# Cellular Immune Responses

4

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**Abstract**

Generally speaking, cellular immune responses are comprised of innate and adaptive cell-based immune mechanisms in which all leukocyte subpopulations are involved. This includes effector functions such as phagocytosis, NETosis, and cell-mediated cytotoxicity. The main players are macrophages/monocytes, dendritic cells, granulocytes, NK cells, and cytotoxic T cells. These effector functions can only be executed and controlled through receptor/ligand interactions and by humoral factors produced by leukocytes or somatic cells. Thus, it is hard to draw a static line between cellular and humoral components of the immune systems since one system cannot exist independently from the other. Similarly, adaptive responses cannot be efficiently induced without innate triggers.

This chapter describes cellular immune mechanisms in teleost fish and relates them to mammalian immunology.

**Keywords**

Cellular immunity · Phagocytosis · NETosis · APC · Cytokines · Receptors · Cell-mediated cytotoxicity · CTL · NK cell

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## Definitions

| | |
|---|---|
| somatic cell: | bodies own cell |
| tissue: | aggregate of cells having the same structure and function |
| antigen: | from anti, Greek = against; gennan, Greek = produce; in the original sense: substances that the body recognizes as foreign via antibodies; in a broader sense: substances against which an immune response is mounted |

## Abbreviations

| | |
|---|---|
| aa | amino acid |
| ADCC | antibody-dependent cell-mediated cytotoxicity |
| APC | antigen-presenting cell |
| CD | cluster of differentiation |
| CMC | cell-mediated cytotoxicity |
| CTL | cytotoxic T cell |
| DC | dendritic cell |
| HSC | hematopoietic stem cell |
| IFN | interferon |
| IL | interleukin |
| MHC | major histocompatibility complex |
| NET | neutrophil extracellular trap |
| NK | natural killer |
| PBL | peripheral blood leukocytes |
| rb | reviewed by |
| TCR | T-cell receptor |
| Th | T helper |

## 4.1 Introduction

One of the most important requirements for the survival of multicellular organisms is to maintain its integrity and to prevent the invasion by other organisms or foreign (to the body) material. The first challenge consists of distinguishing somatic cells from nonself cells to avoid autoaggression. Secondly, the organism has to make a decision whether to tolerate, or to reject foreign cells, pathogens (parasites, bacteria, fungi, and viruses) or altered somatic cells. In mammals, immune response elements are historically divided into cellular and humoral (Greek: juice or sap = liquid) and innate and adaptive. However, these components cannot exist or act independently, thus defining them is difficult. While some definitions for cellular immune responses include only adaptive cellular elements, others

are more global, also incorporating innate immune cells. In this book chapter, cellular immune responses are defined as the reactions of leukocytes where the innate players are granulocytes, macrophages (monocytes), thrombocytes, dendritic cells as well as natural killer (NK) cells and where the adaptive cell-mediated responses are executed by cytotoxic T lymphocytes (CTLs). Fundamental functions of myeloid leukocytes are extracellular trapping and phagocytosis followed by the destruction of pathogens and altered material. Another important function associated with phagocytes is the processing of incorporated antigenic material in a way so that their components can efficiently be presented to, and recognized by cells of the adaptive immune system. While extracellular pathogens can be directly attacked by immune cells, intracellular pathogens, such as viruses and some bacteria, can only indirectly be recognized and their replication hindered, e.g., through cell-mediated cytotoxicity (CMC). To mount an efficient cellular immune response, leukocytes communicate among each other and with nonlymphoid cells through cytokines/cytokine receptors and through receptor/ligand interactions. These interactions are of interest when shaping vaccines that address cellular adaptive immune response mechanisms.

The aim of this chapter is to provide the latest information on cellular defence mechanisms of fishes, and put this knowledge into a comparative context of what we know from tetrapods.

## 4.2 Cell-Mediated Immune Functions

This subchapter refers to cells that directly act against pathogens, allogeneic/xenogeneic or altered and infected somatic cells.

### 4.2.1 Phagocytosis

After a pathogen or foreign material has passed epithelial barriers, the immediate cellular immune response is initiated by cells of the innate immune system. One main innate response mechanism is phagocytosis. Phagocytosis was first described by Metchnikoff in 1883 in frogs as the first cell-mediated immune mechanism ever discovered.

Phagocytosis is a variation of endocytosis, where endocytosis is characterized by one common characteristic: after contact with extracellular material, the cell membrane invaginates around the material, forming a pocket and finally an intracellular vesicle or a vacuole. Phagocytosis is defined as the internalization of particulate material (including bacteria and other “foreign” cells or their debris) by professional phagocytes, usually leading to the destruction of the ingested material.

Several cell types are able to execute phagocytosis in vertebrates. Professional phagocytes are monocytes (blood “macrophages”), macrophages (called so in tissues; for more information on macrophages see Chap. 6), dendritic cells (DC), neutrophils, and mast cells. Nonprofessional phagocytes such as epithelial cells, endothelial cells, fibroblasts, and

mesenchymal cells can also engulf foreign material. Some organs harbor certain specialized professional phagocytes in mammals as well as in teleosts: osteoclasts in bone tissue (rb Witten and Huysseune 2009), microglial cells in nervous tissue (rb Cuoghi and Mola 2007), and Langerhans cells in the skin (He et al. 2018). Despite the evidence for the existence of specialized macrophages, the so called Kupffer cells, in the fish liver (Moller et al. 2014), most scientist avoid the use of this term in fish and instead refer to "liver resident macrophages" (rb Shwartz et al. 2019).

Although phagocytes belong to the innate part of the cellular immune response, this distinction is somehow insufficient since B cells, as representatives of the adaptive immune system, are also able to phagocytose as shown in fish, amphibians (Li et al. 2006), and in mice (Parra et al. 2012; Martínez-Riaño et al. 2018).

Mammalian neutrophils and macrophages are myeloid cells which originate from the multipotential hematopoietic stem cell (HSC) in the bone marrow. Their final maturation occurs in the blood and tissues. However, bony fishes do not have bone marrow and the origin of myeloid cells is suggested to be the kidney (rb Katzenback et al. 2012; Kobayashi et al. 2016).

Mature mammalian neutrophils and macrophages are attracted to the site of pathogen growth or inflammation by chemotaxis. Several chemokine orthologs have been described in teleosts that are able to attract fish myeloid cells (rb Alejo and Tafalla 2011) in vitro and in vivo (Torraca et al. 2017). Goldfish neutrophils exhibit chemotaxis toward mycobacteria and efficiently internalize them (Hodgkinson et al. 2015). A critical role in the recruitment of mammalian neutrophils is performed by the chemokine CXCL8 (also known as IL-8), and several sequences similar to mammalian CXCL8 have been found in teleost fish (rb Havixbeck and Barreda 2015).

Uptake of particulate material into mammalian phagocytes, e.g., of bacteria (rb Kaufmann and Dorhoi 2016), small parasites (Ueno and Wilson 2012), and apoptotic cells (rb Li 2012) is receptor-mediated. Receptors are multifold. Bacterial uptake and killing by macrophages and neutrophils can be mediated through pattern recognition receptors (PRRs), or through opsonization by elements of the complement cascade via complement receptors (rb Lukácsi et al. 2020). This process can also be more antigen-specific, when antibody-opsonized bacteria are bound to the surface of macrophages (rb Hamdan et al. 2020) and neutrophils (rb Rosales 2017) via Fc receptors (FcR). Fish neutrophils have also been described as scavengers of dead cells. In goldfish, neutrophils internalize dead or dying macrophages during bacterial infection (Havixbeck et al. 2017).

Thrombocytes are nucleated cells in nonmammalian vertebrates, and along with their main function of blood clotting they also show phagocytic activity. Putative phagocytic activity of chicken thrombocytes was first described in 1968 (Carlson et al. 1968). However, true phagocytosis was often not clearly distinguished from uptake into the canalicular system, which forms an open network of interconnected channels of the outer cell membrane to broaden the cell surface of thrombocytes. Thus, phagocytosis by thrombocytes in lower vertebrates was questioned for a long time (e.g., Meseguer et al. 2002). The first serious evidence of phagocytosis by thrombocytes in fish was demonstrated in common and ginbuna crucian carp, in goldfish and in Japanese flounder

(Nagasawa et al. 2014; rb Esteban et al. 2015). Since goldfish and ginbuna crucian carp thrombocytes express the major histocompatibility complex-encoded thrombocyte marker G6F (Ohashi et al. 2010) and since thrombocytes from several fish species express MHC class II they are also regarded as important in antigen presentation of processed phagocytosed antigens (rb Stosik et al. 2019).

### 4.2.2 Extracellular Trapping

Another strategy of neutrophils (and less importantly in macrophages) to neutralize bacteria is the formation of extracellular traps (ETs). ETs are extracellular matrices formed by DNA and granular proteins that immobilize and kill bacteria (rb Kaufmann and Dorhoi 2016).

