals, given the augmented evolutionary divergence and the contrasting modalities of terrestrial and aquatic lifestyles.

Despite the aforementioned differences, teleost fishes, in general, share several features with higher vertebrates such as innate immune cells (e.g., neutrophils, macrophages, and NK-like cells), MHC I and II, as well as B and T cells. In teleosts, the primary immune organs are the head kidney and the thymus, and the secondary immune organs the spleen, head kidney, and MALT. The teleost MALT can be subdivided according to the anatomical localization such as the gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT), nasopharynx-associated lymphoid tissue (NALT), interbranchial lymphoid tissue (ILT), and amphibranchial lymphoid tissue (ALT) (Dalum et al., 2021; Salinas, 2015). Recently, a study highlighted a unique pharyngeal mucosal lymphoid organ in zebrafish and other teleost fish, which is suggested to be reminiscent of mammalian tonsils (Resseguier et al., 2023). It appears that the cellular and humoral facets of the innate immune system retain a high degree of conservation when compared to their mammalian counterparts. However, when it comes to the adaptive immune system, notable distinctions arise. Notably, teleosts, unlike their higher vertebrate counterparts, lack lymph nodes, germinal centers, Peyer's patches, and the discourse on the presence of lymphatic vessels remains an open debate (Bjørgen & Koppang, 2022).

1.3.1 The innate immune system of teleosts

The epithelial and mucosal barrier of the skin, gills and alimentary tract comprise a very important barrier against pathogens and inert particles in fishes, particularly for being immersed in an environment containing a huge amount of potentially harmful agents (Pandey et al., 2021). Part of this first line of defense is conferred by the presence of scales in some of them. They protect fish as a physical barrier from the invasion of potential pathogens and parasites as well as other particles that can damage their skin. Moreover, some species of teleosts possess specialized skin structures, such as venomous spines or electric organs, which serve as additional deterrents to predators and parasites (Akat et al., 2022). The gills comprise another important barrier that is highly vascularized and exposed to the surrounding water. Their highly folded structure creates a large surface area, essential for gas exchange and ion transport, but also exposes them to potential hazardous entities. The gill's epithelial cells, specialized cells and mucus layer form a protective

barrier, while resident immune cells provide an immediate response to invading pathogens (Koppang et al., 2015).

There is a prevailing argument suggesting that, in general, fish depend more heavily on their innate immune system as compared to their adaptive immune system. While this notion may not be definitive, the significance of the innate immune system in defending fish against pathogens is certainly prominent (Magnadóttir, 2006a). This is particularly true considering the presence of an extensive surface area made up in conjunction by the skin, gastrointestinal tract, and gills that is in constant exposure to pathogens. Different studies have described the presence and functions of lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides, protease, peroxidase, esterase, alkaline phosphatase, antiproteases and bactericidal activities, and natural antibodies in the mucus of teleosts (Guardiola et al., 2014). These molecules play a crucial role in preventing the entry and tackling of pathogens at mucosal sites.

Many aspects of the teleosts immune system have not been investigated as extensively as in humans and other mammals. Despite this, in teleosts, innate immune molecules and cells homologous to their mammalian counterparts have been described. PRRs such as TLRs, NLRs, RLRs, lectins, and complement receptors, are present in teleosts. Due to genome duplication events in teleosts, some PRRs can be found duplicated or expanded in their genomes, depending on the fish species. Other members of these families are speculated to have neofunctionalizations (Y. Li et al., 2017).

Research has shown that teleosts have a diverse range of TLRs. While some are homologous to mammalian TLRs, others seem unique to fish. Some of the TLRs present in mammals have not been identified in teleosts. However, there has been an expansion of TLRs in teleosts, and it has been suggested that different TLRs are found only in teleosts some of the duplicated TLR encoding genes have undertaken new functions to defray putative functions of mammalian TLRs. For example, teleosts possess TLR22, which is not present in mammals. TLR22 in fish can recognize double-stranded RNA, suggesting a role in viral immunity (Liao & Su, 2021). Through the examination of molecular phylogeny and expression of NLR subfamilies in zebrafish, three distinct NLR subfamilies have been recognized (Y. Li et al., 2017). The first subfamily (NLR-A) comprises eight genes akin to mammalian NODs. The second subfamily (NLR-B) encompasses nine genes resembling mammalian NACHT-, LRR- and PYD-containing proteins (NLRP). Lastly, the third subfamily (NLR-C) includes 405 NLR genes that are exclusive to teleost fish (Howe et al., 2016; Laing et al., 2008).

C-type Lectin Receptors (CLRs) are integral to the immune systems of vertebrates, including teleost fish. Zebrafish genome has been found to house multiple C-type lectin genes involved in their immune response to infections. This group seems to be highly diverse and, in many cases, specific CLRs have been found only in some groups (Ahmmed et al., 2022).

The mammalian RLR family consists of RIG-I, melanoma differentiation-associated factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). These genes play an important role in initiating antiviral immunity against RNA virus infection (Loo & Gale, 2011). The RLR genes family is well conserved among vertebrates. Most of the signaling components of the RLR pathways, including RIG-I, MDA5, LGP2, MAVS and TBK1, have been identified in fish (M. X. Chang, 2021; Lazarte et al., 2019). Intriguingly, Atlantic cod, flounder, chicken and the tree shrew lack RIG-I (*ddx58*) (Jin et al., 2020). In Atlantic cod, it has been suggested that RIG-II (*ifih1*) is involved in the activation of the RLR pathway (Jin et al., 2020). Orthologs of ARLs have not been identified in teleosts.

The complement system is well conserved in vertebrates and even some components are present in invertebrates. In teleost fishes, a majority of complement components have been described. These components bear structural similarities to mammalian counterparts, with all three activation pathways being present in the teleost complement system (M. Li & Zhang, 2022). Teleosts exhibit multiple isoforms of various complement components. The existence of these isoforms including C3, C7, C8, and CFH indicates duplication of these genes within teleosts (M. Li & Zhang, 2022). The functional understanding of these multiple isoforms of complement components remains somewhat elusive and more fundamental research is needed to understand their role under specific conditions.

Innate immune cells such as thrombocytes, NK-like cells, neutrophils, and monocytes/macrophages are well-documented in teleosts (Mokhtar et al., 2023). Other cell types, including eosinophils, basophils, and mast cells, have also been scarcely reported in fish, but their presence and functions are still subject of debate (Reite & Evensen, 2006). In addition to these cells, other cell types like epithelial, melanomacrophages, and rodlet cells have been reported to contribute to the innate defense mechanisms in teleosts fish (Magnadóttir, 2006b).

1.3.2 Adaptive immune system of Teleosts

1.3.2.1 T cells

T cells have been identified in all fish species investigated. Multiple studies on fish have demonstrated the thymus's pivotal role in the maturation of T cells, progressing from early thymocyte progenitors to fully functional T cells (Nakanishi et al., 2015). Unfortunately, the absence of monoclonal antibodies (mAb) has historically hindered the study of T cells in fish as well as the investigation of other immune cell populations. However, a handful of mAbs have been developed and have facilitated the study of T cells to a certain extent in selected fish species. While some of these mAbs do not specifically target T cell markers, they have nonetheless become invaluable tools for exploring this cell population in various species including carp (WCT, WCL9, WCL38), seabass (DLT15), trout (30A8), Japanese flounder (*Paralichthys olivaceus*), and Atlantic salmon (*Salmo salar*) (Sasa CD8 F1-29) (Hetland et al., 2010; Laing et al., 2007; Rombout et al., 1997, 1998; Scapigliati et al., 2000; Secombes et al., 1983; Xing et al., 2017).

Different fish species are observed to have a significant number of T cells in the intestines and gills. In gills, particularly, T cells have been described to form aggregates located along each side of the gill arches as well as in interbranchial site (Dalum et al., 2021). The functional involvement of CD4+ and CD8+ T cells in allograft rejection and graft-versus-host reactions has also been described (Abelli et al., 1999; Nakanishi & Ototake, 1999). Additionally, alloantigen and virus-specific cytotoxicity has been demonstrated in species like ginbuna and rainbow trout (Somamoto et al., 2014). A number of studies suggest that CD4+ helper T cell functions are conserved among teleost fishes, as indicated by the hapten-carrier effect (Shibasaki et al., 2015). Moreover, CD4+ T cells have been clearly identified in teleosts; a study on sea bass demonstrated that all major CD4-related genes — Th1, Th2, Th17, and Treg — are expressed in the gills (Nuñez Ortiz et al., 2014). It has also been reported that cell-mediated immunity, as opposed to humoral immunity, plays a significant role in defending against intracellular bacterial infections (Yamasaki et al., 2013).

The activation of T cells in mammals requires costimulatory molecules (CD80 and CD86) on APCs to interact with their respective T cell receptors (CD28 and CD152/CTLA-4). In contrast, teleost fish, with the exception of salmonids, possess CD80/86 as their sole primordial costimulatory molecule, suggesting a structural and functional difference in their TCR engagement

and activation mechanisms (T.-Z. Lu et al., 2022). Moreover, a duplicated CD80/86 gene, termed rtCD80/86B, in rainbow trout is suggested to possess a distinct function, as indicated by its expression pattern, particularly in B cells serving as APCs (X.-J. Zhang et al., 2018).

Interestingly, due to whole genome duplication events in teleosts, two types of CD4 molecules have been identified in fish. One is known as CD4-1 (also referred to as CD4, CD4L-1), and it contains four extracellular Ig domains in all the fish species examined so far, similar to CD4 in higher vertebrates (Dijkstra et al., 2006; Edholm et al., 2007; Laing et al., 2006; Nonaka et al., 2008; Suetake et al., 2004). The second molecule, named CD4-2 (also known as CD4L-2, CD4REL) (Dijkstra et al., 2006; Laing et al., 2006), has significant similarities with CD4 sequences in other vertebrates but has only two Ig domains in rainbow trout and three Ig domains in channel catfish (Edholm et al., 2007). These CD4 transcripts were reported to be expressed primarily in IgM-negative lymphocytes (Laing et al., 2006; Edholm et al., 2007).

Scapigliati et al. (2018) has suggested that, based on evidence gathered from different studies, teleost fish T cells are functionally closer to mammalian γδ T cells. In mammals, γδ T cells, mucosa-associated invariant T cells (MAIT), and natural killer T cells (NKT) are the primary lymphocyte subpopulations recognized for their innate-like activities, predominantly functioning within mucosal tissues (Garner et al., 2018). Mucosal tissues are also where a large proportion of teleost fish T cells have been described, particularly in the gills and gut.

1.3.2.2 B cells

B cells have been recognized in teleosts as well. Research on B cells has been somewhat more straightforward compared to T cells, owing to the process of isolating IgM and producing mAbs against them. This ease has enabled a more thorough exploration of not only the secreted antibodies but also B cells bearing the same surface antibodies isotype. As mentioned earlier, teleost do not have bone marrow nor lymph nodes and they do not develop germinal centers after encounters with pathogens or immunizations. Hence, B cell populations in teleost differ from their mammalian counterparts. There are important aspects related to B cells that need to be observed. So far, all studied teleosts have shown B cell bearing either IgM, IgD, IgT/Z, or both IgM and IgD. For example, channel catfish have three populations of B cells, IgM+/IgD-, IgM+/IgD+, and IgM-/IgD+ (Edholm et al., 2010). In Rainbow trout on the other side, only two subsets have been described, IgM+/IgD+/IgT- and IgM-/IgD-/IgT+ (Y.-A. Zhang et al., 2010). Importantly, they do not present immunoglobulin isotype switching as mammals do.

It has been suggested that B cells in teleost resemble B-1 B cells in mammals. As a trait of this is the high numbers of mitochondria found in teleost B cells and in B-1 B cells in mouse as well as their functions in vivo and in vitro (Abrahão et al., 2003; Scapigliati et al., 2018). These B cells particularly present a high phagocytic activity and it is higher in the blood compartment than in the head kidney (J. Li et al., 2006).

The humoral component of the adaptive immune system is composed of secreted either IgM, IgT, or IgD. Teleost IgM is predominantly a tetrameric molecule, as opposed to the pentameric mammalian IgM and they are formed without intermolecular interactions made by the immunoglobulin joining (J) chain (Flajnik & Kasahara, 2010). This form exists in different oxidation states, which are thought to play a significant role in their function (Kaattari et al., 1998). Memory IgM has been described in several fish species although the affinity maturation is poor when compared to mammals (Stosik et al., 2021). These immunoglobulins are secreted by plasmablasts and plasma cells to the bloodstream, having a more prominent systemic function although they are also secreted at mucosal sites.

