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Teleost cytotoxic T cells

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ABSTRACT

Cell-mediated cytotoxicity is one of the major mechanisms by which vertebrates control intracellular pathogens. Two cell types are the main players in this immune response, natural killer (NK) cells and cytotoxic T lymphocytes (CTL). While NK cells recognize altered target cells in a relatively unspecific manner CTLs use their T cell receptor to identify pathogen-specific peptides that are presented by major histocompatibility (MHC) class I molecules on the surface of infected cells. However, several other signals are needed to regulate cell-mediated cytotoxicity involving a complex network of cytokine- and ligand-receptor interactions. Since the first description of MHC class I molecules in teleosts during the early 90s of the last century a remarkable amount of information on teleost immune responses has been published. The corresponding studies describe teleost cells and molecules that are involved in CTL responses of higher vertebrates. These studies are backed by functional investigations on the killing activity of CTLs in a few teleost species. The present knowledge on teleost CTLs still leaves considerable room for further investigations on the mechanisms by which CTLs act. Nevertheless the information on teleost CTLs and their regulation might already be useful for the control of fish diseases by designing efficient vaccines against such diseases where CTL responses are known to be decisive for the elimination of the corresponding pathogen. This review summarizes the present knowledge on CTL regulation and functions in teleosts. In a special chapter, the role of CTLs in vaccination is discussed.

1. Introduction

Organisms continuously encounter non-self noxious substances and invasive pathogens, and their immune systems have acquired through evolution the ability to distinguish between self and non-self, but also between dangerous and tolerable non-self or modified self. These distinctions help immune systems to decide whether to tolerate or to mount an aggressive response against the respective antigens [1]. The immune systems of jawed vertebrate species, including teleost fish, show similarities with non-vertebrate species in regard to innate immunity while they are characterized by unique, recombination-based, adaptive immunity. The text below is written from the viewpoint of the jawed vertebrate immune system.

Pathogens can be divided into extracellular, facultative intracellular, and obligate intracellular pathogens. While most bacteria prefer an extracellular lifecycle, others may proliferate facultatively within infected cells. In contrast, viruses are obligate intracellular pathogens and can only replicate by using the metabolic machinery of their host cells. Intracellular pathogens provide special challenges to the immune system, because as long as they are not exposed extracellularly they cannot be contacted directly by immune cells or by humoral factors such as antibodies.

Intracellular pathogens can be detected through pathogen and danger associated molecular patterns (PAMPs and DAMPs, respectively). PAMPS and DAMPS are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors, aka NLRs). Examples of PAMPS are bacterial lipopolysaccharides (LPS) and (viral) double-stranded RNAs. Examples of DAMPS are DNA outside the nucleus and metabolites generated during cell stress. Once receptors have sensed danger they trigger different signal transduction cascades that can result in the expression/activation of NF-κB and other transcription factors, type I interferon (IFN) and other cytokines, antimicrobial peptides and chemokines [2–4].

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In order to avoid unlimited replication of intracellular pathogens in infected cells the host has a vital interest in eliminating those infected cells. In mammals, this killing involves cell-mediated cytotoxicity (CMC) which can be executed by specialized lymphocytes, namely natural killer (NK) cells and $CD8^+TCR\alpha\beta^+$ cytotoxic T lymphocytes (CTLs). CTLs and NK cells can not only kill pathogen-infected cells, but also tumour cells. CTLs are part of the adaptive immune system and, by means of their T cell receptors (TCR), recognize fragments from digested protein antigens presented by major histocompatibility complex (MHC) class I on potential target cells. NK cells, on the other hand, belong to the so-called innate lymphoid cells (ILC) and are stimulated to kill cells in absence of (self) MHC class I expression or if cells express stress-induced molecules, PAMPS, or antibody-opsonized membrane-associated proteins. The latter involves the Fc receptor of NK cells and this mechanism is called antibody-dependent CMC (ADCC). NK cells are thus important for killing of foreign, stressed and tumour cells, and they can kill tumour and infected cells in which the MHC class I expression has been downregulated as a strategy by the tumour or pathogen to escape from CTL killing. Due to their different recognition mechanisms, together CTLs and NK cells are usually able to ensure that problematic cells are eliminated. CTLs and NK cells are similar in that they both use the molecules perforin and granzyme for killing target cells [5].

In order to understand CTLs in evolutionary terms, they should not only be compared with NK cells, but also with B cells, helper/regulatory $CD4^+TCR\alpha\beta^+$ T cells, and with the rather enigmatic $TCR\gamma\delta^+$ T cells. Both B and T cells are characterized by somatically rearranged and clonally selected surface receptors, BCR (B cell receptor aka antibody) in case of B cells and TCR in case of T cells. Different between B and T cells is the site of receptor gene rearrangement and initial clonal selection, which for T cells is the thymus [6]. Different between $TCR\alpha\beta^+$ T cells and $TCR\gamma\delta^+$ T cells is that for TCR-mediated stimulation only the first category fully relies on antigen presentation by MHC molecules. Different between CTLs ($CD8^+TCR\alpha\beta^+$ T cells) and $CD4^+TCR\alpha\beta^+$ T cells is that CTLs recognize complexes of peptide antigens with MHC class I, and that $CD4^+TCR\alpha\beta^+$ T cells recognize complexes of peptide antigens with MHC class II. MHC class I is expressed by most cells and is important for presenting peptides derived from intracellular proteins, whereas MHC class II expression is mostly restricted to professional antigen presenting cells (APCs) and is important for presenting peptides from endocytosed/phagocytosed antigens (reviewed by Refs. [7,8]).