The formation of ETs is a phylogenetically ancient mechanism and not unique to the vertebrate kingdom, including fish. It can also be found in invertebrates and even plants. The formation of neutrophil ETs (NETs) is called NETosis. In teleost fish, NETosis can be induced by stimuli mimicking fungal, viral, and bacterial pathogens, and like in mammals, degradation of entrapped pathogens is executed by myeloperoxidase (MPO), nitric oxide (NO), histones, chymotrypsin-like elastases, and nucleases (rb Neumann et al. 2020). However, there are species-specific differences in the inducibility of NETosis in fish. While Phorbol 12-myristate 13-acetate (PMA) is a potent inducer of ET release in common carp (Pijanowski et al. 2013) and tongue sole (Zhao et al. 2017), PMA triggers only a weak ET release in fathead minnow, zebrafish (Palić et al. 2007), and rainbow trout (Van et al. 2020).

### 4.2.3 Cell-Mediated Cytotoxicity (CMC)

#### 4.2.3.1 Myeloid Cells

Since neutrophils belong to the innate immune system, their recognition mechanisms are not antigen-specific. In addition to phagocytosis, mammalian neutrophils can destroy bacteria by degranulation and release into the extracellular environment of antimicrobial substances such as cathepsin G, defensins, lysozyme, MPO, NE, and proteinase (Gullberg et al. 1997) as well as of antimicrobial peptides. They also produce and release reactive oxygen (ROI) and nitrogen intermediates (RNI) which further contribute to the destruction of extracellular bacteria, foreign cells, and accidently, also somatic cells (rb Kumar and Dikshit 2019; rb Korhonen et al. 2005). Neutrophils are long known to play a decisive role in pus formation. Pus usually consists of bacteria, neutrophils, and cellular debris from bacteria as well as debris of damaged somatic cells. Damage of somatic cells in phlegmons due to injury and wound infection arises not only from the cytotoxic activity of bacteria but also of neutrophils. To protect the surrounding tissues, a capsule is usually built around the pus to form an abscess. Although abscesses are common findings in ornamental fish

practice, scientific articles describing their cellular and molecular mechanisms are rare in fish. A zebrafish larval model for mammalian abscess formation has been described by Prajsnar et al. (2012). While bacteria (*S. aureus*) were shown to survive within phagocytes in infection foci, no capsule formation was described around abscess-like structures, probably due to the early stage of fish development in this study.

The destructive role of mammalian neutrophils is also critical in many other acute inflammatory diseases, e.g., in sepsis (e.g., rb Shen et al. 2017) or organ damage (e.g., rb Jaeschke and Hasegawa 2006). Sepsis is defined as a systemic inflammatory response syndrome (SIRS) in response to an infectious process (rb Soong and Soni 2012). It is largely initiated by bacterial endotoxins and although pattern recognition is different between mammals and teleost fishes, e.g., due to different TLR signaling, a sepsis model has been described in zebrafish (Philip et al. 2017).

Another adverse effect of mammalian neutrophils affects the outcome of transplantation. Neutrophils are recruited to the graft leading to the risk of allograft rejection (e.g., rb Oliveira et al. 2018). Similarly, in carp (Kurata et al. 1995) and rainbow trout (Sasaki et al. 2002), neutrophils kill xenogeneic cells in a nonspecific manner.

Graft-versus-host reaction is characterized by an immune response of grafted cells against the recipient. Although the main effector cells in fish have been described as CD8+ lymphocytes (Shibasaki et al. 2010), the involvement of neutrophils in this process cannot be ruled out (Nakanishi and Ototake 1999). A kind of nonspecific autoaggression has also been described for neutrophils in ginbuna where neutrophils have killed isogeneic erythrocytes in vitro requiring cell to cell interaction (Fischer et al. 1998). However, since the neutrophil effector to erythrocyte target cell ratios in these in vitro studies were much higher (up to 40:1) than those usually found in blood under physiological conditions (approx. 1:1000; Fänge 1992), the probability of neutrophil effectors to encounter an erythrocyte was much higher than under physiological conditions. Further, some senescent or damaged erythrocyte targets might have activated neutrophils which in turn killed additional intact erythrocytes.

#### 4.2.3.2 Natural Killer Cells

Mammalian NK cells are large, granular, and cytotoxic lymphocytes with the ability to recognize and kill tumor cells, stressed cells, and cells infected with viruses and other intracellular pathogens. NK cells do not express antigen-specific receptors, such as the B-cell or T-cell receptors, with highly variable regions. However, they possess members of various families of activating and inhibitory receptors to monitor an aberrant expression of cellular stress markers, MHC class I, and MHC class I-related molecules. Thus, NK cells play important roles in the clearance of altered cells, especially in the early innate response to viral infections, before adaptive immune responses by cytotoxic T cells take part. NK cells can also sense the missing expression of self MHC class I on foreign allogeneic cells without prior sensitization (rb Kärre 2002).

### Origin and Distribution of NK Cells

The majority of our knowledge on NK cells originates from studies on mice and humans. NK cells are widely distributed in most murine organs, and represent only a minor population of total lymphocytes in most tissues (e.g., ~2% and ~ 10% in murine spleen and lung, respectively) (rb Grégoire et al. 2007). In humans, NK cells (CD3−/CD56+) are relatively abundant, comprising 5%–20% of lymphocytes in blood, bone marrow, spleen, and lung with low frequencies in the lymph nodes, tonsil, and gut (rb Freud et al. 2017; rb Dogra et al. 2020).

In adult mammals, the bone marrow is the major site of early phase NK cell development (rb Yu et al. 2013). Common lymphoid progenitor cells (CLPs) committed from HSCs are the earliest lymphoid progenitor cells and can generate T cells, B cells or innate lymphoid cells (ILCs) including NK cells. Stromal cells in bone marrow produce certain cytokines, Fms-like tyrosine kinase 3 ligand (FLT3L), and stem cell factor (SCF), crucial for NK cell differentiation by inducing the common IL-2 receptor subunit β (CD122) shared by IL-2 and IL-15 signaling pathways (rb Leonard et al. 2019). The acquisition of CD122 directs CLPs to become immature NK cells (Carotta et al. 2011). Committed immature CD122+ NK cells in humans and mice further develop through distinct stages, which differ in their cell surface receptor repertoires, such as the activation receptors, natural killer group 2D (NKG2D; alias CD314), natural cytotoxicity receptor 1 (NCR1; alias CD335, NKp46, Ly94), and NK1.1 (NKR-P1C, CD161) (rb Goh and Huntington 2017). Precursor and immature NK cells migrate into secondary lymphoid organs, and these sites are considered as reservoirs for NK cell maintenance (rb Huntington et al. 2007; rb Abel et al. 2018). In humans, the appearance of CD56 (NCAM, neural cell adhesion molecule) further indicates a final transition of immature to mature NK cells (rb Freud et al. 2017; rb Di Vito et al. 2019). Most immature NK cells develop into a minor CD56-bright population and then convert into a major CD56-dim population with the acquisition of the antibody binding-Fc receptor CD16. Human CD56-bright NK cells exhibit reduced lytic capacity while being potent producers of inflammatory cytokines. These subsets reside primarily in lymph nodes and in the intestine. In contrast, the CD16 expressing CD56-dim NK cells represent the major NK cell population in peripheral blood, bone marrow, spleen, and lung, and are potent cytolytic effector cells, rapidly secreting cytotoxic mediators (granzymes and perforin) following receptor-mediated activation (rb Freud et al. 2017).

In mice, mature NK cells are defined by the acquisition of C-type lectin-like Ly49 receptors (rb Goh and Huntington 2017). Since murine NK cells show no expression of CD56, mature NK cells are further classified into three functionally distinct subsets by their expression of CD27 and CD11b (Hayakawa and Smyth 2006; Chiossone et al. 2009). In detail, mature CD11b-/CD27+ NK cells, representing the first maturation stage, are abundantly present in the bone marrow and lymph nodes. Intermediate CD11b+/CD27+ NK cell stages are equally distributed in the lymphoid organs, liver, and lung while most mature CD11b+/CD27- NK cells are found in blood, spleen, lung, and liver. This maturation step is associated with the functional development of mature NK cells (Walzer et al. 2007; Chiossone et al. 2009).

Mammalian NK cells use activating and inhibitory receptors to recognize both healthy and altered cells, such as infected, stressed, and tumor cells. Within one individual, NK cell activation is largely regulated by MHC class I molecules which are expressed by most nucleated cells marking these cells as "self" (rb Höglund and Brodin 2010). When the NK cell inhibitory receptors recognize self MHC class I molecules on another cell, the NK cell's cytotoxicity function is inactivated to prevent killing of nonaltered self cells. To acquire the capacity to recognize "non-self" target cells with "wrong" MHC class I expression (in the case of grafted cells) and self target cells with down-regulated or altered MHC class I expression (e.g., during virus infection or in tumor cells), mammalian NK cells must be educated to detect host MHC class I molecules using their inhibitory receptors. Only NK cells that have engaged their inhibitory receptors with self MHC class I molecules during development are functionally mature and competent (rb Sun and Lanier 2011; rb Shifrin et al. 2014). This process of NK cell development is termed NK cell licensing, NK cell tuning or classical NK cell education. NK cell licensing brings forth two types of self-tolerant NK cells. The licensed cells effectively patrol for missing MHC class I targets. In contrast, unlicensed NK cells lacking the expression of inhibitory MHC class I-specific receptors diminish the capacity to respond to MHC class I-deficient targets (rb Anfossi et al. 2006). Thus, the unlicensed NK cells have a low potential to attack normal cells and are not autoreactive. In addition to classical MHC class I molecules, nonclassical MHC class I, or even other than MHC class I molecules are involved in NK cell education (rb He and Tian 2017).