IgT has been described to play an important role at mucosal sites where it is secreted on and where most of the IgT+ B cells are homed. It acts mainly by coating bacteria in the gut but also on the gills. IgT, and IgZ which are homologous, were first described in trout and zebrafish, respectively (Danilova et al., 2005; Hansen et al., 2005). Since then, this isotype has been reported in several fish species like Atlantic salmon, common carp, and three-spined stickleback. Different subtypes (IgT1, IgT2, IgT3, IgT4, IgT5) as well as a chimeric (IgM-IgT) have been reported to be present in some of these species (Buonocore et al., 2020; Y.-A. Zhang et al., 2011).

IgD has been studied at the genomic level in different species and cloned in multiple teleost species (Edholm et al., 2011). It has been characterized as a membrane-bound immunoglobulin, but a secreted monomeric form has been described in channel catfish (Bengtén et al., 2006). In this species, IgD is suggested to lack a variable region, and its function in immunity is still unclear.

Adaptive humoral immune responses have been studied in some teleost species using antigens that classically induce TD and TI-1 responses in mammals. In trout this has been addressed looking at primary and secondary antibody responses to TI and TD antigens. While there was not a significant difference between the TI and TD antibody responses, the secondary responses to both were longer compared to the primary responses. During the primary responses, both antigens showed increases in titers and affinity, whereas only slight increases were observed during the secondary responses (Ma et al., 2013). The presence of a true secondary response in teleosts is still a matter of debate. Some studies as the latter have described an enhanced antibody response after a second exposure to the same antigen while other studies have shown its absence.

1.4 The immune system of Atlantic cod

1.4.1 The innate immune system

The innate immune system in Atlantic cod plays a crucial role right from the earliest stages of this fish's life. Even before fertilization, the defense system is active, evident from the presence of maternally transferred transcripts that contribute to its early immune development (Seppola et al., 2009). In these unfertilized eggs, maternal immune gene transcripts as well as functional proteins have been documented. However, as the embryo develops, these transcripts are progressively replaced by those the embryo transcribes from its own genome. Maternal transfer of transcripts encoding antibacterial products such as lysozyme and cathelicidin were identified in these unfertilized eggs. The presence of lysozyme activity suggested a functional protein even at this stage (Seppola et al., 2009).

The transcription of innate-related genes begins around the gastrula period, and there is a significant increase in the transcription of these innate immune genes around hatching and the first feeding. This surge prepares the fish for heightened pathogens exposure. As the fish undergo morphological changes, another notable surge in transcript levels occurs, marking further maturation of the immune system (Seppola et al., 2009). The adaptive immune system of Atlantic cod does not fully mature during embryonic and larval phases, making their immune competence somewhat limited. Moreover, the detection of IgM and IgD and transcripts and protein in the ovarian fluid has been suggested to offer passive protection to eggs (Seppola et al., 2009). This could potentially be leveraged in broodstock vaccination strategies to ensure the well-being of new progeny.

The Atlantic cod's external mucus and the mucosal surfaces of its alimentary tract, skin, and gills carrier important humoral immune factors. This mucus serves as a primary physical

barrier against infections, trapping, and immobilizing pathogens. The mucus contains immune-relevant components like proteases and anti-proteases, lysozyme, alkaline phosphatase, cathepsin B, antimicrobial peptides (AMPs), lectins, and immunoglobulins (Schroder et al. 1998a, Subramanian et al. 2007). The external mucus has shown bactericidal activity against different bacteria, including those known to be important pathogens of farmed Atlantic cod such as *Vibrio anguillarum* and *Aeromonas salmonicida* (Ruangsri et al., 2010). Interestingly, when compared to tissue extracts from immune organs like the head kidney and spleen, the bactericidal efficiency of the mucus extract was low. Natural non-specific IgM antibodies are also found on the Atlantic cod's mucosal surfaces. These natural antibodies can act in many ways such as neutralization and promote microbiota homeostasis. Mucosal surfaces cover a vast area of the individual, immunological homeostasis at these sites is critical to contain potential pathogens from spreading systemically.

In Atlantic cod, both membrane-bound and secreted pattern recognition receptors (PRRs) are present. These factors display individual variations, influenced by factors such as age, temperature, and season. When the cod was experimentally infected with *Aeromonas salmonicida*, its hemolytic activity showed changes, with environmental factors like temperature and fish size playing a role in its activity variation (Magnadóttir, 2014). Although lysozyme activity is typically minimal or absent in Atlantic cod serum, it becomes pronounced in mucus under experimental conditions. The cod's serum anti-protease activity shows seasonal fluctuations and is reduced in warmer environments. Interestingly, infections and acute phase responses have a limited effect on this activity. Conversely, the levels of two pentraxins in Atlantic cod, CRP-PI and CRP-PII, decrease during infection and acute phase stimulation (Magnadóttir et al., 2011). These two proteins undergo post-translational deimination and display different tissue localizations from 3 to 84 days post-hatching (Magnadóttir et al., 2011). Deimination is a post-translational modification where arginine residues are converted to citrulline residues, which can influence the structure and function of proteins (György et al., 2006). Both CRP-I and CRP-II are found in the liver but at varying levels over time. CRP-I is primarily associated with nervous tissue, while CRP-II is more prevalent in the mucosal tissues of the gut and skin. This differential expression suggests unique roles for each pentraxin in immune responses and tissue remodeling, with CRP-I potentially playing a novel role in the nervous system (Magnadóttir et al., 2018). Magnadóttir and collaborators (2019) highlighted the presence of innate immunity components, such as C3 and C-reactive protein, within extracellular vesicles (EVs) in Atlantic cod mucus. Interestingly, a segment of the C3 protein in these EVs undergoes deimination. While the contribution of EVs to mammalian innate immunity is well-established, their role in fish is less delineated. Given that deimination can modify protein structure and function, this alteration in C3 might have significant implications for its role within these vesicles.

Cytokines play a pivotal role during the onset of inflammation and regulation of the immune response at the different stages of this process. Gene expressions of cod interleukins, after exposure to viral and bacterial infections or stimulants like Poly I:C, and bacterial LPS, have been studied across different tissues and experiments. A significant change in the expression of inflammatory genes has been documented in Atlantic cod, both under natural and experimental conditions against different pathogens such as *Francisella noatunensis*, *Vibrio anguillarum* and *Aeromonas salmonicida* (Ellingsen et al., 2011; Magnadóttir, 2014). Also, increased levels of IL-

1β and IL-8 (but not IL-10) were noted in different Atlantic cod whole blood and gill cells after infection with *V. anguillarum* and *A. salmonicida* (Caipang et al. 2008c, 2010; Seppola et al., 2008). In one of these studies, the serum from vaccinated and non-vaccinated Atlantic cod had antibacterial activity against *V. anguillarum* but this antibacterial activity increased over several days after the infection only in the vaccinated group. There are also differences in induced gene expression associated with the pathogen itself, sampled tissue, and environmental factors during experiments performed *in vivo* and *in vitro* (Seppola et al., 2008).

It has been described that Atlantic cod injected with Poly I:C increased IFN-γ expression (Furnes et al., 2009). In contrast, a less pronounced response was observed in cod injected with *V. anguillarum*. This suggests that IFN-γ is active in the Atlantic cod's innate immune defense more prominently against viruses. Three distinct homologues of IFN-stimulated gene 15 (ISG15), a ubiquitin-like interferon-induced protein that has been implicated as a central player in the host antiviral response, were found in Atlantic cod, suggesting potential variations in their roles in the antiviral defense mechanisms (Seppola et al., 2009). The expression of ISG15 increased after a Poly I:C injection, but it remained unaltered by the injection of formalin-killed *V. anguillarum* or bacterial LPS, supporting a role for ISG15 in antiviral, but not antibacterial defense in Atlantic cod. This would further suggest that IFN-γ, which was also induced albeit not as much by *V. anguillarum* and LPS activates different effector genes in antibacterial defense (Furnes et al., 2009).

Studies have characterized genes in cod that are involved in the activation of non-specific immune cells of teleosts. For instance, Seppola et al. (2007a) has characterized the non-specific cytotoxic cell receptor (NCCRP)-1 gene in cod. The gene expression of NCCRP-1 was observed to increase in peripheral blood cells post-vaccination with heat-killed *V. anguillarum*. The Atlantic cod genome has unveiled a distinctive array of TLRs. Atlantic cod lacks most surface TLRs found in mammals, notably those identifying bacterial molecular patterns (TLR 2, 4, 5, and 11) (Solbakken et al., 2016). However, they have kept the intracellular counterparts (TLR 3, 7, 8, and 9) (Solbakken et al., 2016). Uniquely, they possess the teleost-specific surface TLRs, namely TLR 21, 22, and 23 (Sundaram et al., 2012). Of these, TLR 22 is particularly intriguing due to its multiple paralogues. These paralogues can detect bacterial molecular patterns, thereby offsetting the lack of a typical mammalian TLR system. They appear to be consistently expressed in cod's immune organs and tissues (Star et al., 2011; Sundaram et al., 2012). Sundaram et al. (2012) further highlighted that after exposing cod to *V. anguillarum*, there was a varied expression of these teleost specific TLRs, contingent upon the organ in focus and the TLR paralogue in question.

Lange et al. (2004) used immunohistochemistry to detect C3 in Atlantic cod larvae from 1 day post hatching (dph) to 57 dph. The researchers discovered that C3 was detectable in the yolk sac membrane starting from 1-day post-hatching (dph), and from 2 dph, it was present in the liver, brain, kidney, and muscle. Additionally, C3 was identified in various other organs including the eye, notochord, stomach, intestines, pancreas, heart, and gills at diverse stages of larval development. These findings suggest that the complement system is crucial for immune defense in Atlantic cod larvae from the very early stages of development. The presence of C3 in a variety of organs also suggests that the complement system may also play a role in other developmental processes such as organogenesis (Hawksworth et al., 2017; McLin et al., 2008).

A different investigation conducted by Lange et al. (2019) outlined the presence of C4-like protein in serum, mucus, and extracellular vesicles (EVs) derived from both serum and mucus. This protein was also detected in different organs of cod larvae, including the liver, kidney, gut, muscle, skin, mucus, brain, spinal cord, and eye. Additionally, the authors identified a deiminated form of the C4-like protein in cod serum and EVs. The authors propose that the C4-like protein might have roles in immune defense and also on tissue remodeling in Atlantic cod. They further suggest that the post-translational deimination of the C4-like protein could modify its function.

Guslund et al. (2020) utilized single-cell transcriptomics technology to provide insights into immune cell populations residing in the spleen of the Atlantic cod. The identification of macrophages was based on the marker genes macrophage colony-stimulating factor 1 receptor (M-CSF1R) and macrophage mannose receptor 1-like (MRC1). These macrophages were found to express numerous genes associated with the complement system, including properdin (CFP) and complement factor B (CFB). Additionally, a distinct, smaller cell population was primarily located in the spleen. These cells were identified as dendritic cells and were observed to express a range of innate immune genes. These genes include toll-like receptor 22 (TLR22), the chemokine receptor chemokine XC receptor 1 (CXCR1), bactericidal permeability-increasing proteins (BPI), and complement genes such as complement C1q-like protein 2 (C1QL2).

1.4.2 The adaptive immune system

Genomically, the presence of IgM has been documented in Atlantic cod, and experimentally, it has been demonstrated that Atlantic cod harbors IgM+ B cells. However, the presence of IgD has been documented solely at the genomic level and interestingly, δ domain exhibits several major differences from the catfish and salmon δ (Stenvik & Jørgensen, 2000). Included are the lack of the δ3, δ4, δ5, and δ6 domains, the repeated duplication of the δ1 and δ2 domains, the presence of an incomplete δ7 domain downstream of the δTM exons, and a short exon (δy) positioned between the δ2A and δ1B domains. The latter shows homology to a conserved part of the TM1 domains of certain H-chain isotypes (Stenvik & Jørgensen, 2000). The Atlantic cod IgM has been described to be tetrameric and has a natural binding capacity to a variety of antigens used for immunization (Pilström & Petersson, 1991).