This review within the special issue "Virus Defences" will summarize common and more recent knowledge on CTL-mediated cytotoxicity in general and in fish. Attention will be given to the regulation of CTL functions, to effector mechanisms and to the role of CTLs in vaccine development.

2. Regulation of CTL activity

By suggesting a third signal for activation of naive $CD4^+$ and $CD8^+$ T cells, Curtsinger et al. (1999) [9] introduced the three signal model where an optimal activation of $CD8^+$ lymphocytes is accomplished by the following three signals: TCR engagement with peptide-MHC class I complex (signal 1), co-stimulatory signals through co-stimulatory receptors on T cells and their corresponding ligands on APCs (signal 2), and a stimulus via cytokines (signal 3) [10]. Overall speaking, the components involved in these signals appear to be conserved in teleost fish. However, while the basic immune strategy is representative of the shared ancestral situation, at a more detailed level diversification between teleost fish and mammals can be observed, for example, in differences in the number of genes per gene family [11–13]. In this section, cell surface and soluble components involved in CTL activity in teleost fish are reviewed.

2.1. Signal 1 interactions

$CD8^+$ lymphocytes utilize their TCR complexes to recognize antigenic peptide–MHC class I (pMHC-I) complexes on target cells. Orthologous genes for molecules of the TCR complex (e.g. TCR chains, CD3 subunits and CD8 co-receptors), MHC class I and relevant peptide loading pathway molecules [e.g. tapasin, proteasome subunit beta (PSMB) molecules of the immunoproteasome, and transporters associated with antigen processing 1 and 2 (TAP1 and TAP2)] have been identified in many fish species [14–17]. Although signalling cascades mediated by these molecules are largely unexplored in teleost fish, signature motifs and domains of each molecule are rather conserved in teleost fish. Therefore, it is believed that teleost $CD8^+$ lymphocytes utilize similar signalling cascades to mammalian $CD8^+$ lymphocytes. Known teleost signal 1 molecules are summarized in Table 1.

2.1.1. TCR and CD3 molecules

T cell receptors are the defining markers of T cells. T cells that express a heterodimer of TCRα and TCRβ chains represent conventional T cells that can only recognize antigens if presented by MHC molecules ("MHC-restriction") [18]. In contrast, heterodimers of TCR-γ and TCR-δ chains are expressed on γδ T cells, which can form an important mucosal tissue-resident T cell subset and recognize various structurally different ligands [19]. Recently, zebrafish (*Danio rerio*) γδ T cells have been shown to be distinct from αβ T cells by using combinations of anti-TCRα, TCRβ, TCRγ and TCRδ antibodies and RT-PCR analysis [20], and teleost fish probably possess two distinct major T cell subsets as in mammals. There have been several studies on the genetics of teleost fish TCR α, β, γ and δ genes showing/suggesting, in essence, similar genomic organization and somatic recombination as in Refs. [21–25], and for a convenient summary of zebrafish TCR sequences we refer to the International ImMunoGeneTics (IMGT) database [26]. Teleost fish can express highly diverse repertoires in TCRα and β chains, which are similar to or even more diverse than mammalian TCRα and TCRβ repertoires [15]. It has been shown that teleost fish mount strong public and private clonal responses of TCRβ upon infection with pathogens [15,27], and repertoire analysis for trout $CD8^+$ lymphocytes showed significant skewing of TCRβ CDR3 length profiles following VHSV infection, which is suggestive for clonal expansion of teleost $CD8^+$ CTLs upon pathogen infection [28].

The CD3 complex of T cells transmits and propagates TCR signals into the cytosol to subsequently activate signalling cascades [29]. Teleost fish contain three types of CD3 subunits CD3-γδ, CD3-ϵ, and CD3-ζ chains. CD3-γδ is present in non-mammalian vertebrates as the common ancestor of mammalian CD3-γ and CD3-δ [30–32]. Although the association between CD3 subunits and signal transduction through CD3 molecules remains to be reported in teleosts, amino acid sequences of the CD3 subunits show similar overall structures with conserved ITAM motifs essential for TCR signal transduction. Moreover, transcripts of the CD3 subunits in Atlantic salmon (*Salmo salar*) and halibut (*Scophthalmus maximus*) were highly expressed in thymus in association with high expression levels of TCR and CD8 genes [33–36]. Recent studies with antibodies against CD3ϵ in several fish species have revealed that $CD3^+$ cells consist of T cells, but not B cells [37–41]. Interestingly, $CD4\text{-}1^+$ and $CD8\alpha^+$ T cells from the kidney of ginbuna crucian carp (*Carassius auratus langsdorfii*) express no CD3ε proteins in contrast to the presence of $CD3\varepsilon^+$ T cells in other tissues. Thus, resting mature T cells which express no or little CD3ε might accumulate in the kidney in some fish species [41].