In teleost fish, two types of homologous cells to mammalian NK cells have been described: nonspecific cytotoxic cells (NCCs) and NK-like cells (rb Fischer et al. 2013). NCCs, which have been well characterized in catfish, are small and agranular lymphocytes that spontaneously kill a variety of xenogeneic targets and can be recognized by a monoclonal antibody, 5C6, specific to the NCC receptor protein 1 (NCCRP1) (rb Shen et al. 2002). NCCRP-1+ cells in catfish are present at concentrations of ~30%, ~40–50%, and ~ 2% in pronephros, spleen, and blood, respectively (Evans et al. 1988). Given NCCRP-1 is a marker of NCCs in teleost fish, expression analysis of NCCRP-1 transcripts in carp and grouper suggests its ubiquitous distribution in the fish body (Sakata et al. 2005; Huang et al. 2014). In contrast to fish NCCs, NK cells have not been isolated in fish yet, as no specific markers or reliable antibodies are available, although cell lines with NK cell activity have been isolated from catfish (discussed below). However, large granular lymphocytes similar to mammalian NK cells were identified in rag1−/− mutant zebrafish (Muire et al. 2017). Moreover, single cell transcriptomes in several fish species clearly identified NK-like cell populations (Moore et al. 2016; Carmona et al. 2017; Guslund et al. 2020). These data are valuable not only for analysing the function of fish NK cells, but also to isolate them in the future using suitable reporter genes (e.g., GFP) and/or antibodies targeting NK cell-specific molecules.

### Induction and Regulation of NK Cell-Mediated Cytotoxicity

Mammalian NK cells play a crucial role in immune responses against viral infections and tumors. However, the killing activity of NK cells must also be tightly regulated to assure tolerance to healthy self tissues. NK cells monitor the surface of each host cell using germline-encoded receptors. Some cell surface markers are commonly associated with healthy cells, and others are expressed mainly by damaged or infected cells. NK cells have receptors for each of these types of molecules: one type, called activating receptors, recognizes the "unhealthy" markers, and another type, called inhibitory receptors, recognizes the "healthy" markers (rb Pegram et al. 2011). Whether NK cells kill a target cell or not is determined by the overall balance of signaling by these inhibitory and activating receptors. If more activating receptors are stimulated than inhibitory receptors, the NK cell decides to attack the target cells which are infected or badly damaged, and releases cytoplasmic granules which destroy the target cells (rb Vivier et al. 2008).

In mammals, genes encoding NK cell receptors (NKRs) are clustered in two main gene complexes: the natural killer complex (NKC) encoding C-type lectin-like molecules, and the leukocyte receptor complex (LRC) encoding the immunoglobulin-like receptors (rb Carrillo-Bustamante et al. 2016). These NKR gene clusters show species-specific expansion resulting in divergent activating and inhibitory receptors. The main NK cell receptors for MHC class I in humans are the killer cell immunoglobulin-like receptors (KIRs), which are located in the LRC. Mice lack KIR genes and instead predominantly harbor Ly49 receptors encoded in the NKC to regulate their NK-cell activity. These Ly49 receptors show high polymorphism between different strains of mice. In contrast, humans lack functional Ly49 genes (rb Kelley et al. 2005; rb Rahim et al. 2014).

Human KIRs have either two or three extracellular immunoglobulin-like domains and contain either long cytoplasmic tails with immunoreceptor tyrosine-based inhibition motifs (ITIM) or short cytoplasmic tails comprising a charged residue in their transmembrane regions that associate with signaling adaptor molecules with immunoreceptor tyrosine-based activating motifs (ITAM), such as DAP12, or FcγR (rb Purdy and Campbell 2009; rb Pegram et al. 2011). Most KIRs are inhibitory receptors with cytoplasmic tails containing ITIMs. The ligands of human KIRs are classical MHC class I molecules, such as HLA-A, B, and C molecules. The ligand binding to either activating or inhibitory KIRs induces the phosphorylation of tyrosine residues in ITAMs or ITIMs, respectively. This activation process through activating receptors leads to the release of cytotoxic granules. In contrast, the phosphorylation of ITIMs in inhibitory receptors recruits phosphatases and neutralizing activating signals (rb Long 2008). As regular and healthy cells express MHC class I, the recognition of KIRs on NK cells inhibits its killing process (Fig. 4.1).

In mice, instead of KIR receptors, a family of NKC-encoded lectin-like receptors called Ly49 is used for recognition of polymorphic classical MHC class I and related proteins. Similar to KIRs, inhibitory Ly49 receptors have an ITIM in their cytoplasmic tail although activating Ly49 receptors use the DAP-12 molecule for signaling. An important feature of the NK-cell population is that not all NK cells in an individual are identical and NK cells express only a subset of the receptors in its potential repertoire. The decision which KIRs

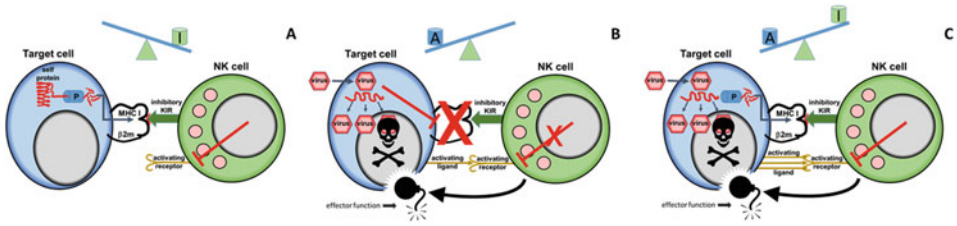


**Fig. 4.1** Human NK cells possess inhibitory and activating receptors. In a normal situation, KIRs recognize self-peptide-loaded self MHC class I molecules on the surface of somatic cells which program NK cells into an inert state (**a**). Some viruses down-regulate MHC class I expression and induce the expression of activating ligands in infected cells. While the inhibitory function of KIRs is missing, activating receptors are engaged and drive NK cells into an activated stage (**b**). Even if MHC class I expression is not affected or up-regulated during certain virus infections, the balance might still be shifted toward NK cell activation through interaction between activating ligands expressed on infected cells and activating receptors displayed on NK cells (**c**). The inert state of NK cells can be shifted into an activated state in any situation where the signals from activating receptors are stronger than those from inhibitory receptors

and Ly49s are expressed on each NK cell seems to be random and is regulated by the methylation of KIR gene loci (Santourlidis et al. 2002; Liu et al. 2009).

Mammalian NK cells also express C-type lectin-like receptors, CD94 and NKG2 as heterodimeric receptors with activating or inhibitory effects. NKG2 consists of several members (A-F, H) (rb Pegram et al. 2011, rb Wilk and Blish 2018). NKG2A, 2C, and 2E are displayed as covalently linked heterodimers with CD94. NKG2B and NKG2H are alternative splice isoforms of the NKG2A and NKG2E genes, respectively, and are also expressed as heterodimers with CD94. These heterodimers interact with nonclassical (also nonpolymorphic) MHC class I-like molecules, including HLA-E in humans and Qa1 in mice. NKG2A and 2B contain two ITIMs and are important in educating NK cell tolerance to self cells and contribute to the inhibition of NK cell-mediated immunity to infections and tumors. Whilst CD94/NKG2C and CD94/NKG2E can associate with DAP12 and work as activating receptors, NKG2D makes homodimers but not heterodimers with CD94, and functions as an activating receptor. It binds several MHC class I-like molecules that are induced by various types of cellular stress (rb Waldhauer and Steinle 2008).

Natural cytotoxicity receptors (NCRs) are an additional group of mammalian activating receptors. This group belongs to the Ig-superfamily and includes NKp46 (NCR1/CD335), NKp44 (NCR2/CD336), and NKp30 (NCR3/CD337) (rb Barrow et al. 2019). Among NCRs, NKp46 is conserved in both humans and in mice; however, NKp30 and NKp44 in mice are pseudogenes. Thus, NKp46 is the most selective marker of NK cells in mammalian species. In humans, activated and resting NK cells express NKp46 and NKp30, which can associate with the transmembrane regions of the ITAM-containing CD3ζ-homodimers (Khakoo et al. 2004). Moreover, NKp46 can also engage with FcεRIγ-CD3ζ heterodimers, both of which possess ITAM motifs. In contrast, NKp44 is expressed on some NK cells upon IL-2 stimulation and can directly associate with DAP12. Some of the ligands

recognized by the NCRs are virus-derived molecules, such as influenza virus hemagglutinin (HA), that activates effector functions of NK cells against infected cells. Intracellularly localized proteins from tumors and stressed cells are also possible ligands for NCRs. Nuclear proteins (e.g., BAG6, MLL5, and PCNA) can be transported to the cell surface on stressed and tumor cells via exosomes. The cytosolic protein proliferating cell nuclear antigen (PCNA) released by mammalian tumor cells can inhibit the function of NKp44 (Rosental et al. 2011). NKp30 can bind to B7-H6 which is mainly expressed on cancer cells, triggering NK cell activation (Brandt et al. 2009). Interestingly, NKp30 can interact with BAG6 secreted by tumor cells in an exosomal or soluble form, which in turn can activate or inhibit NKp30-mediated NK cytolytic functions, respectively (Reiners et al. 2013). The NKp30 gene is the most ancient among the NKR family and is also found in cartilaginous fish (Flajnik et al. 2012). Sharks also possess B7H6 genes, suggesting that B7H6-NKp30 interaction arose early in vertebrate evolution. However, animals including bony fish which have lost NKp30 genes have also lost B7H6 genes.