Although phagocytosis has been described to be a process taking part in cells of the innate immune system, it has also been described to be present in B cells with important roles in antigen presentation such as in B and T cell activation (Parra et al., 2012). In a comparative study by Øverland et al. (2010) looking at the phagocytic index (number of particles per cell) and phagocytic percent (number of phagocytosing cells) of Atlantic cod B cells, the authors observed that B cells' phagocytic percent from the Head Kidney Leukocytes (HKL) was higher than for B cells from peripheral blood leukocytes (PBL). Interestingly, neutrophils had a significantly higher phagocytic percent than B cells in both HKL and PBL, while the phagocytic index of cod B cells were higher than for neutrophils in both HKL and PBL.

Experimental immunizations with different type of antigens and haptens coupled to soluble proteins (TNP-BSA, TNP-LPH, DNS-LPH, NIP-LPH, LPH) or sheep red blood cells have also been performed (Espelid et al., 1991; Israelson et al., 1991; Magnadóttir et al., 2001; Schrøder et al., 1992; Stenvik et al., 2001). These studies corroborated and highlighted the unusual response of the Atlantic cod's adaptive immune system compared to other fish species like Atlantic salmon. In many studies using ELISA assays, researchers recorded a null or poor specific antibody response against the immunizing antigen, when compared between immunized and non-immunized groups

antibody levels were not significantly different between them. This phenomenon intrigued researchers for several years leading to many studies trying to decipher the underlying causes. In a study from 2005, Pilström et al. mentioned that according to his own experiments using cross-hybridization and RT-PCR approaches with degenerate probes representing consensus fish MHC II sequences, he was not able to detect cod MHC-II. They hypothesized that maybe Atlantic cod has deleterious MHC II axis (Pilström et al., 2005). In 2011 (Star et al.), a study described the loss of the entire MHC II locus and the absence of a functional CD4 in Atlantic cod confirmed Pilström's hypothesis. CD4 was found to be a pseudogene and the invariant chain (Ii) functionally associated with the MHC II-CD4 antigen presentation pathway was also not present in the genome of Atlantic cod. This peculiarity has been reported in all species of the Gadiformes lineage. Interestingly, genomic studies have revealed that other fish species such as pipefish and monkfish have also lost the MHC II (Malmstrøm et al., 2016).

Atlantic cod's unique immune system has raised the interest to study it as a model for evolutionary aspects. The functional study of fish lymphocytes has been hindered by the lack of reliable reagents such as mAbs to study B and T cells. In the lack of mAbs, new technologies like single-cell RNA sequencing (scRNA-seq) have invaluably contributed to the study and understanding of the Atlantic cod's immune system. Based on scRNA-seq, Guslund et al. (2020) described the presence of major immune cell populations in Atlantic cod including lymphocytes, and later on describing different T cell subsets in this species, among which a large subpopulation that did not express CD8 (Guslund et al., 2022).

Most research on the Atlantic cod immune system has been done in the context of aquaculture, in efforts to make efficacious vaccines to prevent diseases outbreaks. This research was spurred by the collapse of the Atlantic cod stock in the western Atlantic, specifically the Canadian banks, in the late 1980s (Harris, 1998). Since then, this species has been the subject of experimental vaccinations against different pathogens such as *Vibrio anguillarum*, *Aeromonas salmonicida* and *Francisella noatunensis* (Mertes et al., 2021; Schrøder et al., 2009). These experiments have shown variable results based on factors like the bacteria itself, adjuvants, and environmental factors like temperature and other seasonal conditions. Besides all this, the common and widely accepted belief is that Atlantic cod has a poor or null antibody response (Magnadóttir, 2014; Pilström et al., 2005). This aspect is highly significant since specific antibody titers is an important parameter that correlates with the efficacy of the vaccination and with protection. Intriguingly, in many of these studies the vaccinations were still protective and produced immunological memory.

1.5 Aquaculture and vaccine strategies

1.5.1 Current status of Atlantic cod aquaculture

The Food and Agriculture Organization (FAO) of the United Nations has reported that aquaculture is the fastest expanding food sector globally (FAO, 2021). This surge in demand is projected to continue, with farmed aquatic species anticipated to be the primary source of fish for human consumption by 2030 (FAO, 2021). However, the global expansion of farmed fish species presents considerable sustainability challenges. Issues such as infectious diseases outbreaks, inefficient feed conversion, early sexual maturation, environmental risks, and in some cases, slow and inadequate genetic improvement, pose long-term risks to the aquaculture industry (Puvanendran et al., 2022; Sonesson et al., 2023).

1.5.2 Challenges and diseases

Infectious diseases pose a significant challenge to the aquaculture industry. Rising global temperatures, diversification of farmed fish species, and intensified production directly contribute to the increased susceptibility of farmed species to diseases (Cascarano et al., 2021). The impact of infectious diseases on the industry is multifaceted. Firstly, it reduces the feed intake of sick fish, slowing down their growth curve, which necessitates keeping them longer than expected and therefore delays readiness for market entry (Lieke et al., 2020). Secondly, the outbreak of bacterial diseases leads to increased use of antimicrobials, which have a clearance period that must be met, again delaying market entry. Excessive use of antimicrobials also harbors potential risks to human health, as it could contribute to the development of resistant strains of human pathogens. Moreover, infectious diseases in aquaculture raise serious concerns regarding fish welfare as well as its possible impact on wild populations (Johansen et al., 2011).

1.5.3 Vaccine development and strategies for disease management

Prophylactic methods are preferred over treatment methods for disease prevention. Thus, vaccines are the best-known method to prevent and reduce the impact of infectious diseases. The efficacy of using vaccination in teleosts has been demonstrated since the late 1930s and early 1940s (Gudding & Van Muiswinkel, 2013). This has also been demonstrated by the fact that vaccination significantly prevents the use of antimicrobials. In Norway, the significant reduction of antibiotics use in Atlantic salmon farming is largely attributed to the introduction and effective use of vaccines (Bondad-Reantaso et al., 2023). During the decade of the 1990, a notable shift from antibiotics to vaccination occurred across Norwegian fish farms, thanks to a collaborative effort involving the government, academy, farmers, and the industry. This change was facilitated by an automated process of injecting vaccines into the salmon during their freshwater phase, ensuring uniform and effective vaccination across populations (Sommerset et al., 2005). This prophylactic method also benefits the sustainability of the global aquaculture industry in terms of the environment, society, and economy.

Most of the current vaccines used in aquaculture target viral and bacterial pathogens. The types of vaccines include attenuated or inactivated pathogens, subunit, recombinant live vector, recombinant protein, and nucleic acids vaccines. Delivery routes vary depending on the type of the vaccine. They can be delivered through intraperitoneal or muscular injection, immersion (bath, dip), or orally (Ma et al., 2019). Future fish vaccines aim to be eco-friendly and cost-effective, continuing to offer broad-spectrum protection against infectious agents.

Live-attenuated vaccines are desirable because they can mimic natural invasion by the pathogen and usually trigger a strong and protective immune response (Lauring et al., 2010; Wise et al., 2015). This type of vaccine is suitable for farmed species where the high costs associated with other types of vaccines (e.g., subunit, recombinant vaccines) and vaccination strategies (e.g., intramuscular, intracoelomic injection) that are not cost-effective. However, live attenuated vaccines carry an inherent risk of reversion to virulence, and may cause severe disease which can become lethal in immunocompromised individuals (Ben Hamed et al., 2021).

Inactivated vaccines comprise chemically (e.g., formalin) or thermally killed organisms. These vaccines are used to target several bacterial and viral diseases prevalent in aquaculture. Usually administered via intracelomic or muscular inoculation, although other methods like

immersion can also be employed in some cases. Their use dates back to 1942 when Duff first applied an inactivated oral vaccine against *A. salmonicida* on cutthroat trout with significant progress marked by the development of vaccines since then (Duff, 1942). While they can trigger protective immune responses, effectiveness varies with pathogen type and vaccine formulation, fish species, and adjuvant presence. The low-cost production of these vaccines makes them a great fit for the aquaculture industry (Sommerset et al., 2005). However, their preparation can compromise the integrity of some pathogens, potentially reducing effectiveness.

Modern vaccinology includes alternatives such as subunits vaccines, which contain specific pathogen component(s) (e.g., protein), reducing side effects and safety concerns associated with live attenuated vaccines. Subunit vaccines are stable and can target unique antigens for enhanced immunogenicity and specificity. Thus, subunit vaccines can strike a balance between safety, specificity, and stability, making them valuable tools in disease prevention. However, the substantial costs associated with production can hinder its use in some species due to economic feasibility concerns. Furthermore, it has been shown that, depending on the fish species and subunit used, some subunit-based vaccines have weak immunogenicity and multiple immunizations are recommended (Ji et al., 2020).

Another strategy that that has been developed more recently is nucleic acid-based vaccines. The best know is the mRNA vaccine successfully used during the covid pandemic. In these vaccines, a piece of DNA or RNA encoding the target protein derived from the pathogen is delivered to a host cell, either as naked nucleic acid or by a vehicle such as a harmless virus (Evensen & Leong, 2013). The delivered nucleic acid is transcribed (if it is DNA) and translated by the host cell, which then produces the antigenic protein that triggers an immune response. Enhancing efficacy has been attempted by targeting the vaccines to antigen-presenting and dendritic cells using molecular adjuvants and carriers. However, challenges like low encapsulation efficiencies and DNA degradation persist. Safety, public perception and acceptance, regulatory concerns, and the lengthy, costly market approval process pose hurdles, necessitating more research to improve DNA vaccine efficacy against viral diseases in fish (Hølvold et al., 2014). DNA vaccines have had success in combating specific fish viral diseases like IHNV (Infectious Hematopoietic Necrosis Virus) and VHSV (Viral Hemorrhagic Septicemia Virus) (Marsella et al., 2022), but can be less effective against other vaccine presentations due to issues like low immunogenicity, suboptimal immune responses, and DNA degradation (Byon et al., 2006; Traxler et al., 1999). DNA vaccines targeting IHNV and VHSV have been commercially launched in Canada, and more recently, Clynav, a DNA vaccine formulated to combat salmon pancreatic disease (caused by salmonid alphavirus-3, SAV3), received marketing authorization in the European Union (Jose Priya & Kappalli, 2022).

Due to immunological and physiological differences among the variety of fish species employed in aquaculture, vaccine strategies should be adjusted according to each specie's needs. As mentioned earlier, Atlantic cod lacks the CD4-MHC II antigen-presentation pathway of the adaptive immune system thus different strategies for vaccinating cod should be considered compared to the ones used in Atlantic salmon, channel catfish, or any other fish species. Malmstrøm et al. (2013) described that cod possesses an exceptionally high count of MHC I genes and that the analyzed MHC I sequences present two different endosomal sorting motifs. They suggested that these two signal motifs enable cross-presentation, similar to MHC II functionality,

and allow for a more versatile use of MHC I. Thus, the novel type of MHC I molecules is suggested to have acquired the antigen-presenting functions typically associated with MHC II (Malmstrøm et al., 2013). Professional APCs present antigens to CD4+ T cells and are generally identified by high expression of MHC II in most species. Expression of genes involved in antigen presentation has been identified in splenic macrophages and DC-like cells in cod (Guslund et al., 2020), but no functional studies have been performed with these cells and it is currently not known how to best activate both humoral and cell-mediated immunity in cod.

Importantly, as well as scientifically sound, vaccination strategies should also be economically viable. For instance, although intracelomically delivered vaccines showed good efficacy in some species, this method might not be feasible to be applied due to its high cost-effectiveness.

1.5.4 Uncovering the immune systems of teleosts through genome editing: the advent of the CRISPR/Cas9 technology

By far, the best understood vertebrate immunologically is the inbred laboratory mouse. Numerous carefully controlled studies with wild-type and gene-modified mice have yielded a mechanistic understanding of the immune system and how this can be controlled or harnessed in various ways. Unfortunately, these findings do not always translate easily to human medicine, and they translate even less well to fish, which show numerous evolutionary differences in their immune system with mammals. Furthermore, teleosts constitute a vast taxonomic group, that diverged approximately 350 million years ago and have undergone whole-genome duplication events, leading to significant differences in the evolution of their immune systems (Pasquier et al., 2016). Therefore, to get a comprehensive understanding of the immune system of a particular species, studies in that specific species are necessary. One way of achieving this is through functional methods using targeted genome editing as has been done with great success in the mouse. This will enhance our comprehension of their immunological responses, which may lead to the development of targeted vaccination strategies according to each specie's unique needs. Understanding the ways that many different fish species react to different pathogens or stressors enables us to establish preventive health measures and improve their productivity. This knowledge is particularly crucial in aquaculture, where disease outbreaks could result in catastrophic economic consequences. Moreover, studying the immune system of fish species used in biomedical research that serves as comparative models for human diseases is also essential. It will define basic immunological mechanisms through the different vertebrates' clades as well as differences among them. This will help to determine the extent of comparability of the immune system between them and their viability to answer specific scientific questions.

A variety of technologies have been employed to alter the genomes of diverse organisms. These technologies include the utilization of meganucleases, transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs), clustered regularly interspaced short palindromic repeats (CRISPR) systems, alongside other innovative genome-editing instruments stemming from CRISPR, like base editors and prime editing (Gaj et al., 2013). Among these, the CRISPR-based genome editing systems have been a game changer and stand out due to precision, versatility, and cost-effectiveness (Hillary & Ceasar, 2023). CRISPR technology allows for the precise knock-in and knock-out of DNA sequences within the genome of any organism, providing unprecedented access to understand and modulate them genetically and hence functionally. As the

practicality and economic feasibility of these genetic technologies have improved, they have become increasingly accessible to researchers worldwide. This has led to a significant surge in studies exploring the function of coding and non-coding DNA sequences of several species, including fish. As researchers continue to exploit this technology, we can expect an increase of new insights into teleosts immune systems, potentially revolutionizing aquaculture health management and disease prevention strategies. This proliferation of knowledge not only benefits the aquaculture industry but also contributes to the broader scientific understanding of immune systems and genetic manipulation across diverse species.

CRISPR-Cas9/Cas12a is a revolutionary genome editing tool derived from a bacterial immune mechanism against bacteriophages (Doudna & Charpentier, 2014). This system consists of two key components: either the Cas9 or Cas12a enzyme, which is an endonuclease and acts as molecular scissors, and a guide RNA, designed to match the specific DNA sequence targeted for editing. The guide RNA directs the Cas enzyme to the desired DNA sequence location. Once there, Cas induces a double-stranded break in the DNA. This break triggers the cell's own DNA repair machinery, either through the error-prone non-homologous end joining (NHEJ), often leading to gene mutation via insertion or deletion of nucleotides, or the more precise homology-directed repair (HDR). The latter can be used to induce a specific insertion of a DNA sequence or mutation when the Cas-guide RNA complex delivered to the cell is supplied with a DNA repair template carrying desired. Thus, by simply altering the guide RNA sequence, CRISPR-Cas9/Cas12a offers unprecedented precision and versatility for genome editing.

The zebrafish model system has led the charge in advancing genome editing technology using CRISPR/Cas9. Early works demonstrated the efficient induction of small indels in injected zebrafish embryos (Hwang et al., 2013). Since then, this technology has been rapidly developed and widely applied in numerous organisms, including fish species like grass and common carp, Atlantic salmon, tilapia, and catfish (Roy et al., 2022).

2 Aims

The fundamental goal of this doctoral research was to deepen the understanding of the humoral adaptive immune response in Atlantic cod. Utilizing traditional immunological assays, the humoral immune response of Atlantic cod was assessed following immunizations with either model antigens or a natural pathogen-derived vaccine. Another goal of this thesis was to set the stage for immune studies of gene-modified Atlantic cod.

The specific objectives were:

- To analyze the adaptive humoral immune response of Atlantic cod post-immunization with hapten-carriers known to employ different pathways of antibody response in mammalian systems.
- To determine the IgM antibodies from immunized Atlantic cod function as a specific protective factor.
- To develop a protocol for genome editing in Atlantic cod, employing the CRISPR-Cas9/Cas12 system as a platform to further investigate functional aspects of its immune system.

2.1 Summary of papers I-III

2.1.1 Manuscript I: Hapten Carrier-Induced Antibody Responses in a Naturally MHC II-Deficient Teleost Fish

A B cell epitope, often referred to as an antigenic determinant, is the specific portion of an antigen that is bound by a BCR. Haptens are small molecules that serve the function of an epitope, but on their own cannot elicit an immune response. Only when attached to a larger carrier molecule can haptens become immunogenic and induce B cell activation, proliferation, and the production of antibodies that specifically recognize it. We aimed to describe the specific antibody response of Atlantic cod against different haptenized carriers. For this purpose, we screened for the presence of natural antibodies in cod against various haptens. We selected 4-Hydroxy-3-nitrophenylacetyl (NP) for further studies as we found little to no pre-existing antibodies targeting this hapten in sera from non-immunized fish. Atlantic cod were immunized with NP covalently conjugated to different carriers known to elicit T cell-dependent (TD), T cell-independent type 1 (TI-1), and T cell-independent type 2 (TI-2) immune responses in mammals. We found that Atlantic cod did not develop antibody immune response against TD nor to TI-1 antigens, but they did exhibit NP-specific antibodies when immunized with TI-2 antigens. These titers persisted at elevated levels for up to six months post-immunization with a single vaccination. Contrary to the widely accepted notion that Atlantic cod produce a null or poor specific antibody response, our findings reveal that this teleost, which naturally lacks the MHC class II-CD4 axis, can generate a potent and long-lasting antibody response triggered by an antigen know to employ a TI-2 cellular and molecular mechanism in mammals.

2.1.2 Manuscript II: Protective antibody response in Atlantic cod with evolutionary losses of MHC class II and CD4

Historically, the Atlantic cod Gadus morhua was believed to exhibit weak antibody responses after immunizations, likely due to its evolutionary absence of the MHC class II locus and CD4, both crucial for T cell assistance in antibody responses. However, when we exposed Atlantic cod to formalin-fixed *Vibrio anguillarum* serotype O2a through bath immunization, we observed notable serum IgM levels interacting with the *V. anguillarum* antigens. This method of immunization conferred robust immunity against both mucosal (bath) and systemic (intracoelomic injection) infections by the virulent *V. anguillarum* O2a and offered partial protection against a mucosal infection by the non-isogenic *V. anguillarum* O2b. This protection persisted for over a year post-immunization. To further understand the protective role of immune IgM, we transferred serum or purified IgM from vaccinated donors to naïve recipients, using serum from unvaccinated donors as a control. The results showed that only the immune serum or immune IgM purified from vaccinated donors offered protection against a lethal *V. anguillarum* O2a infection, not the naïve serum. Western blot analyses revealed that the immune serum IgM recognized protein antigens in the O2a serotype and displayed some cross-reactivity with O2b. Notably, unlike rabbit sera against *V. anguillarum* O2a or O2b, the immune cod serum did not recognize the LPS O-antigen. Even without MHC class II and CD4, Atlantic cod can be effectively vaccinated to produce protective antibodies against a gram-negative bacterial pathogen. These antibodies seem to primarily target protein antigens rather than the LPS O-antigen presented by the bacteria.

2.1.3 Manuscript III: CRISPR-Cas9/Cas12a-based genome editing in Atlantic cod *Gadus morhua.*

In Manuscript III, our objective was to create a platform for knocking out genes associated with Atlantic cod's adaptive immune responses, enabling detailed functional studies in subsequent experiments. To this end, we chose to target the *slc42a* gene as homozygous mutants are phenotypically presented as albino-like. We evaluated five CRISPR formats to ascertain the most effective method for generating knockouts in Atlantic cod. The Cas9 protein was introduced in preformed ribonucleoprotein (RNP) complexes with either single guide or duplex guide RNAs. Alternatively, an mRNA encoding Cas9 was paired with the same two guide RNA formats. Cas12a was also assessed in RNP complexes with single guide RNAs. Our findings indicated that the combination of Cas9 mRNA with a single guide RNA was the most efficient in knocking out both alleles of the *slc45a2* gene. This led to an albino-like phenotype in up to 75% of the surviving larvae. DNA analysis of individual larvae showed mosaic genotypes. However, the high mortality rate of injected eggs meant the overall efficiency was low. Despite this, our study provides a foundational step for future genetic and functional research using the CRISPR/Cas9 genome editing system in Atlantic cod.

3 Methodological considerations

3.1 Hapten-carriers

It has been reported that Atlantic cod may have natural antibodies reacting with different synthetic haptens and proteins commonly used in immunological research. We therefore screened serum from naïve fish and from fish immunized with formalin-fixed bacteria *Vibrio anguillarum* (an irrelevant immunogen in this context) against a panel of different haptens (4-Hydroxy-3-nitrophenylacetyl (NP), Azobenzenearsonate (ABA), 2,4-Dinitrophenyl (DNP), 4-Hydroxy-3-iodo-5-nitrophenylacetyl (NIP), Phosphorylcholine (PC), 2,4,6-Trinitrophenyl (TNP)) coupled to BSA to evaluate the presence of natural antibodies against these haptens. Pooled sera from 12 fish showed the lowest reaction against NP, with similar OD values of the same sera against non-conjugated BSA. There was an important biological variability among the individual sera used in the screening, with some fish showing high natural antibodies against all the screened haptens. We chose the NP hapten because this showed lowest reactivity with natural antibodies, making it more likely we could track changes NP-specific antibodies in each individual tagged fish over time.

We selected standard TD and TI commercially available haptenated carriers. As a protein carrier , we chose KLH given that previous studies have used different proteins with no discernible response. It could have been beneficial using TD and TI type I carriers. Unfortunately, logistics was a limiting factor. For proteinaceous antigens, other protein carriers have been used in previous studies with the same conclusions we got. For the LPS, to our knowledge, no studies have been conducted to address differences of antibodies responses against haptens coupled to different LPS carriers and it should be addressed in future studies.

3.2 Hapten immunization protocols

Immunization was conducted based on previous studies involving other fish species and Atlantic cod. The intracoelomic (IC) injection route was chosen due to the low amount of NP-carrier used, and because it is commonly employed in many aquaculture vaccination settings. For the adjuvant, we selected Imject™ Alum (Thermo Fisher Scientific), an aqueous solution of

aluminum hydroxide and magnesium hydroxide. This choice was made because oil-based adjuvants have been observed to cause significant persistent granulomatous inflammation and peritonitis in Atlantic cod when injected IC. The choice to use 10-16 fish across different treatments was primarily driven by animal welfare concerns and statistical considerations. Prioritizing the well-being of our subjects, we aimed to minimize the number of animals exposed to treatments, adhering to ethical standards that advocate for the least number of subjects without compromising the integrity of the research. Statistically, while larger samples offer more power, our design was based on prior studies and preliminary data suggesting that our chosen sample size was sufficient to detect meaningful differences. This approach ensured both ethical responsibility and methodological rigor.

Boosting was performed 6 weeks after the initial immunization. In teleost fish, the timing and peak of antibody production can vary based on factors like temperature, sex, fish species, the antigen used, adjuvants, and the specifics of the immunization protocol. Litle is known regarding to this in Atlantic cod given its previous reported poor antibody response. While some reports indicate that antibodies in teleost fish peak around 3 weeks post-immunization, this is not universally true and in some fish species this might take longer periods. This notion is further supported by the second trial where a group was given only a single immunization without a subsequent boost and showed a peak at 9 weeks post-immunization. Generally, boost immunizations are administered when antibody levels start to wane to enhance antibody titers and strengthen long-term immunity. It is still a matter of debate if fish have a real boost effect and if so to what extent it is applicable in terms of fish species. However, given differences among the teleost fish and mammals immune systems, if boost effect is present, the optimal interval for boosting might differ.

3.3 Sampling time points

Timepoints for sampling were also defined based on different studies showing differences in specific antibodies after 3 weeks post-immunization. Sampling blood every three weeks was considered appropriate to detect any changes in the specific IgM level over time, as well as considerations for the fish welfare. The last time point was 25 weeks post initial immunization. This time point was set to serve as a cut off to look for long-lasting specific IgM antibodies. This also could have been done for a 1-year period in order to evaluate a longer lasting specific response over different seasons, temperatures, and other seasonal fluctuations and also about how much it can be affected this seasonality.

3.4 *Vibrio anguillarum* immunization and infection challenge protocol

The bath immunization protocol used in our studies was chosen based on previous studies using this method given that it simulates the natural infection route of water-borne pathogens at mucosal sites. Vaccination doses were based on previous studies and infectious doses were titrated with our strains. We aimed to avoid oil-based adjuvants via intracoelomic injection, which are known to induce peritonitis and persistent inflammation in Atlantic cod. We titrated the doses to achieve a mortality rate between 50% and 80%, enabling a robust statistical analysis. This approach aligns with different studies looking at vaccines efficacy in aquaculture settings and makes it easier to discern differences among vaccinated and non-vaccinated groups. Two different *V. anguillarum* serogroups (O2a and O2b) during the challenges. This allowed us to evaluate for

specificity by looking at antibody cross-reaction and survivability after challenge with isogenic bacteria.