Although NK-like cells have been identified in teleost fish, there are no orthologous genes of mammalian NKRs (e.g., KIR and Ly49 families). However, it is believed that multiple multigene families of immunoglobulin domain-containing innate immune receptors (IIIRs) in teleost fish represent the counterparts of mammalian innate immune receptor families such as the KIRs, leukocyte immunoglobulin-like receptors (LILRs), Fc receptors, triggering receptors expressed on myeloid cells (TREMs), and CD300s (rb Wcisel and Yoder 2016). These IIIR families in bony fish include the novel immune-type receptors (NITRs); diverse immunoglobulin domain containing proteins (DICPs); polymeric immunoglobulin receptor-like proteins (PIGRLs); novel immunoglobulin-like transcripts (NILTs); and leukocyte immune-type receptors (LITRs).

Novel immunoglobulin-like transcripts (NILTs) which show structural similarities with mammalian TREM and NKp44 receptors were identified in carp, trout, Atlantic salmon, and zebrafish, but not in fugu genomes (rb Wcisel and Yoder 2016). NILTs basically encode one or two Ig domains and possess ITIM or ITAM motifs. The number of NILTs have diverged greatly in cyprinids, whereas only a few (<10 genes) have been found in salmonids. The function and ligands of NITRs are still unknown.

IIIRs may function as NKRs in fish, and one of the most investigated IIIRs in teleost fish is the novel immune-type receptor (NITR) family (rb Yoder and Litman 2011). Multiple genes of the NITR family including activating or inhibitory receptors are present in teleost fish and show similar structures to mammalian KIRs. The majority of NITRs have a cytoplasmic ITIM while some NITRs possess a positively charged residue within their transmembrane domain for possible association with adaptor proteins containing ITAMs. In fact, activating and inhibitory NITRs can activate and inhibit the ERK/MAPK pathways in human cell lines, respectively. Moreover, NITR9 representing the only activating NITR in zebrafish can interact with Dap12 resulting in activating signaling (Wei et al. 2007). In addition, cross-linking of a recombinant zebrafish NITR9 on a human NK cell line resulted in increased target cell killing. Importantly, recent single-cell transcriptome analyses of

lymphocytes in zebrafish demonstrate that certain NITRs may be markers for NK cells (Carmona et al. 2017; Tang et al. 2017).

LITRs are teleost's unique innate immune receptors containing two to six immunoglobulin domains. They are well described in catfish and likely have a role in target cell recognition and signal transduction. The structure of LITR Ig domains is similar to that of mammalian FcRs. Catfish LITRs consist of multiple genes and include inhibitory and activating forms. The mAb (CC41) was established to target LITRs, and members of the LITR family were identified as markers for catfish NK cells, alloantigen-specific CTLs, and antiviral cytotoxic cells (Shen et al. 2004; Taylor et al. 2016).

Another NK-like cell subset in teleost fish, NCCs, expresses NCCRP-1. This receptor is a type III membrane protein with functional domains for antigen binding, signaling, and transcriptional activation (Jaso-Friedmann et al. 2001). Cross-linking of NCCRP-1 induces receptor tyrosine and serine phosphorylation (Evans et al. 1999). It binds to the natural killer target antigen (NKTag) of tumor antigens and protozoan parasites resulting in target cell killing (Jaso-Friedmann et al. 1997). NCCRP-1 genes have also been identified in a number of other animal species including axolotl, mouse, and man. However, mammalian NCCRP-1, a member of the lectin-type FBXO gene family, is not specific to immune tissues and is expressed only in the cytosol (Kallio et al. 2011).

NK cells isolated from uninfected mammals can spontaneously kill targets, but this activity is enhanced when NK cells are exposed to interferons or certain cytokines produced by dendritic cells and macrophages during infection by various types of pathogens. Cytokines IL-2, IL-12, IL-15, IL-18, and type I interferons positively control NK cell function, either independently or in cooperation (rb Wu et al. 2017). As CD122 (IL-2Rb) is critical for NK cell development from CLPs, IL-2 and IL-15 are the most extensively studied cytokines for NK cell activation in humans and mice (rb Meazza et al. 2011). IL-2, which was originally found to be a T-cell growth factor (its roles in T-cell function are discussed later), has been shown to induce proliferation and cytokine production of NK cells as well as to activate cytotoxic effector mechanisms in NK cells. Interleukin-15, discovered by its "IL-2-like" stimulatory role, also has important roles in NK cell development and function. IL-2 and IL-15 share not only properties regarding NK cell function, but also with respect to receptor binding. Both can bind to CD122 (IL-2Rβ) and CD132 (IL-2Rγ). The binding of IL-2 and IL-15 to respective receptors is due to their corresponding α chains, IL-2Rα (CD25) and IL-15Rα (CD215), respectively (rb Leonard et al. 2019). Moreover, IL-2 secreted as a free cytokine can bind directly to the IL-2Rαβγ complex with high-affinity or to dimeric IL-2Rβγ with intermediate affinity while IL-15 can function as a cell surface-bound form on IL-15Rα-expressing professional antigen-presenting cells. IL-15Rα binds to IL-15 with high affinity, which effectively activates NK cells at relatively low concentrations when compared to IL-2 (rb Waldmann 2006). JAK-STAT5 signaling through IL-2 or IL-15 regulates NK cell activation, and the IL-15-STAT5 signaling pathway is especially critical for NK cell development and homeostasis (rb Gotthardt and Sexl 2017). Interestingly, and different from mammals, teleost fish have no IL-2Rα gene, meaning that both fish IL-2 and IL-15 require IL-15Rα

for signaling (Dijkstra et al. 2014). In teleost fish, IL-2 and IL-15 genes have been identified, and its biological function analyzed in numerous fish species, including rainbow trout (Wang et al. 2018a, b; Yamaguchi et al. 2020). Trout IL-15/IL-15Rα heterodimers induce the up-regulation of transcription of IFNγ and perforin, and the phosphorylation of STAT5 in trout CD4-CD8α-IgM-splenocytes, a subpopulation of lymphocytes presumably containing NK cells (Yamaguchi et al. 2020). Grass carp IL-15 also induces type 1 immune responses (as concluded from IFNγ and T-bet expression) and NK cell activation (as concluded from perforin and eomesodermin aka EOMES-a expression) (Wang et al. 2020). Thus, teleost IL-15 is also suggested to be a critical factor in NK cell activation.

Mammalian IL-12 was originally named natural killer cell stimulatory factor (NKSF) based on its activities to induce a large amount of IFNγ. Like IL-18, it is mainly produced by professional antigen-presenting cells, such as DCs, monocytes, and macrophages. The cooperative effect of these cytokines, IL-12 and IL-18, is required for proper and efficient IFNγ production by NK cells, and this is crucial in controlling viral infections before cytotoxic T cells become activated (rb Wu et al. 2017).

Viral infections trigger the expression of type I IFNs (IFNα/β). They are one of the most potent regulators of NK proliferation and cytotoxicity. IFNα induces DCs to produce IL-12, IL-15, and IL-18, thereby activating the IFNγ production by NK cells (rb Paolini et al. 2015). The production of IFNγ by NK cells in the early immune response enhances the capacity of macrophages in pathogen killing, activates DCs, and promotes the differentiation of CD4 T cells into the Th1 subset, which further leads to the production of IFNγ.

In addition to cytokines, multiple transcription factors coordinate the development and effector functions of different lymphocyte subsets. In mammals, ID2 (Inhibitor of DNA binding 2) is a critical regulator of all ILCs including NK cells (Boos et al. 2007). Its expression continues throughout early NK cell development and during NK cell maturation while repressing the development of CLPs into T cells (Zook et al. 2018; rb Brillantes and Beaulieu 2019). The transcription factors NFIL3 (Nuclear factor, interleukin 3 regulated), ETS1 (ETS proto-oncogene 1), and TOX (Thymocyte selection-associated high mobility group box) are expressed in early stages of NK cell development and control Id2 transcription. NFIL3 (alias E4BP4) can bind to the EOMES promoter during early stages of NK cell development (Male et al. 2014), while TOX1 is involved in later stages of NK cell maturation (Aliahmad et al. 2010).

The mammalian T-box transcription factors EOMES and T-bet are both required for late NK cell development and effector function (Gordon et al. 2012). The balance of their expression is well regulated during NK cell development (rb Brillantes and Beaulieu 2019). For example, increased T-bet expression in NK progenitors in bone marrow can suppress EOMES expression and stabilize the transition from immature to mature NK cells. EOMES is thought to promote NK cell development and maturation following T-bet in NK cell development. Importantly, EOMES can induce CD122 expression in both NK cells and CD8+ CTLs, resulting in an enhancement of IL-15 responsiveness. Finally, T-bet in association with Zeb2 controls terminal maturation of NK cells. However, tissue-residing NK cells show distinct expression patterns of EOMES and T-bet in various tissues,

suggesting unique developmental pathways (rb Simonetta et al. 2016). T-bet and EOMES genes have been identified in a few fish species. In rainbow trout, T-bet and EOMES are expressed in both CD8+ T cells and IgM-/CD8- lymphocytes (possibly including NK cells) (Takizawa et al. 2011, 2014). Overexpression of Atlantic salmon EOMES induces the expression of IFNγ and granzyme A in salmon splenocytes (Kumari et al. 2013). These results indicate that teleost T-bet and EOMES may also play a critical role in cell-mediated cytotoxicity.

#### Effector Functions of NK Cells

In mammals, both NK cells and CTLs contain lytic granules in their cytoplasm including perforins, granzymes, and NK-lysin (alias granulysin) to eliminate pathogens and altered cells. Perforin can polymerize to form transmembrane pores in the membrane of target cells, and granzymes belonging to the family of serine proteases mediate apoptosis of target cells. This can effectively induce target-cell apoptosis of virus-infected cells to inhibit viral spread. NK-lysin is an antimicrobial peptide, destroying cholesterol-poor microbial membranes, and NK-lysin and granzyme can cooperatively kill intracellular bacteria and parasites after disruption of host cell membranes by perforin (Walch et al. 2014; rb Dotiwala et al. 2016). In addition to lytic granules, mammalian NK cells also utilize death receptor-mediated apoptosis with Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) to kill viral-infected cells and altered cells (rb Prager and Watzl 2019).

Although a variety of leukocytes are involved in nonspecific cell cytotoxicity in teleost fish, two types of NK cell homologs with nonspecific cytotoxicity have been well described in channel catfish thus far: NCCs and NK-like cells. Homologous genes to cytolytic granules, FasL and TRAIL have been cloned in many fish species, and their functions are likely conserved between mammals and teleost fish (Yamaguchi et al. 2019a; Tafalla and Granja 2018). However, the association of these molecules to teleost NK cells is unknown in several fish species. Catfish NCCs possess granzyme-like serine proteases (Praveen et al. 2004) and express transcripts of perforin and granulysin genes (Praveen et al. 2006), indicating that teleost NCCs use cytotoxic mechanisms similar to mammalian NK cells. Tilapia and catfish NCCs do not express the membrane-bound type of FasL, but constitutively release soluble FasL proteins (rb Evans et al. 2001; Bishop et al. 2002). NCC activity has been found in various fish species, such as rainbow trout (Hayden and Laux 1985; Greenlee et al. 1991), carp (Bielek 1988; Meseguer et al. 1994), damselfish (McKinney and Schmale 1997), and tilapia (Faisal et al. 1989). In channel catfish, a subpopulation of peripheral blood leukocytes (PBL) was found to exhibit NK cell activity against allogeneic cells (Yoshida et al. 1995; Stuge et al. 1995) and virus-infected cells (Hogan et al. 1996). Later, several NK-like cell lines were established from catfish PBL that were shown to be negative for TCR, as well as neutrophil and monocyte marker genes, but also negative for mAb 5C6, a specific marker for catfish NCCs, indicating the existence of distinct populations of NK-like cells in catfish (Stuge et al. 2000, Shen et al. 2004). Moreover, these NK-like cells contain cytoplasmic granules and kill allogeneic target cells by induction of apoptosis via perforin/granzyme mechanism (Hogan et al. 1999;

Shen et al. 2004). Current research from single cell transcriptomes identified additional candidates of marker and effector molecules in teleost NK-like cells (Moore et al. 2016; Carmona et al. 2017; Guslund et al. 2020). However, further progress is needed to understand the mechanisms by which fish NK cells recognize altered target cells and are activated.

Antibody-dependent cell-mediated cytotoxicity (ADCC) is another mechanism by which mammalian NK cells can kill their target cells. ADCC is based on the interaction of cytotoxic Fc receptor (FcR)-expressing cells with the Fc part of antibodies followed by direct recognition of the respective antigens on target cells by FcR-bound antibodies (rb Gómez Román et al. 2014) (Fig. 4.2). Alternatively, surface antigen-expressing cells are already opsonized by antibodies, and NK cells bind to opsonized cells by means of their FcR. In mammals, where ADCC can be critical in viral infection and cancer clearance, IgG antibodies represent the major ADCC-mediating Ig isotype. Mammalian NK cells generally express CD16 (FcγRIII) and upon recognition of IgG on target cells, NK cells are activated and kill opsonized target cells by cytotoxic granule release and Fas signaling. NK cell-mediated ADCC is also used as effector mechanism for immunotherapy against tumor cells using antitumor antibodies (rb Wang et al. 2015). In channel catfish, NK-like cells armed with IgM were shown to kill target cells through ADCC (Shen et al. 2003, 2004). However, orthologous genes of FcμR and FcαμR (CD351) in fish, amphibians, birds, and reptiles have not been identified yet (rb Akula et al. 2014; Kortum et al. 2014). Strangely, although catfish possess an FcμR homolog that can bind to catfish IgM, this molecule is only expressed as soluble form (Stafford et al. 2006). Therefore, it remains to be explored which receptor is utilized in teleost fish to induce ADCC.

#### 4.2.3.3 Cytotoxic T Lymphocytes (CTLs)

##### Origin of CTLs

Mammalian cytotoxic T cells have their origin in the thymus, and T-cell precursors are recruited from HSCs in the bone marrow. While traversing the thymus cortex and medulla, progenitor T lymphocytes develop from immature CD4/CD8 double-negative (DN) into CD4/CD8 double-positive (DP) and finally either CD8 or CD4 single-positive (SP) T cells, depending on their preference to either bind to MHC class I or II molecules, respectively. During mammalian T-cell maturation, two important processes occur: T-cell receptor (TCR) rearrangement and selection. TCR rearrangement of the variable (V), diversity (D), and joining (J) gene segments occurs during the DN and DP stages yielding TCRs with great diversity. The TCR finally defines the antigen specificity of T cells enabling CD8 SP and CD4 SP T cells to recognize antigenic peptides presented by MHC class I or II. In a strict selection process, during which only a small fraction (<5%) of developing T lymphocytes survive, both weak interaction of the TCR with self-peptide–MHC ligands as well as excessive signaling result in T-cell apoptosis (negative selection). Negative selection ensures that mature T cells are not committed to react against the body's own cells. Only T cells showing an intermediate level of TCR signaling are allowed to further

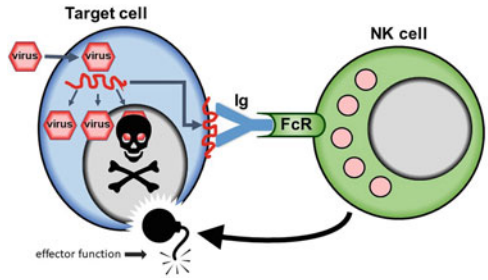


**Fig. 4.2** NK cells can kill target cells by Antibody-Dependent Cell-mediated Cytotoxicity (ADCC). During certain virus infections, viral proteins are incorporated into the membrane of infected cells. Antibodies can bridge the contact between Fc receptors (FcR) expressed on the surface of NK cells and the Fc part of antibodies opsonizing virus-infected cells

mature (positive selection) (rb Germain 2002). Mature naïve SP T lymphocytes leave the thymus and recirculate through secondary lymphoid tissues such as lymph nodes, Payer's patches, and spleen. In these tissues, CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T helper (Th) cells are activated through binding of their TCRs to antigenic peptides displayed by MHC class I or MHC class II molecules on the surface of antigen-presenting cells (APC), respectively. Activated T cells undergo additional development and differentiate into effector and memory cells (rb Boes and Durham 2017). However, there are also examples where DP T cells can be found outside the thymus, e.g., in pigs (Saalmüller et al. 1987).

The T-cell development in teleost fishes is similar to that in mammals although there are still many gaps in our knowledge (rb Bajoghli et al. 2019). In the zebrafish embryo, HSCs originating from dorsal aorta migrate to the caudal hematopoietic tissue (CHT) and finally home to the thymus and the kidney. Two days post fertilization (dpf), the zebrafish thymus region is colonized by HSC and T lymphopoiesis is initiated following the expression of RAG1 and later on by the formation of cortex and medulla. Based on the consecutive expression of RAG1, Ikaros, TCRα, TCRβ, TCRγ, and TCRδ and other T-cell markers in the zebrafish thymus, it may be concluded that CD8α is first transcribed at 6 dpf (rb Ma et al. 2013). In rainbow trout, CD8α is expressed as early as between 7 and 10 dpf, while first TCR expression can be seen between 14 and 40 dpf (Fischer et al. 2005; Heinecke et al. 2014, respectively). In sea bass, CD8 transcription was first recorded 51 days after hatch (Picchietti et al. 2009). In ginbuna crucian carp and rainbow trout, thymocytes traverse a stage where they are CD4/CD8 DP cells while such cells cannot be found outside the thymus. Thus, DP cells reach a SP state prior to leaving the thymus (Toda et al. 2011; Takizawa et al. 2011, 2016).