3.5 CRISPR-Cas9/Cas12a genome editing system protocol

Due to the breeding seasonality of Atlantic cod and associated logistical issues, obtaining their eggs throughout the year is not feasible. Consequently, we successfully utilized zebrafish to validate the microinjection technique and to evaluate the quality of the in vitro transcribed mRNA by observing eGFP expression in this species. A significant challenge was the pronounced mortality rate in microinjected cod eggs at the 1-2 cell stages. This was largely due to the rigidity of the Atlantic cod egg's chorion at these stages. This hardness, which only increased as the eggs matured, made needle insertion for delivering the CRISPR-Cas9/Cas12a components challenging. Attempts to microinject at this stage yielded nearly 100% mortality, even without injecting the construct and only PBS. We tested several reagents to soften the chorion, but these attempts were unsuccessful. As a result, we shifted to performing microinjections immediately post-fertilization, rather than waiting for the fertilized eggs to reach the 1-2 cell stage, with the cost that a considerable proportion the of the injected eggs were not actually fertilized (16%). Additionally, microbial contamination posed a threat to the eggs and embryos at these stages and microinjection might facilitate pathogens invasion. While we implemented sea water sterilization with filters, we refrained from using antimicrobials to avoid potential toxic effects on the Atlantic cod eggs. This is a particular issue that should be addressed in future research to enhance embryo survival.

It was possible to perform only two sessions of microinjection due to logistics constraints (including travel restrictions due to the covid pandemic). This hampered the opportunity to try different concentrations of the different treatments. It has been documented that different concentrations have a direct effect on mortality of the eggs, while high concentrations are toxic, too low concentrations affect efficiency of the method. Although concentrations used were calculated based on previous studies with different fish species, it certainly needs to be optimized for Atlantic cod in particular. By optimizing these factors survivability might improve significantly.

When using the CRISPR-Cas9/Cas12a genome editing system, there's a potential risk of unintended off-target modifications. Our study did not conduct an exhaustive screening for these off-target indels. We primarily aimed to develop a protocol tailored for Atlantic cod, so our analysis was confined to on-target indels in larvae. Technical constraints in maintaining CRISPR knockouts beyond the feeding stage meant that our DNA samples were limited, making a comprehensive genome-wide off-target evaluation unfeasible. Furthermore, off-target modifications are largely guide-RNA dependent. However, for future efforts to breed Atlantic cod with specific knockouts to establish genetic lines, rigorous off-target screening will be essential.

4 Results and discussion

Previous studies have shown that antibody responses developed by Atlantic cod after immunizations are variable and mostly showing either a weak or null response. Although Atlantic cod possesses a high amount of IgM in blood, up to 10 times higher than the levels found in Atlantic salmon, specific antibodies do not increase after vaccination and are not a reliable parameter to measure the effectiveness of a vaccine, even when successful protection has been achieved in some experiments. This is remarkable and it raised the questions such as how does Atlantic cod’s

immune system develop specific immunity after vaccination or immunization? Which humoral and cellular factors are involved and are more crucial in this immune response in the long term? How does its immune system discriminate between serotypes after immunization with a serotype-specific vaccine?

In mammals, several antigen types have been established to study antibody responses after immunizations. These antigens have been categorized mainly on the basis of the involvement of T cells. If they are involved directly in cell-to-cell contact, the antigen is categorized as T cell-dependent antigen and the induced antibody response as TD immune response. On the other hand, if T cells are not involved, the antigen is categorized as T cell independent. Furthermore, the latter can be subdivided into TI type 1 (TI-1) and TI type 2 (TI-2). In TI-1 immune response, an antigen, LPS typically, is recognized by the BCR as the first signal, and also by TLRs which delivers a secondary signal. These signals trigger a polyclonal proliferation of the B cells. In TI-2 immune response, the typical antigen possesses highly repetitive motifs. These highly repeated units are recognized by several BCRs on the same B cell. This cross-links the BCR, which delivers a strong activation signal to the B cell resulting in clonal proliferation. We used hapten NP conjugated to representative carriers to evaluate if Atlantic cod responds to any of these classical antigens. The Atlantic cod did not develop a specific immune response against NP when conjugated to the TD antigen KLH, as previously reported, nor when conjugated to the TI-1 antigen LPS. Strikingly, Atlantic cod did respond against TI-2 antigens in a strong fashion. Moreover, this immune respond lasted for at least six months after the initial immunization. It seems that there is no true boost effect in this species, indeed, in the group of fish immunized and boosted with NP-ficoll in the second trial, the specific antibodies after the boost went transiently down, but rebounded and reached comparable levels to those registered in fish immunized only once with NP-ficoll 12 weeks after the boost. Also, the Atlantic cod IgM half-life was determined by *in vivo* injection of labelled and IgM and calculated to be 4 days. This means that the levels of the antibodies are maintained over time by active IgM secretion by antibody secreting cells.

After being immunized, the Atlantic cod developed a long-lasting and specific immune response against the *Vibrio anguillarum* (*Va*) serotype O2a. The immune response and protection conferred by immunizations was specific, being more robust against this vaccine's parental serotype than a different serotype, *Va*-O2b. Specific IgM antibodies play an important role in immune protection. Protective anti-*Va*-O2a IgM antibodies were proven by passive immunization of naïve fish with whole serum and purified IgM from immunized fish. Moreover, specific adaptive immune responses activated through mucosal immunization confers both mucosal and systemic protection. Therefore, the Atlantic cod IgM response targets in a specific way *Va*-O2a and cross-react against *Va*-O2b. It is important to notice that *Va*-O2a and *Va*-O2b harbor other differences than the terminal LPS O-antigen sugars. Protease sensitivity shows that the antigenic targets are protein-based, either with or without modifications. This discovery reshapes our understanding of Atlantic cod's adaptive immune system, especially given its absence of specific genes like MHC class II and CD4+ T cells.

We have successfully applied the CRISPR-Cas9 genome editing system to Atlantic cod, specifically targeting the slc45a2 gene, which is involved in melanin production. Sequencing results showed indels in 16 specimens, which were chosen based on noticeable changes in phenotypic pigmentation. Notably, the combination of Cas9 mRNA and a single-guide RNA was

the most effective in both the number of specimens exhibiting indels and the efficiency of indel generation. It's important to note that the actual rate of indels may be significantly underestimated. This is because we only selected embryos with a visually albino phenotype for sequencing, which requires indels in both alleles. As a result, minor albino-like phenotypes might have gone undetected. While these initial results are foundational, they pave the way for further studies on the immune system of Atlantic cod. As other studies have highlighted, functional studies using the F0 generation can be valuable, especially when, for various reasons such as a lengthy timeframe to reach sexual maturity and breeding seasonality, using the F1 generation is less feasible or impractical. This is true for Atlantic cod, whose breeding might necessitate a 3–4-year timeframe.

5 Concluding remarks and future perspectives

Throughout our studies, we have delved deep into the distinctive immune response mechanisms of the Atlantic cod. This exploration spans its antibody response to the development of innovative tools for a more profound understanding of its fundamental immune system processes.

In Manuscript I, focusing on the adaptive humoral immune response, we present findings that challenge widely held beliefs about the immune capacities of the Atlantic cod. Despite its inherent lack of the MHC class II-CD4 axis, the Atlantic cod exhibits a remarkably long-lasting, strong, and specific antibody response. This highlights that the cod's antibody-driven immune response is not just robust, but also intricately fine-tuned in its defensive strategies.

In Manuscript II, we delved deeper into the functional role of Atlantic cod's IgM, examining it under conditions that more closely reflect natural scenarios. We confirmed earlier findings: after vaccination with *V. anguillarum*, the Atlantic cod generates specific IgM antibodies and protection against the homologous serotype, and cross-protection against heterologous serotype, accentuating the refined specificity of the cod's antibody response. Significantly, our data illustrated that both the whole serum and its isolated IgM from *V. anguillarum*-immunized fish were capable of protecting naïve fish from *V. anguillarum* infections. This emphasizes the pivotal protective contribution of the humoral response in this fish species. Strikingly, this protection targets a protease-sensitive component of *V. anguillarum*, defying the common belief of protection being tied to lipopolysaccharide (LPS).

However, as we transition from immunology to genomics in Manuscript III, we recognize the early stages of genome editing in Atlantic cod. While the CRISPR-Cas9 system's efficiency remains modest, the establishment of a foundational protocol for gene knockout in this species paves the way for more advanced genetic manipulations and investigations.

Overall, the findings presented in these three manuscripts provide a strong foundation for future research on the Atlantic cod adaptive immune system. The development of new tools and technologies, such as CRISPR-Cas9 genome editing, will enable researchers to further elucidate the complex mechanisms of immunity in this important teleost fish species.

Looking forward, we identify several prospective research paths:

- Characterize Atlantic cod's adaptive immune response further. This involves the detailed study of different B cell subpopulations, antibodies, and the supportive roles of T cells and other immune cells in the humoral response.

- Examine protective mechanisms against a range of pathogens. Determine which specific antigens are targeted by the immune system and assess the contributions of antibodies, complement proteins, and phagocytes.
- Develop innovative vaccines for Atlantic cod. Utilize the insights from our foundational research on cod's immunity to create potent protective strategies against diseases.
- Optimize genome-editing techniques. Building on our initial research, there's significant scope to enhance the CRISPR-Cas9 methodology, improving its efficiency and embryo survival rates in Atlantic cod. Additionally, streamline methods for robust genome editing in Atlantic cod, facilitating the creation of fish lines with targeted gene mutations for in-depth immune system studies.
- Conduct in-depth gene function studies. Given the established platform for genome editing, the next steps should delve into the roles of specific genes in cod's immune system, providing insights into evolutionary nuances and adaptations.

In summary, the prospects for research into the Atlantic cod's immune system are promising. The advent of new tools and technologies, coupled with the expanding knowledge base on this species, will empower researchers to achieve substantial advancements in understanding the Atlantic cod’s adaptive immune mechanisms. These insights can subsequently be used to formulate innovative and effective strategies, such as vaccines, to shield Atlantic cod from diseases, a crucial step for the sustainability of this import cultural and aquaculture species in the North Atlantic.

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MANUSCRIPTS

CRISPR-Cas9/Cas12a-based genome editing in Atlantic cod (*Gadus morhua*)[†]

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Abstract

Aquaculture is the fastest-growing food sector worldwide but faces sustainability challenges that need to be addressed in many ways, including genetic enhancement. Atlantic cod has re-emerged as an aquaculture species and tools for genetic manipulation are needed. Thus, we compared five formats of CRISPR to determine which was most efficient to generate knock outs in Atlantic cod. Cas9 protein was presented in preformed ribonucleoprotein (RNP) complexes with single guide or with duplex guide RNAs or an mRNA encoding Cas9 was used with the same two formats of guide RNAs. Cas12a was tested as RNP complexes with single guide RNAs. We found Cas9 mRNA with single guide RNA to be the most efficient format to knock out both alleles of the *slc45a2* gene, which results in an albino-like phenotype in up to 75% of surviving larvae. DNA analysis of individual larvae revealed mosaic genotypes. The mortality of injected eggs was high, resulting in low overall efficiency. Nevertheless, this study lays the foundation for further genetic and functional research using the CRISPR/Cas9 genome editing system in Atlantic cod.

Keywords: Atlantic cod, CRISPR/Cas9, CRISPR/Cas12a, genome editing, knock out, albino-like

1 Introduction

Aquaculture is the fastest growing food sector worldwide as reported by the Food and Agriculture Organization (FAO) of the United Nations (FAO, 2020). This demand is expected to continue to grow, and farmed aquatic species are predicted to be the main source of fish destined for human consumption by 2030 (FAO, 2020). However, the global rise of fish farming faces challenges threatening its sustainability, including disease outbreaks, suboptimal feed conversion, early sexual maturation, environmental issues, and lack of breeding programs for fish. (Puvanendran et al., 2022; Sonesson et al., 2023).

Aquaculture is the second most important economic activity in Norway, with Atlantic salmon as its flagship. Nevertheless, Atlantic cod (*Gadus morhua*) has re-emerged in recent years as a promising farmed species. It has had an important historical and cultural impact on societies of the north Atlantic. With its farming starting in the 1980s, Atlantic cod production rose during the next two decades reaching a maximum production in 2010 and collapsing in the period of 2011-2014 (Nardi et al., 2021). One of the factors that contributed to this collapse was the emergence of infectious diseases such as Francisellosis, which caused devastating outbreaks (Nylund et al., 2006; Ottem et al., 2008). A steady recovery of the Atlantic cod farming has been reported in the past years with a production of about 5000 tons in 2022 and more than 7000 tons first six months of 2023 (The Norwegian Directorate of Fisheries, 2023).

Infectious diseases are one of the biggest threats to aquaculture sustainability. Vaccination is an efficient prophylactic method against diseases thereby improving animal health and reduction in the use of antimicrobial agents. The Atlantic cod possess a unique immune system when

compared to mammalian and other vertebrates including other fish species like Atlantic salmon and rainbow trout. The absence of major histocompatibility complex class II (MHC II), invariant chain, and the CD4 molecules (Star et al., 2011) in cod is highly relevant to consider for the development of vaccination strategies. The Atlantic cod has a poor or null antibody response after vaccination even when protection was conferred (Magnadottir, 2014). Hence, understanding how the cod immune system works is crucial to secure effective vaccination strategies. Therefore, genome editing needs to be developed as a research tool in this species.

Genetic improvement has been shown to be a bulwark in the sustainability of several aquaculture species. This improvement has relied on methods including selective breeding, hybridization, chromosome manipulation, genomic selection, transgenics, and genome editing technologies (Bentsen et al., 1998; Ponzoni et al., 2011; Tonelli et al., 2017). Genome editing approaches can help to understand the basic functional aspects of the Atlantic cod immune system, enabling the enhancement of immunological competence, the increase of disease resistance, and the improvement of other productivity parameters. In the last years, the use of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) has gained popularity as a genome-editing tool because it is relatively simple to implement and cost-effective compared to other methods. This system provides a powerful tool for targeted gene editing, which enables scientists to selectively delete, modify, or insert genes in an organism's genome (Doudna & Charpentier, 2014).

Cas9 is a dual RNA-guided DNA endonuclease that allows precise, targeted modifications of genes by cutting DNA at specific locations recognized by a protospacer adjacent motif (PAM) sequence, followed by endogenous DNA repair mechanisms (Doudna & Charpentier, 2014). There are several Cas9 orthologues, thus PAM sequence also varies. For instance, Cas9 recognizes the NGG motif while Cas12a, a single RNA-guided DNA endonuclease, recognizes TTTX (Tóth et al., 2020). This technology is a revolutionary genome editing tool that has been rapidly developed and widely applied in numerous organisms, including fish like zebrafish, Atlantic salmon, tilapia, and catfish (Roy et al., 2022). Researchers have used CRISPR/Cas9 to generate disease-resistant strains of fish by knocking out or modifying genes associated with pathogens infections (Simora et al., 2020); improve food intake (Kishimoto et al., 2018); and it has also been used to study and control the sex of fish by disrupting or manipulating the genes involved in sex determination and differentiation (Lau et al., 2016; Li et al., 2014; Wargelius et al., 2016).

Although the use of this technology has been increasing in the last years, differences among fishes' biology intrinsically pose challenges in the implementation of this technology for some species. Thus, standardization and establishment of protocols need to be addressed according to each species' needs and characteristics.

The goal of this paper is to compare, describe, and establish a CRISPR-based genome editing method for Atlantic cod. This tool can be used to further investigate functional aspects of this species' physiology and immunology, with the potential to enhance production-related traits.

2 Materials and Methods

2.1 Ethics statement

Experiments were conducted according to the guidelines of the Regulation on the Use of Animals in Experiments of Norway.

2.2 Plasmids

The pT3TS-nCas9n plasmid (Addgene plasmid # 46757) was used to produce Cas9 mRNA (Jao et al., 2013). This plasmid encoding a zebrafish codon-optimized Cas9 protein was used to

create the pT3TS-EGFPn (enhanced green fluorescent protein) by standard molecular biology methods. Briefly, the vector was prepared by partial digestion of pT3TS-nCas9n with BglII and complete digestion with NcoI (New England Biolabs; NEB) to excise the Cas9 gene and purified by gel electrophoresis (QIAquick Gel Extraction Kit; Qiagen, 28704). The gene encoding EGFP was PCR amplified with Pfu polymerase (NEB) from pEGFP-1 (Clontech Laboratories) with primers EGFP-NcoI.for: GTACCATGGTGAGCAAGGGCGA and EGFP-BglII.rev: GCGAGATCTTCCCTTGTACAGCTCGTCCAT (recognition sites for NcoI and BglII underlined). The PCR product was digested with NcoI and BglII, gel purified (QIAquick Gel Extraction Kit) and inserted by DNA ligation into the pT3TS vector. Plasmid construction was verified by DNA sequencing (Eurofins Genomics).

2.3 Design of gRNAs

The *slc45a2* gene was selected for knockout as loss of both alleles of this gene causes an easily identifiable albino phenotype (Edvardsen et al., 2014). Four gRNAs were designed and selected with the web based Benchling's CRISPR analysis tool (https://benchling.com) and CRISPOR tool (http://crispor.org; Concordet & Haeussler, 2018) with the gadMor3.0 Atlantic cod genome as template. For the targets of Cas9, the website tools returned several target sites in exons 1 and 6. Three gRNA target site sequences with high CFD scores (CRISPOR) and with no potential off-target and one sequence homologous to the site previously described to produce knockouts in Atlantic salmon (Edvardsen et al., 2014) were selected (Table 1). For each of the four Cas9 gRNAs, the same gRNA sequence in two different formats were used: duplex guide RNA (crRNA:tracrRNA, henceforth called dg) and single guide RNA (henceforth called sg). For the Cas12a, due to the more restrictive PAM sequence, fewer targets were retrieved and no targets within exon 1 were obtained. Thus, targets for Cas12a found were one in exon 2, one in exon 4, and two in exon 6 (Table 1). Since Cas12a requires only a single-component guide RNA, only the single-guide crRNA (henceforth called cr) format was used. All gRNAs were purchased from Integrated DNA technologies (IDT).

2.4 In vitro transcription of Cas9 and EGFP mRNA

The pT3TS-nCas9n and pT3TS-EGFPn ("n" denotes nuclear localization signal) plasmids were linearized by XbaI (NEB) digestion and purified with QIAprep Spin columns (Qiagen) prior to *in vitro* transcription with mMessage mMachine T3 kit (Ambion). Cas9 and EGFP mRNAs were purified with the RNeasy MiniKit spin columns (QIAGEN) and the integrity of the mRNA was evaluated by gel electrophoresis according to the method described by Aranda et al. (2012).

2.5 Production of Cas9/Cas12a-guide RNA Ribonucleoprotein Complexes

Target-specific Alt-R crRNA and common Alt-R tracrRNA were used to prepare the crRNA:tracrRNA duplex according to manufacturer's instructions. Cas9 protein (Alt-R® S.p. Cas9 nuclease, v.3,) and Cas12a (Alt-R™ A.s. Cas12a (Cpf1) V3) were purchased from IDT and used to prepare duplex or single guide RNP complexes according to manufacturer's protocol. Prior to microinjection, the RNP complex solutions were incubated at 37°C for 5 min and then placed at room temperature.

2.6 Fertilization of Atlantic cod and zebrafish eggs

Atlantic cod eggs and sperm obtained from the breeding program at NOFIMA (Tromsø, Norway) were freshly collected and transported to the NOFIMA laboratory. Eggs were fertilized with sperm in a petri dish by swirling, adding filtered (0.2µM mesh) natural seawater and kept for 4 minutes at 6°C. Afterwards, the eggs were rinsed with filtered natural seawater and incubated for 15-30 minutes before microinjection.

Adult zebrafish (*Danio rerio*) stocks (Norwegian Center of Molecular Medicine, Oslo, Norway) were kept at standard aquaculture conditions (i.e., 28.5 °C). Fertilized eggs were collected via natural spawning. Embryos were reared under constant light conditions in embryo medium. All embryos and larvae were kept in an incubator, at 28.5 °C.

2.7 Microinjection procedures and screening of albino-like larvae

Glass capillaries (Narishige) were pulled using a micropipette puller (Narishige, PC-100) and subsequently beveled with a micropipette beveler (Narishige, EG-400) to make microneedles. To standardize the microinjection protocol, 1-cell stage zebrafish and fertilized Atlantic cod eggs were microinjected with 1.5 nL of EGFP mRNA (150 pg). The zebrafish embryos were kept at 28°C and Atlantic cod embryos at 6±2°C until hatching. Cas9 mRNA (300 ng/µL) and either duplex or single gRNA(s) (100 ng/µL) mixtures, or 20 µM preformed Cas9 (or Cas12a):gRNA RNP complex solutions were injected into the fertilized cod eggs using the FemtoJet® 4i microinjector (Eppendorf) under a stereomicroscope (Motic, SMZ168). The injected volume was approximately 1.5 nL. After microinjection, Atlantic cod eggs were kept at 6±2°C through the hatching period until larvae were harvested shortly prior to the onset of the feeding stage. Zebrafish and cod larvae were screened for fluorescence and images were taken using a Leica M205 FA stereomicroscope. The Atlantic cod larvae microinjected with the different treatments were screened for albino-like phenotype under a stereomicroscope and images were taken with an Euromex 4K Sony Ultra HD camera and analyzed using the Euromex ImageFocus Alpha. Selected larvae were euthanized with an overdose of MS-222, preserved in RNAlater® (Invitrogen™) and stored at -80°C until further analysis.

2.8 Larvae DNA extraction and sequencing

DNA was extracted from individual larvae that exhibited an albino-like phenotype and from control larvae. The larvae were rinsed with PBS to remove excess RNAlater and DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen, 69506) and eluted in 30 µL. PCRs to amplify the targeted exons were performed with 2.5 µL of the extracted DNA as template in a 50 µL-reaction with Phusion® High-Fidelity PCR Master Mix with HF Buffer (NEB, M0531S) and 2.5 µL of each primer (10µM) in the following primer pair combinations (exon1F: TGCTAGTGGAAGACCAGTCC vs. exon1R: GTTGAGGAAGAGGCTGATGC [product size 446 bp], exon2F: TGTGTGTCTTGTCTTGCAGC vs. exon2R: TTTACGGATCCTCGAAGGCA [product size 304 bp], exon4F: CATGCCTGGTGTTGTCTCCA vs. exon4R: ACATACGTGCACGGAACTCG [product size 597 bp], exon6F: CTCATTAGCTCACGGTTCGC vs. exon6R: GGCGAAGGAGACCACTCCAA [product size 405 bp]). PCR settings were as follows: initial denaturation at 98°C for 30 seconds; 35 cycles of denaturation at 98°C for 15 seconds, annealing at 68°C for 30 seconds, and extension at 72°C for 15 seconds; final extension at 72°C for 5 minutes; hold at 4°C. Resultant amplicons were purified by gel electrophoresis with the QIAquick Gel Extraction Kit (Qiagen, 28704) following the manufacturer's instructions. The forward primer from each unique PCR primer set was used to sequence the PCR amplicons. Sanger sequencing for each sample was conducted using the TubeSeq service provided by Eurofins Genomics, in accordance with the provider's instructions for sample preparation.

2.9 Assessment of CRISPR edits

The Sanger sequencing output files (.ab1 extension) for the forward strand of each selected larva were uploaded to Synthego's ICE (https://ice.synthego.com; Conant et al., 2022) and DECODR (https://decodr.org/analyze; Bloh et al., 2021) web-based tools for potential insertion

and deletion (indel) analysis. Sequences derived from control group larvae were included as a wild-type template for comparison. The sequences obtained from wild type larvae were validated by aligning them to sequences retrieved from the Atlantic cod genome available in the NCBI database.