Although CD8+ cells can be found in all trout and ginbuna organs, their distribution is unequal both when comparing organs, and within the same organ (Table 4.1).

In contrast to humans where the percentage of CD8α+ cells among PBL is between 16 and 38% (rb Virella 2007), the corresponding share in teleost fish is remarkably low

**Table 4.1** Percentages of CD8α+ (ginbuna crucian carp and rainbow trout) and CD8β+ (Japanese flounder) lymphocytes in different organs of adult fish

| Lymphocyte source | Rainbow trout (%) | Ginbuna crucian carp (%) | Japanese flounder % | References |
|---|---|---|---|---|
| Thymus | 70 | 35 | nd | |
| Intestine | 54 | nd | nd | rb Nakanishi et al. (2015) for trout and ginbuna |
| Gill | 25 | nd | nd | Xing et al. (2017) for flounder |
| Spleen | 2 | 3.5 | 2.8 | |
| Pronephros | 4 | 7 | 3.2 | |
| PBL | 0.3 | 1.7 | 2.1 | Takizawa et al. (2011) for trout<br>Toda et al. (2011) for ginbuna<br>Xing et al. (2017) for flounder |

(Table 4.1). While trout CD8α+ lymphocytes are equally distributed in most tissues, they are found in higher concentrations in the internal (basal) zones of trout thymus (Takizawa et al. 2011), and in the cortical zones of sea bass thymic lobes (Picchietti et al. 2009). Although teleost fishes lack lymph nodes, Payer's patches, and distinct T-cell and B-cell zones with germinal centers, CD8α+ lymphocytes form follicle-like structures in the trout spleen (Takizawa et al. 2011). According to investigations by Leal et al. (2016), abundance of CD8α+ T lymphocytes among skin lymphocytes is higher in its anterior parts (12%) than in posterior sections (6%).

### Induction and Regulation of CTL-Mediated Cytotoxicity

Before mammalian CTLs (like NK cells) use their effector molecules perforin, granzyme, and FasL for target cell killing, they need to be activated and proliferated. In order to create a systematic approach of CTL (and Th cell) induction, two main activation signals were initially suggested. According to this model, signaling starts with the binding of TCR complexes on T cells to the peptide-MHC complex on APCs (signal 1). Generally speaking, MHC class I is expressed by all nucleated cells (in fish this includes erythrocytes and thrombocytes!) and presents peptides derived from intracellularly produced proteins, while MHC class II expression is restricted to professional APCs and is important for displaying peptides from endocytosed/phagocytosed antigens. While CD8 serves as a coreceptor of TCR to stabilize contact of CTLs to MHC class I, CD4 supports binding of Th cells to MHC class II expressing APCs (rb Murphy and Weaver 2017). Signal 2 is characterized by costimulatory signaling between additional receptors on T cells and their corresponding ligands on APCs. To more comprehensively explain additional activation of

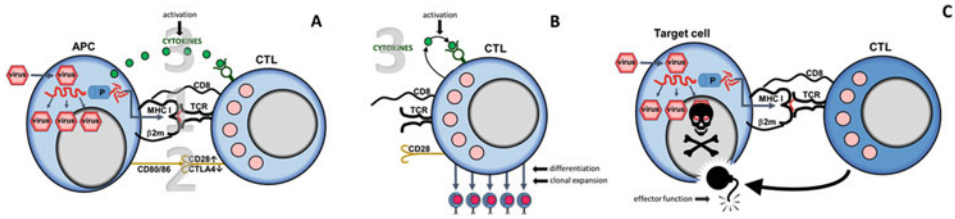


**Fig. 4.3** Three signals are needed for CTL activation in mammals, differentiation, clonal expansion, and finally effector function: (1) Presentation by APCs of antigenic peptides through MHC class I to TCR of CTLs; (2) Accessory receptor/ligand interactions; (3) Paracrine cytokines produced by APCs (**a**). After this, autocrine cytokine signaling leads to further CTL activation resulting in CTL differentiation, clonal expansion (**b**), and effector function (CMC) (**c**)

T cells by cytokines, Curtsinger et al. (1999) introduced signal 3 (rb Mescher et al. 2006). This signal theory does not fully explain the complex activation system for T cells, but it reflects its most important steps that appear to be conserved throughout vertebrates (Fig. 4.3).

Before mammalian CD8+ CTLs utilize their TCR complexes to recognize peptide–MHC class I complexes on target cells, proteins intracellularly synthesized by viruses or by intracellular bacteria are proteolytically cleaved to peptides of around 10 amino acids (aa) in the proteasome. Peptides are then translocated into the endoplasmic reticulum (ER) by transporters associated with antigen processing (TAP). In the ER, the MHC class I heavy chain folds into three domains (α1, α2, and α3) and associates with a fourth domain, the β2-microglobulin (β2M). The α1 and α2 domains of MHC class I form the peptide-binding groove where antigenic peptides of 8–10 aa in length are loaded. Peptide-loaded MHC class I molecules are finally transported to the cell surface of APCs and presented to the corresponding specific TCR complex on CD8+ lymphocytes (rb Hewitt 2003). However, peptide specificity does not imply that a certain TCR can only bind to peptides with identical aa sequence. TCRs can also cross-react with other peptides of different avidities depending on the presence of anchor residues in the respective peptides (rb Singh et al. 2017).

Orthologous genes for MHC class I and peptide-loading pathway molecules, e.g., tapasin, proteasome subunit beta molecules of the immunoproteasome, and TAP (TAP1 and TAP2) have been described in a number of fish species (rb Yamaguchi and Dijkstra 2019). While signature motifs and domains of these molecules are conserved among teleost fishes, MHC class I signaling cascades remain largely unexplored. Orthologous genes of MHC class I, the TCR complex (consisting of the TCR chains and the CD3 subunits), and the TCR coreceptor CD8 are known in teleost fish, while in grass carp, CD8αα homodimers were reported to bind specifically to the pMHC-I complex (Wang et al. 2018a, b). Additionally, genes associated with TCR complex signaling namely the zeta-chain-associated protein kinase (ZAP)70 and lymphocyte-specific protein tyrosine kinase (LCK) have been reported in teleosts. However, the Lck-binding motif of the teleost CD8

molecules is different from tetrapods. While in tetrapods, a bicysteine Lck-binding motif (CxC) is only present in the cytoplasmic tail of CD8α chains, both teleost CD8α and CD8β chains possess the possible Lck-binding motif CxH suggesting that both CD8 chains can be involved in Lck signaling. However, since Lck binding to CxH motif is zinc-dependent in CD8α but not CD8β, CD8α seems to be the main TCR coreceptor to recruit Lck molecules in teleost CTLs.

CD8 is the main CTL marker, however does not reveal the antigen specificity of CTLs. MHC class I multimer technology utilizes labeled and multimerized recombinant MHC class I proteins that are loaded with peptides of a known aa sequence and that are stabilized with recombinant β2m, for staining of antigen-specific CTLs expressing a certain (peptide-) specific TCR mimicking signal 1 during CTL activation. Such multimers have been used in grass carp to identify antigen-specific CTLs against grass carp hemorrhagic virus (GCHV) and in rainbow trout against infectious hematopoietic necrosis virus (IHNV) (rb Yamaguchi et al. 2019a).

There are several studies on the regulation of CD8 expression in virus-infected teleost fish. In rainbow trout, the expression levels remained unchanged or down-regulated after IPNV infection, while in Atlantic halibut infected with nodavirus and in Atlantic salmon inoculated with infectious salmon anemia virus (ISAV), CD8 expression was decreased. The reasons for such down-regulations are unknown. In mammals, herpesviruses, pox viruses, and the human immunodeficiency virus suppress MHC class I pathway components to prevent antigen presentation to CD8+ lymphocytes thereby suppressing signal 1 of CTL activation. Other reasons for CD8 suppression could be simply a cytolytic infection of CD8+ cells or an attrition of CTL responses by increased type I interferon expression (rb Yamaguchi et al. 2019a).

Monoclonal and polyclonal antibodies against CTL markers, particularly against CD8 are valuable tools to study CTL responses. They are available for ginbuna, rainbow trout (rb Nakanishi et al. 2015), and for olive flounder (Xing et al. 2018). Five days after infection with viral hemorrhagic septicemia virus (VHSV), the number of CD8α+ cells in the spleen was found to be decreased while a concurrent increase occurred in the liver (Castro et al. 2014). VHSV infection also increases the number of CD8+ cells in the adipose tissue (Pignatelli et al. 2014). CD8α+ cells separated from trout spleen showed a skewed TCR repertoire 3 weeks after a secondary VHSV infection suggesting clonal expansion of CD8+ CTLs upon viral infection. Several papers describe the attraction of CD8+ cells to the site of infection. In trout, interaction of IHNV with olfactory sensory neurons in the nasal cavity triggered the recruitment of CD8+ T cells to the olfactory mucosa (Sepahi et al. 2019) and an influx of CD8+ cells was also seen during ichthyophthiriasis in infection foci of rainbow trout gills (Olsen et al. 2011), while in another parasite infection (proliferative kidney disease) of the same species, no major changes among CD8+ cells were recorded (Bailey et al. 2020). During red mouth disease (caused by *Yersinia ruckeri*), a transient early decrease in densities of CD8α+ cell was seen shortly after infection of vaccinated trout followed by an increase at 30 days after infection (Deshmukh et al. 2013).