3 Results

3.1 Microinjection of Zebrafish and Atlantic cod eggs with mRNA encoding enhanced green fluorescent protein

In order to evaluate the efficacy of the translation of injected mRNAs in the fertilized egg as well as the microinjection skills and technical set up, *in vitro* transcribed EGFP mRNA was microinjected in zebrafish at the 1-2 cell stage and Atlantic cod eggs at the same stage or earlier and followed up until they were about to hatch. All injected zebrafish eggs expressed EGFP in the animal pole of the eggs five- and 48-hours post fertilization (Fig. 1A&1B). For zebrafish, there was no appreciable loss of individuals during the follow-up period, which was until before the required feeding stage of larva. Microinjection of Atlantic cod eggs at 1-2 cell stage was difficult due to the hardness of the chorion. To circumvent this, we began the microinjection process prior to the appearance of the first cell. Furthermore, we noted that survival was poor compared with microinjected zebrafish eggs by the same researcher. Nevertheless, some injected Atlantic cod eggs clearly expressed EGFP stably up to 20 days post fertilization (Fig. 1C&1D).

3.2 Microinjection of Atlantic cod eggs with Cas9/Cas12a and guide RNAs

Four different gRNAs for both Cas9 and Cas12a were tested individually, or all combined in a single mix. For Cas9, both single guide (sg) RNAs and duplex crRNA:tracrRNA (dg) guides with identical target specificities were tested, i.e., 10 combinations of gRNAs were used. Furthermore, Cas9 gRNAs were tested by co-injection with mRNA encoding Cas9 protein as well as injection of preformed RNP complexes made from Cas9 protein and gRNAs. For preformed RNP complexes, we tested all sg RNAs as well as dg1-1 RNAs and the combination with all four tracers (dgAll). Cas12a guides were only tested as preformed RNP complexes with Cas12a, i.e., five combinations in total (Table 1).

A total of 8,356 fertilized Atlantic cod eggs were microinjected in this study. Very high mortalities were recorded in all the microinjected groups. The overall mortality was 96.3 % and some groups showed 100% mortality (Table 2). Compared to control groups of fertilized eggs (18.4%), high mortality must be related to the microinjection process itself. Additionally, given that fertilized Atlantic cod eggs were injected prior to the 1-2 cell stage, some of injected eggs were likely not fertilized. This observation is based on the fact that 16% of the eggs from the non-microinjected control groups did not appear to be fertilized (data not shown).

3.3 Albino-like phenotypes and knockouts

Among the 305 microinjected eggs that survived until the end of the experiment, 15 specimens demonstrated an evident albino-like phenotype (Table 2; Fig. 2A&2B). Of the 22 treatment groups, only four produced albino-like larvae. Of these four groups, three were injected with Cas9 mRNA and gRNAs, and one with Cas12a protein and gRNAs. No pigmentation changes were observed in embryos injected with preformed Cas9 gRNA RNP complexes. The most albino-like individuals were found in treatment groups “Cas9 mRNA-dgAll” and “Cas9 mRNA-sg1-1”, both yielding 6 individuals with this phenotype. In the former group, 6 of 8 surviving larvae were albino-like, while in the latter group 6 of 18 had this phenotype. Thus, 75% and 33% of surviving larvae in these groups, respectively, showed an albino-like phenotype. However, 660 eggs were injected in group “Cas9 mRNA-dgAll” and 312 eggs in group “Cas9 mRNA-sg1-1”, so the overall efficiency

of albino-like phenotype obtained per egg injected was only 0.9% and 1.9%, respectively (Table 2).

To assess the CRISPR edits at the DNA level, we extracted DNA from all larvae with albino-like phenotype and some control larvae. PCR amplified the region of interest and subjected it to Sanger sequencing (Fig. 3). While mosaicism is a typical occurrence in the F0 generation and may manifest genetically rather than phenotypically, we opted to sequence only the specimens with reduced pigmentation, instead of sequencing all surviving microinjected embryos from the different treatments. Mutations in the targeted *slc45a2* gene were found in all larvae that were selected for displaying albino-like phenotype. A notable variation in indel levels was found among larvae within the same group and between distinct groups. Embryos from the "Cas9 mRNA+sg1-1" group displayed the highest indel percentage based on analyses with the ICE tool, ranging from 59% to 95% (Fig. 3; Table 3). The CRISPR edits from the "Cas9 mRNA-dgAll" group displayed lower levels of indels in exon 6 as well as in exon 1. The albino-like larvae treated with Cas12a and all four guide RNAs (Cas12a-crAll) showed a high level of deletions. These larvae were analyzed with the DECODR website tool since the ICE website tool does not support analysis of sequences targeted with Cas12a.

4 Discussion

In this study, we established a protocol for CRISPR/Cas9 and CRISPR/Cas12a genome editing system in Atlantic cod. We first validated the effective production and viability of *in vitro* transcribed mRNA and microinjection settings using zebrafish and Atlantic cod embryos. This *in vitro* transcribed EGFP mRNA yielded EGFP protein expressed shortly after injections and present for a relatively long period of time. Fluorescence was still evident at the time the experiment was terminated, before the feeding stage, and at both high (28.5°C, zebrafish) and low temperatures (6°C, Atlantic cod). This also gave us the opportunity to standardize the microinjection settings as well as to evaluate the procedure itself before using the actual CRISPR/Cas9 and CRISPR/Cas12a systems in Atlantic cod fertilized eggs. These conditions are particularly important to establish in advance to decrease the negative impact that technical challenges intrinsic to the targeted species can have on the efficacy of gene editing. For instance, Atlantic cod breeding is seasonal, and the quality of the eggs and sperm varies over the season, impacting the efficiency of fertilization throughout the season. Therefore, these are limiting factors in the sense that embryos cannot be obtained all year and with efficiency outcomes varying through the season. Following fertilization, zebrafish eggs were microinjected at 1-2 cell stages, a procedure that is very well established in this species. In contrast, in Atlantic cod, due to the rapid initiation of chorion hardness, eggs were microinjected right after fertilization and before they reached the 1-cell stage.

To our knowledge, in this study we report the first targeted gene knockout in Atlantic cod using the CRISPR technology. Thus Atlantic cod joins an expanding list of fish species successfully edited with this technology, which includes zebrafish (*Danio rerio*) (Hwang et al., 2013), medaka (*Oryzias latipes*) (Ansai & Kinoshita, 2014), tilapia (*Oreochromis niloticus*) (Li et al., 2014), Atlantic salmon (*Salmo salar*) (Edvardsen et al., 2014), and common carp (*Cyprinus carpio*) (Zhong et al., 2016). Our approach entailed microinjection of single or duplex guide RNAs with endonuclease protein (Cas9 or Cas12a) or mRNA encoding Cas9, in each instance targeting the *slc45a2* gene, into Atlantic cod fertilized eggs. This approach generated 15 phenotypically apparent *slc45a2* knockout F0 larvae, which were mutated at different levels by insertions and deletions resulting in frameshifts. Mortality of injected eggs was the main obstacle to obtaining a high overall efficiency, which was only 0.9% and 1.9% in the two best groups. However, the efficiency of gRNA-mediated DNA targeting was much higher in both groups as 75% and 33% of

surviving larvae displayed an albino-like phenotype. The knockout efficiency (percentage of survivors with the desired gene modification) was therefore similar to that reported for other fish species such as zebrafish (80%; Hwang et al., 2013), medaka (44.8-100%; Ansai & Kinoshita, 2014), tilapia (13-52%; Li et al., 2014), Atlantic salmon (22-40%; Edvardsen et al., 2014), and common carp (up to 100%; Zhong et al., 2016). Further efforts should emphasize the optimization of factors related to the high mortality including chorion softening, timing of the microinjection after fertilization, chemical- or antimicrobial saltwater treatments. Improving each of these variables might increase the survival and ultimately the overall efficiency of this system.

An important difference between the groups microinjected with Cas9 protein and Cas9 mRNA was observed. While the groups treated with Cas9 protein did not effectively produce any albino-like Atlantic cod larvae, those treated with Cas9 mRNA yielded the highest number of albino-like specimens. In general, each delivery format comes with its own set of advantages and disadvantages in terms of overall effectiveness and present unique challenges for successful delivery (Glass et al., 2018). Considering that the conditions in which Cas9 mRNA or protein are delivered can impact their performance, it is important to note that Cas9 protein is immediately active from the time it is delivered, whereas its activity might be more short-lived compared to Cas9 produced from mRNA. Also, Cas9 mRNA has to be translated into protein, which can result in a longer window of Cas9 activity. This prolonged activity could increase the chances of successful gene editing, which may explain the higher number of albino-like specimens in the Cas9 mRNA group.

Targeting the first exon of the *slc45a2* gene with Cas9 mRNA-sg1-1 resulted in the highest indel levels in larvae with albino-like phenotype. Interestingly, although the Cas9 mRNA-dgAll combination resulted in a higher proportion of albino-like survivors than Cas9 mRNA-sg1-1 (75% vs. 33%), the percentage of indels in the former group was lower than that of the latter. While these findings are not conclusive in determining which method is superior due to the small sample size, both approaches can be utilized. However, it is important to consider that using fewer gRNAs can help decrease off-target effects.

While most of the larvae processed for DNA sequencing exhibited an albino-like phenotype, there were also larvae showing varying degrees of mosaicism, as well as a few with deformities that were not genotyped (Fig. 2C&2D). In this sense, our study took a more conservative approach by focusing solely on those exhibiting the albino-like phenotype. Considering that both alleles of *slc45a2* must be non-functional to obtain the albino-like phenotype, it is possible that other larvae not displaying this phenotype carried mutations that could have been passed on to their offspring to produce albino-like phenotypes, as has been described in previous studies (Wu et al., 2018). Future attempts to establish a genetic line by selecting F0 specimens should expand the screening to include all surviving specimens. Ideally, attempts should be made to obtain and screen F1 progeny. Unfortunately, the challenge lies in the fact that Atlantic cod only reaches sexual maturity at around 3 years of age, making it impractical for this purpose. In this sense, the CRISPR genome editing system provides an efficient strategy for gene knockout in F0, enhancing productivity and efficiency by reducing the time and resources required to breed mutant alleles to homozygosity for experimental purposes. This method proves to be particularly beneficial for animal models with extended periods to sexual maturity, seasonal breeding, or short breeding season, where breeding to homozygosity is unfeasible or challenging.

5 Conclusions

As aquaculture rapidly emerges as the primary source of fish for human consumption, it also presents new challenges that need to be addressed by different approaches. The increasing

accessibility and affordability of CRISPR genome-editing technologies makes it one of the more important tools to address some of these challenges in an efficient and effective fashion. For a species of considerable commercial and ecological importance in the North Atlantic such as Atlantic cod, despite the modest efficiency shown in this study, the outcomes here described represent a significant milestone in applying new technologies to study this species. Our results contribute to the existing and expanding body of knowledge on applying genome-editing technologies in non-model organisms and open new avenues for future comparative studies to optimize CRISPR/Cas9 efficiency in Atlantic cod and other fish species. This technology opens up an avenue to investigate physiology, immunology, and genetics of Atlantic cod at the cellular and molecular level.

6 CRediT authorship contribution statement

Adrián López-Porras: Conceptualization, Methodology, Writing - Original Draft, Visualization, Investigation. **Ragnhild Stenberg Berg:** Investigation, Visualization. **Erik Burgerhout:** Resources, Funding acquisition. **Øyvind J. Hansen:** Funding acquisition. **Ádám Györkei:** Conceptualization. **Shuo-Wang Qiao:** Conceptualization, Writing - Review & Editing, Funding acquisition. **Finn-Eirik Johansen:** Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

7 Declaration of interest statement

The authors declare that they have no competing interests.

8 Data availability

Data will be made available on reasonable request.

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Table 1. List of guide RNAs (gRNA) used for CRISPR/Cas9 and CRISPR/Cas12a genome editing. The table displays the oligo names, the targeted exon of the *slc45a2* gene, the corresponding gRNA sequences, and the nuclease used for each oligonucleotide.

gRNA name	Target	gRNA Sequence (5'-3')	Nuclease	CFD score	Off-targets for 0-1-2-3-4 mismatches
sg1-1/dg1-1	Exon 1	CGGCCGCGAGTTCTGCTACG	Cas9	97	0 - 0 - 0 - 2 - 31
sg1-2/dg1-2	Exon 1	CCACCGCGTAGCAGAACTCG	Cas9	97	0 - 0 - 1 - 4 - 19
sg1-3/dg1-3	Exon 1	ACGCCGGTGCTGCTGAGCGT	Cas9	97	0 - 0 - 1 - 1 - 40
sg6-1/dg6-1	Exon 6	GGGGAAGAGGCCGATGAGGC	Cas9	85	0 - 0 - 2 - 17 - 270
cr2-1	Exon 2	ACTTTGCGGCCGACTTCATTG	Cas12a	5	0 - 0 - 0 - 0 - 1
cr4-1	Exon 4	TCTGCAACATGCTCTTCTTCA	Cas12a	22	0 - 0 - 0 - 0 - 8
cr6-1	Exon 6	TGTTCGGCCTGGGCACCGGCC	Cas12a	5	0 - 0 - 0 - 0 - 2
cr6-2	Exon 6	GCGTGATGTCGAGCACGCTGT	Cas12a	12	0 - 0 - 0 - 0 - 2

Note: the structure of the oligo names is as follows: sg indicates single-guide RNA for Cas9, dg indicates duplex-guide RNA for Cas9, and cr indicates CRISPR RNA for Cas12a. The number following the prefix refers to the exon targeted by the guide. The number following the hyphen indicates the specific guide variant for that exon. Scores are based on the CDF specificity score (a value of 0 indicates no predicted off-target activity whereas a value of 100 indicates a perfect match, higher scores indicate a higher likelihood of successful and specific DNA targeting) obtained from the CRISPOR analysis (http://crispor.org; Concordet & Haeussler, 2018). Based on this same tool, in the last column, for each number of mismatches, the number of off-targets is indicated. As an example: 1-3-20-50-60 means 1 off-target with 0 mismatches, 3 off-targets with 1 mismatch, 0 off-targets with 2 mismatches, etc.

Table 2. Treatments used and treatment outcome in the present study. Cas9 was presented either injected as preformed ribonucleoprotein complexes (RNP) with either duplex guide (dg)RNA or RNPs with single guide (sg)RNA or as mRNA encoding Cas9 co-injected with the same guides. Cas12a was injected as preformed RNP with different crRNA guides. See table 1 for details of the guide RNAs. Treatment conditions that resulted in phenotypically albino-like lavae are highlighted in bold.

Treatments	**Microinjected eggs**	**Survivors**	**Albino-like**	**Survival**	**Albino-like survivors**	**Overall efficiency**
Cas9 mRNA-dg1-1	261	1	0	0.4 %	0 %	0.00 %
Cas9 mRNA-dg1-2	**289**	**30**	**1**	**10.4 %**	**3 %**	**0.35 %**
Cas9 mRNA-dg1-3	291	2	0	0.7 %	0 %	0.00 %
Cas9 mRNA-dg6-1	223	14	0	6.3 %	0 %	0.00 %
Cas9 mRNA-dgAll	**660**	**8**	**6**	**1.2 %**	**75 %**	**0.91 %**
Cas9 mRNA-sg1-1	**312**	**18**	**6**	**5.8 %**	**33 %**	**1.92 %**
Cas9 mRNA-sg1-2	211	2	0	0.9 %	0 %	0.00 %
Cas9 mRNA-sg1-3	190	3	0	1.6 %	0 %	0.00 %
Cas9 mRNA-sg6-1	195	33	0	16.9 %	0 %	0.00 %
Cas9 mRNA-sgAll	546	0	NA	0.0 %	NA	NA
Cas9 Protein-dg1-1	415	0	NA	0.0 %	NA	NA
Cas9 Protein-dgAll	265	5	0	1.9 %	0 %	0.00 %
Cas9 Protein-sg1-1	241	4	0	1.7 %	0 %	0.00 %
Cas9 Protein-sg1-2	680	9	0	1.3 %	0 %	0.00 %
Cas9 Protein-sg1-3	513	25	0	4.9 %	0 %	0.00 %
Cas9 Protein-sg6-1	461	4	0	0.9 %	0 %	0.00 %
Cas9 Protein-sgAll	732	76	0	10.4 %	0 %	0.00 %
Cas12a-cr6-1	378	18	0	4.8 %	0 %	0.00 %
Cas12a-cr6-2	325	5	0	1.5 %	0 %	0.00 %
Cas12a-cr2-1	403	8	0	2.0 %	0 %	0.00 %
Cas12a-cr4-1	78	25	0	32.1 %	0 %	0.00 %
Cas12a-All	**687**	**15**	**2**	**2.2 %**	**13 %**	**0.29 %**
Total	***8356***	***305***	***15***	***3.7 %***	***5 %***	***0.18 %***

Table 3. Indel percentages at different *slc45a2* exons in larvae subjected to different CRISPR/Cas9 and CRISPR/Cas12a treatments. "Larva ID" is the unique identifier for each individual larva within a treatment group. Percentage of indels (insertions or deletions) in the respective exon of the *slc45a2* gene based on analysis performed with the ICE website tool. (-) data were not applicable for the respective exon in the given larva. (*) Specimens were analyzed with DECODR because ICE is not suitable to analyze sequences targeted by Cas12a.

Treatment	**Larva ID**	**Exon 1 indel %**	**Exon 2 indel %**	**Exon 4 indel %**	**Exon 6 indel %**
Cas9 mRNA-sg1-1	A1	75	-	-	-
	B1	93	-	-	-
	C1	86	-	-	-
	C2	32	-	-	-
	D1	95	-	-	-
	E1	59	-	-	-
Cas9 mRNA-dg1-2	G11	3	-	-	-
Cas9 mRNA-dgAll	A3	3	-	-	28
	B3	0	-	-	28
	C3	2	-	-	28
	F3	2	-	-	35
	G3	0	-	-	38
	H3	1	-	-	31
Cas12a-All	G12*	-	0	98	0
	H12*	-	0	98	22

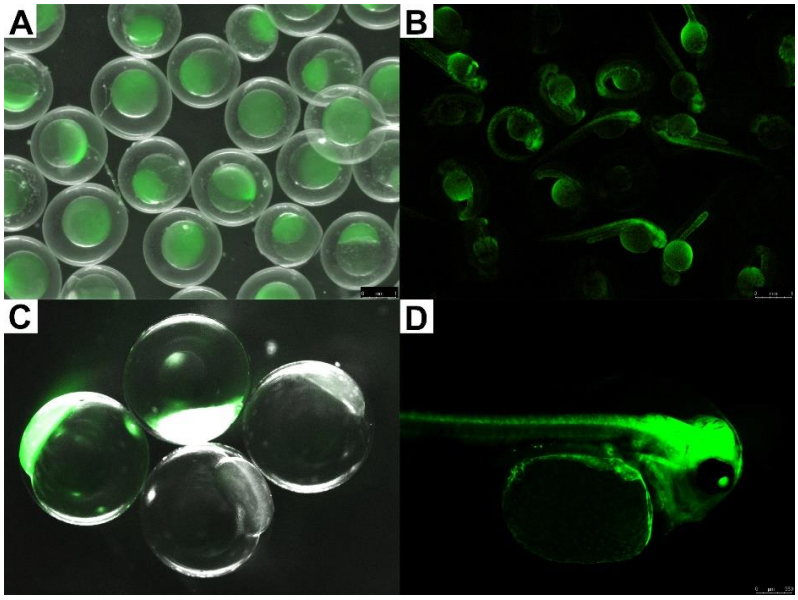

Figure 1. Microinjection of EGFP mRNA into zebrafish and Atlantic fertilized eggs. A) Zebrafish embryos EGFP positive 5 hours post-fertilization (hpf). B) Zebrafish embryos EGFP positive 48 hpf. C) Atlantic cod embryos, 36 hpf. Arrows indicate EGFP positive embryos. D) Atlantic cod larva, 20 days post-fertilization. Images in panels A and C are merged bright-field and green fluorescence channels. Images in panels B and D were taken only with green fluorescence channel.

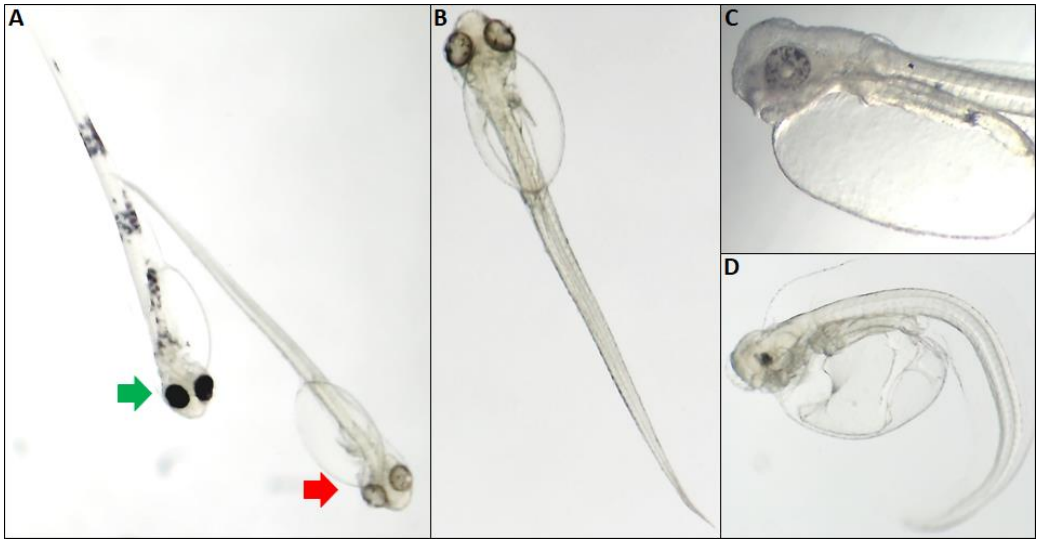

Figure 2. Variations in phenotypes of albino-like Atlantic Cod larvae. A) Atlantic Cod larvae exhibit the normal pigmentation phenotype (green arrow) and the albino-like phenotype (red arrow). B) Close-up of a different Atlantic Cod larva with the albino-like phenotype. C) Albino-like Atlantic Cod larva featuring mosaicism with scattered pigmented cells in both the eye and the body trunk. D) Albino-like Atlantic Cod larva exhibiting deformities.

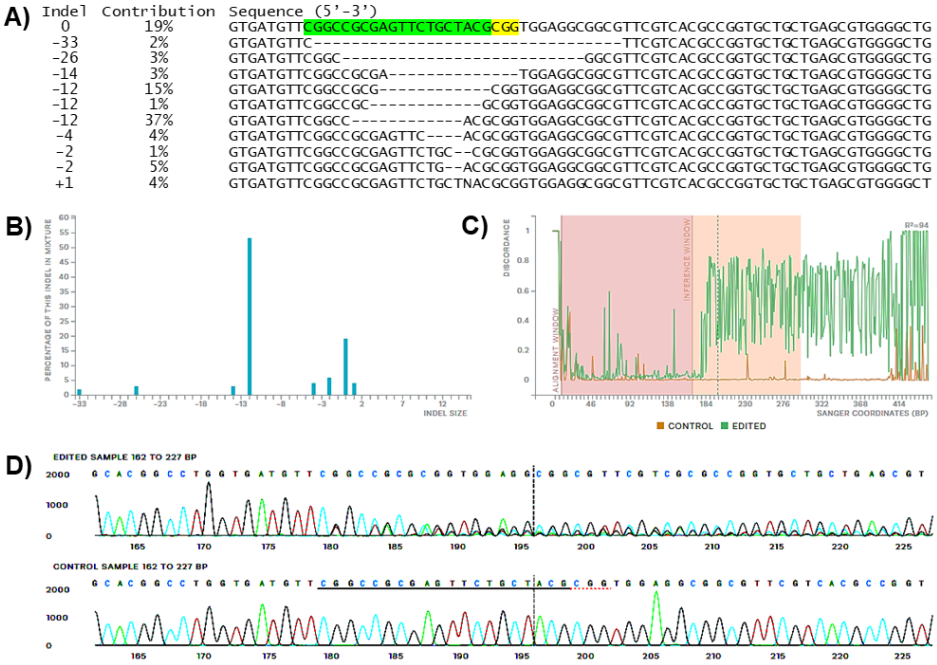

Figure 3. A representation of the outputs from the ICE (Inference of CRISPR Edits) software for a guide RNA targeting the slc45a2 gene in an Atlantic cod larva. (A) Sanger sequencing reveals indels in the Atlantic cod larva (ID=A1) treated with Cas9 mRNA-sg1-1. The target region is shown in green and the PAM site in yellow. (B) The predicted distribution and percentage of indels within the target region. (C) Discordance between the edited (in green) and control (in orange) trace files, illustrating the location and prevalence of editing. (D) Sanger sequencing traces after PCR of both edited and wild-type (control) Atlantic cod larvae in the region surrounding the guide sequence. The guide sequence is highlighted with a black underline, the PAM site with a red dotted underline, and the expected cut site is indicated by a vertical dotted line.