Costimulatory signal 2 interactions between B7 family molecules (B7s and B7-homologous family members expressed on APCs) and CD28 family molecules (expressed on T cells) are essential for the balance between T-cell activation and T-cell tolerance following TCR-mediated signal 1 transduction in mammals. Within the CD28 family, there are two positive costimulatory regulators: CD28 and inducible costimulatory signal (ICOS), and three inhibitors: cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death-1 (PD-1), and B- and T-lymphocyte attenuator (BTLA) (rb Riley and June 2005). Mammals possess two B7 molecules: B7-1 (CD80) and B7-2 (CD86). In teleost fish, a single CD80/86 gene is believed to be the ancestor from which CD80 and CD86 arose by gene duplication during evolution. Trout CD80/86 is expressed on B cells and up-regulates the expression of IL-2 in PBL (Zhang et al. 2009). Since teleost B cells have been associated with phagocytosis and zebrafish B cells were shown to express CD80/86, they are suggested to play a role as professional APCs (Zhu et al. 2014). Additional B7 family members such as H1/DC, B7-H3, and B7-H4, have been identified in teleost fishes too. Although signaling motifs are apparently missing in the cytoplasmic region of teleost CTLA-4, both CD28 and CTLA-4 are structurally highly conserved among vertebrates suggesting similar functions (rb Yamaguchi et al. 2019a, b).

To fully activate naïve mammalian CD8+ CTLs, a third signal provided by cytokines is required. Interferon (IFN)γ is a signature cytokine for this activation. Additional highly relevant cytokines that can activate mammalian CTLs are IL-2, IL-15, and IL-21. IFNγ is expressed by CD8+ CTLs, by NK cells and Th1 cells and is thereby a key inducer of type 1 immunity. Upstream, this type 1 response IFNγ production is stimulated by IL-12, a cytokine produced by DCs. During intracellular infections, mammalian DCs not only provide this signal 3, but also the aforementioned signals 1 and 2 to CTLs (Henry et al. 2008). IFNγ homologs have been described in many fish species. Rainbow trout and zebrafish express two IFNγ genes (IFNγ1-1 and IFNγ1-2). While in zebrafish, IFNγ1–1 expressing cells do not transcribe effector molecules of cytotoxic cells, IFNγ1–2 is coexpressed with granzymes and granulysins. In trout, IFNγ is expressed by CD8α+ lymphocytes and triggers the expression of IL-15, suggesting its role in Th1 responses. However, direct effects of piscine IFNγ homologs on CTL activity still need to be shown. Channel catfish, common carp, ginbuna, goldfish, grass carp, Indian major carp, and zebrafish, additionally express IFNγ-related proteins (IFNγrel). However, the functions of IFNγrel still need to be explored (rb Yamaguchi et al. 2019a).

Another important cytokine stimulating the clonal expansion of mammalian T cells and the growth of antigen-stimulated T cells is IL-2 (rb Nelson 2004). IL-2 plays a crucial role in the differentiation of naïve CD8+ T cells into CTL effector cells expressing perforin, granzyme B, and IFNγ, but also into CTL memory cells (Pipkin et al. 2010). Additionally, IL-2 is involved in the formation of immune tolerance (rb Malek and Bayer 2004). IL-15, another member of the IL-2 cytokine family, stimulates CTLs and has been shown to be even more important than IL-2 in the development and maintenance of CTL memory (rb Sim and Radvanyi 2014). Mammalian IL-2 binds to the IL-2Rαβγ with high affinity or to the IL-2Rβγ with intermediate affinity, while IL-15 is predominantly expressed in

association with IL-15Rα either on the surface of APCs or released as a soluble heterodimer with IL-15Rα. IL-15Rα significantly stabilizes IL-15 which then binds to IL-2Rβγ, e.g., on CD8+ lymphocytes (rb Waldmann 2006). However, teleost fishes do not possess IL-2Rα implying that both cytokines must only rely on IL-15Rα (Dijkstra et al. 2014). Trout and yellow croaker IL-2 trigger gene expression of IFNγ. Trout IL-15 induces IFNγ gene expression in splenocytes (rb Yamaguchi et al. 2019a) and recently, Yamaguchi et al. (2019b) have shown that trout IL-15/IL-15Rα heterodimers possess a higher bioactivity with respect to the activation of CD8+ lymphocytes than IL-15 alone.

Another cytokine that promotes CTL function by induction of perforin, granzyme B, and IFNγ in mammals is IL-21. However, IL-21 rather works in concert with other cytokines such as IL-7 and IL-15 to expand and activate CD8+ T cells (rb Leonard et al. 2019). In rainbow trout, IL-21 triggers gene expression of IFNγ, IL-10, IL-21, IL-22, CD8, and IgM; and it maintains transcription of CD8 at a later stage of in vitro stimulation (rb Wang et al. 2011a, b). Synergistic effects of IL-21 with additional cytokines are still unknown in teleost fishes.

Besides mammalian cytokines that activate CTLs, there are also cytokines that suppress their function. One of them, IL-10, can reduce the production of IFNγ as well as T-cell proliferation and effector activity. By inhibiting signal 1 through decreased antigen presentation by professional APCs, IL-10 can have an additional negative feedback on T-cell responses. However, IL-10 has both immunosuppressive and stimulatory effects in mammals. Although IL-10 triggers the proliferation and effector function of CD8+ T cells against tumors, it could also inhibit CTL activity against tumor cells by decreasing antigen presentation (rb Ouyang and O'Garra 2019). IL-10 activity has been described in several fish species. It down-regulates IFNγ gene expression in goldfish and promotes the survival and proliferation of T cells isolated from immunized common carp. From stimulation experiments in common carp, it was concluded that IL-10 promotes the development of CD8+ memory T cells, while down-regulating CD4+ memory T cells. In infected amberjack, IL-10 was suggested to shift the Th1/Th2 balance toward Th2 (rb Yamaguchi et al. 2019a).

Mammalian cytokines transduce signals through their corresponding receptors resulting in transcription factor induction and finally in the expression of certain effector molecules. The T-box transcription factors EOMES and T-bet are signature transcription factors promoting Th1 and consequently CTL (and NK cell) activation, trafficking and regulation in mammals. They are engaged by IL-2, IL-12, IL-21, IFNγ, type 1 interferons, and even by IL-4. T-bet and EOMES are usually expressed in different ratios. While mammalian effector T cells are rather T-bet$^{high}$ and EOMES$^{low}$, this situation is inversed in memory cells (rb Pritchard et al. 2019 and Dejean et al. 2019). Homologous genes to T-bet and EOMES have been identified in many fish species. In virus-infected grass carp, in bacterial-infected Atlantic salmon, and in vaccinated ginbuna, T-bet and IFNγ expressions were found to be up-regulated. T-bet (along with perforin and granzyme) expression can be induced in allo-grafted ginbuna. In rainbow trout, high expressions of EOMES and T-bet were recorded in CD8α+ cells (rb Yamaguchi et al. 2019a).

Apart from these three signals, chemokines also contribute to CTL recruitment and differentiation in mammals. Several chemokines and their corresponding receptors are involved in CTL priming, effector function, and memory formation in mammals (rb Griffith et al. 2014). In the teleost orange-spotted grouper, recombinant CCL4 was found to induce chemotactic activity in PBL and to up-regulate TNF-α1, TNF-α2, IFNγ, Mx, T-bet, and both CD8α and CD8β expressions, suggesting the induction of a Th1-skewed response. Recombinant CK12a, a CCL19-like chemokine was found to trigger the expression of CD8α, granulysin, and IFNγ while increasing the number of CD8α+ cells at the site of administration in rainbow trout (rb Bird and Tafalla 2015; Sepahi et al. 2017).

### Effector Functions of CTLs

CTL-mediated cytotoxicity is MHC class I restricted in mammals, meaning that the MHC class I of the APC must fit into the TCR of a CD8+ CTLs. However, MHC molecules were not discovered during investigations on immune responses against pathogens, but in transplantation medicine. Dausset, Snell, and Benacerraf were awarded the Nobel Prize in physiology in 1980 for the discovery of the so-called histocompatibility ("tissue matched") antigens during early 1970s. Subsequently, Doherty and Zinkernagel found the MHC class I restriction of CTL-mediated cytotoxicity against virus infection in mice (Nobel Prize in 1996).

The first studies on cell-mediated cytotoxicity in teleosts also used allograft rejection models. However, the initial lack of assay systems requiring MHC class I matched effector and target cells have delayed investigations on antiviral CTL-mediated cytotoxicity. A respective assay system was introduced in clonal ginbuna crucian carp by establishing a syngeneic cell line from the respective fish clone. In clonal rainbow trout, a syngeneic MHC class I matched target cell line was identified after sequencing of well-established trout cell lines. Another approach was used in channel catfish and orange-spotted grouper where autologous target cells isolated from the same individual effector cell donor were applied. The latter system offers the possibility to use CMC assays even if clonal fish are not available. Infected MHC class I mismatched target cells are usually not killed by CTLs. Such killing indicates the contribution of NK cells to cell-mediated cytotoxicity (see subchapter on NK cell responses). Phenomena related to CTL-mediated cytotoxicity in fish are comprehensively reviewed in a recent publication by Yamaguchi et al. (2019a).

Strong suggestion for MHC class I restricted CMC has been shown in ginbuna against two rhabdoviruses (carp hematopoietic necrosis virus—CHNV; eel virus from America—EVA) and against a birnavirus (infectious pancreas necrosis virus—IPNV), in rainbow trout against two rhabdoviruses (VHSV and IHNV) as well as in orange-spotted grouper against nervous necrosis virus (NNV)-infected fish. DNA vaccines are suggested to be potent triggers of CTL responses since they mimic a viral infection in terms of intracellular antigen processing and presentation through MHC class I (rb Wang et al. 2011a, b). In trout, CMC against VHSV was shown after intramuscular administration of DNA encoding the G or the N protein of VHSV (rb Yamaguchi et al. 2019a).

Further characterization of effector cells in MHC class I restricted CMC has brought additional evidence that teleost CTLs are among the effector cells contributing to antiviral and allo-specific cytotoxicity. In ginbuna and rainbow trout, such effector cells express mRNA encoding TCR and CD8. Moreover, CD8+ lymphocytes that were separated from CHNV-infected ginbuna crucian donors were able to kill CHNV-infected MHC class I matched target cells and to protect syngeneic recipients against CHNV infection. By using a system of allo-specific CMC in ginbuna, it was shown that CD8α+ effector cells kill their target cells by utilizing perforin and granzyme pathways (rb Yamaguchi et al. 2019a). Recently, Taylor et al. (2020) succeeded to clone four different antiviral cytotoxic cell lines expressing rearranged TCR genes, perforin, granzyme, and IFNγ. Surprisingly, none of these T-cell lines expressed CD8, while three of them expressed CD4-like molecules.

Mammalian CTLs (and NK cells) use several effector molecules to drive target cells into apoptosis. They store in their cytolytic granules perforin, granulysin (or its ortholog NK-lysin), and serine protease granzymes that are released into the cytotoxic synapse formed by signal 1 related and adhesion molecules. Perforin polymerizes to form transmembrane pores in the target cell membrane resulting in the delivery of granulysin and granzymes into target cells. Another granula-independent mechanism is based on interaction between FasL on activated CTLs and Fas on the target cell membrane. Both mechanisms lead to activation of caspases and finally to target cell apoptosis (rb Voskoboinik et al. 2015).

Genes encoding CTL effector molecules have been sequenced in several fish species and a few studies also investigated their activities such as antimicrobial activity, proteolytic activity, caspase, and apoptosis induction. CD8+ cells have been shown to express perforin and granulysin (rb Yamaguchi et al. 2019a). However, deep insights into mechanisms of CTL effector functions in fish are still missing.

There is little information on CTL memory in fish. Effector cells isolated from ginbuna reinfected with IPNV or CHNV were more effective in killing syngeneic cells infected with the respective homologous viruses than against syngeneic cells infected with a heterologous virus (EVA). The use of syngeneic (clonal) fish offers the possibility to transfer immune cells from one individual to another without risking rejection of donor cells by the recipient. In clonal ginbuna, adoptive transfer of CD4− cells (containing CD8α-expressing cells) from CHNV-infected donors to naïve syngeneic recipients induced an antiviral immune response (Somamoto et al. 2014). Using adoptive transfer experiments, CD8+ cells have also been proven to be important in intracellular bacterial infections in fish such as with *Edwardsiella tarda*. Transfer of CD8+ cells from ginbuna donors sensitized by *E. tarda* to isogenic naïve recipients resulted in up-regulation of IFNγ and perforin and conferred protection against *E. tarda* challenge (Yamasaki et al. 2014). Adoptive transfer of leukocytes from clonal rainbow trout donors that were previously immunized against the bacterium *Yersinia ruckeri* to syngeneic recipients resulted in protection of the latter. However, expression of CD8 versus IgM and IgT in organs as well as IgM levels in sera of recipients suggest a B- rather than a T-cell memory response (Yamaguchi et al. 2019b).

## 4.3 Vaccination Targeting CTL-Mediated Immune Responses

Vaccination is based on B-cell and/or T-cell (including Th and CTL) memory. Due to intracellular processing of antigens during intracellular infections, CTL responses seem to be highly important in antiviral responses in vertebrates. Nevertheless, in several vaccines against human viral diseases, antibodies alone were found to be sufficiently protective. An antibody-based protective response has also been suggested against IHNV in Atlantic salmon, as high antibody levels corresponded with the reduction of postchallenge mortality. However, most licensed vaccines are inactivated and thus antigens are rather taken up by APCs resulting in an MHC class II presentation pathway. Peptides presented by MHC class II, however, are recognized by Th cells but not by CTLs. Part of the peptides produced after uptake may also be cross-presented through MHC class I. Although the existence of cells with a phenotype of cross-presenting DCs in rainbow trout suggests cross-presentation in teleost fish, this phenomenon remains to be demonstrated (rb Yamaguchi et al. 2019a).

DNA vaccines seem to be best suited to mimic a viral infection and to trigger CTL responses through MHC class I pathways. Indeed, a CTL-like response could be shown after DNA vaccination against VHSV in an MHC class I matched system of vaccinated rainbow trout effector cell donors and virus-infected target cells. Moreover, effector cells isolated from the injection site of DNA vaccinated clonal trout donors migrated to the injection sites of the homologous DNA vaccine after transfer to vaccinated fish of the same (isogenic) trout clone suggesting antigen-specific effector cell homing (rb Yamaguchi et al. 2019a).

Our current knowledge on MHC class I restricted cell-mediated cytotoxicity in teleost fish gives rise to the hope of using peptide vaccines. For this, antigenic peptides conforming to the groove of MHC class I need to be initially analyzed or predicted. The correctness of the corresponding synthesized peptides needs to be confirmed by analyzing their capacity to stabilize recombinant MHC class I and β2m heterodimers. This has been demonstrated in grass carp for peptides derived from GCHV and in rainbow trout for IHNV peptides. However, high polymorphism among MHC class I alleles makes the use of MHC class I peptide-based vaccines difficult in an outbred population with high genetic variability (rb Yamaguchi et al. 2019a). Moreover, peptide presentation profiles are even more variable due to the fact that diploid bony fish possess two β2m loci which may lead to structural changes in the peptide-binding groove of the MHC class I α1 and α2 domains (Li et al. 2020).

Very important components of vaccine formulations are adjuvants. In mammals, several adjuvants are known that can support CTL responses. Although adjuvants are widely used in commercial fish vaccination, their capacity to enhance T-cell responses remains unknown. To overcome the side effects of oil adjuvants and to design vaccines that preferentially trigger CTL memory, molecular adjuvants are the focus of current research. Both IL-2 and IL-15 are known to stimulate CTL responses, and IL-15 contributes to T-cell

memory in mammals. Recently, Yamaguchi et al. (2019b) have shown that recombinant IL-15/IL-15Ra heterodimers trigger type 1 immune responses in rainbow trout.

## 4.4 Conclusion/Outlook

Cell-mediated immune functions are an integral part of a concerted immune response. While nonvertebrates rely on pattern recognition only and have evolved without the need of a true adaptive immune system, vertebrates have developed cells that specifically recognize and counteract infectious agents. As in their mammalian counterparts, evolutionary ancient cells of the innate immune system such as macrophages and granulocytes act in concert with TCR bearing Th cells and CTLs of the adaptive immune system in teleost fish. Many of the cells involved in cell-mediated immune responses have been described in fish and the increasing availability of tools to identify them have been developed during the last decades. While evolutionary ancient cell-mediated functions such as phagocytosis and NETosis could clearly be shown in fish, the phenomenon of cell-mediated cytotoxicity requires further research efforts. Currently available tools in fish immunology still do not always allow to clearly distinguish between NK cell and CTL responses, even where MHC class I matched effector and target cell systems are available. Another aspect that should attract our attention is the early stage of fishes in evolution where immune cells might not have reached a stage of specialization as in mammals thus exhibiting several functions. Even in mammalian immunology, former clear textbook borders between innate and adaptive immune responses are not a dogma anymore (cf. innate memory, NKT cells, etc.). Another challenge for future studies is to better understand how cell-mediated immune response mechanisms are embedded in the whole process of disease protection and resistance. Finally, basic and applied research in fish immunology will help to design vaccines that are able to selectively target those immune mechanisms that are responsible for disease protection.

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