2.1.2. CD8 molecules

Conventional $TCR\alpha\beta^+$T cells can be subdivided into CTL versus helper/regulatory T (Treg) cells by the expression of the TCR co-receptors CD8 and CD4 (see below). CD8 molecules, as is known for CD4 molecules, stabilize the binding of TCRs to peptide-MHC complexes as well as orientate the lymphocyte-specific protein tyrosine kinase (Lck)

Table 1
Signal 1-related molecules.

Receptors	In mammals			In teleost fish			Reference
	Expressing cells	Ligands	Main Immune Functions	Orthologous genes	Observations in fish like in mammals	Unique observations in fish	
Classical MHC class I	Ubiquitous	TCRαβ	Peptide antigen presentation to TCRαβ$^+$CD8αβ$^+$ T cells.	Yes	Polymorphic, binds peptide and β2m, ubiquitous expression, linked with allograft rejection, stimulated by virus	Not linked with MHC class II, extreme allelic sequence divergence	[16]
TCRα	αβT cells	Peptide-MHC complex	Interaction with complexes of peptide plus MHC class I or II. Somatic recombination.	Yes	A large diversity of variable region of teleost TCRα and β genes. Somatic recombination. Define αβ T cells.		
TCRβ				Yes		Two or multiple constant genes in several fish species.	[14, 15]
CD3ε	Pan T cells	ZAP70 recruitment	Mediation of activation signal through TCR and regulation of TCR expression on cell surface.	Yes	Single Ig domain and ITAM motif. Expressed on T cells, but not B cells	No or little expression in kidney T cells of ginbuna carp.	[38, 39, 41]
CD3δ				Yes (CD3γ/δ)	Single Ig domain and ITAM motif.	CD3γ/δ represents common ancestor of mammalian CD3γ and CD3δ	[14]
CD3γ							
CD3ζ (CD247)				Yes	Three ITAM motifs		
CD8α	CTLs, IELs	EC: MHC class I CP: Lck	Promotes adhesion to antigen-presenting cells and infected target cells. Signals T cells.	Yes	Expressed on T cell subset, associated with CTL activity.	Cytoplasmic tail of both CD8α and CD8β possess CXH motif, instead of CXC, as Lck binding motif.	[51, 52]
CD8β	CTLs			Yes			
Lck	Pan T cells	CD8, CD4	Phosphorylate ITAMs of the cytoplasmic domains of CD3 subunits.	Yes	Expressed in T cells.	Trout Lck only binds to CD8α but not to CD8β despite a CXH motif in both CD8α and CD8β. Catfish Lck binds to CD2, CD4-1, CD4-2, but neither to CD8α nor to CD8β.	[49, 279, 280]
ZAP-70	Pan T cells	CD3 subunits	Bind to the phosphorylated CD3 chains and phosphorylate other adaptor molecules (e.g. LAT and SLP-76).	Yes	Expressed in T cells. ZAP70-mutant zebrafish show T cell immune deficiency.	Some tyrosine residues are lacking.	[281, 282]

to the TCR/CD3 complex to facilitate early signalling events [42]. Two chains of CD8 molecules, CD8α and CD8β, can form either heterodimers (CD8αβ) or homodimers (CD8αα). In humans and rodents, CD8 heterodimers are expressed exclusively by conventional MHC class I restricted CTLs while the homodimer has a more diverse expression pattern involving distinct lymphoid cells and certain myeloid cells [43]. The detailed functional differences between CD8 homo- and heterodimers are still unclear, but CD8αβ has been shown to be a more effective TCR co-receptor and to be required for an optimal positive selection of $CD8^+$ lymphocytes in the thymus [44,45].

CD8α and CD8β genes have been reported in many fish species and found to encode molecules that are structurally similar to mammalian CD8 [33,46–48]. However, the teleost Lck binding motif, as a key feature of CD8 molecules, is different from tetrapods. A bicysteine motif (CxC, with x denoting any amino acid) is present in the cytoplasmic tail of tetrapod CD8α, but there is no such Lck binding motif in CD8β [42]. Instead of the tetrapod bicysteine motif, both teleost CD8α and CD8β possess another possible Lck-binding motif CxH. Thus, it has been suggested that both CD8 chains can be involved in the translocation of Lck for TCR signalling in teleost fish. However, Hayashi et al. (2010) revealed an Lck binding to CxH motif of CD8α in a zinc-dependent manner, but not to that of CD8β [49]. Therefore, CD8α rather than CD8β is probably the main TCR co-receptor to recruit Lck molecules in teleost $CD8^+$ lymphocytes.

Although other cells besides CTLs can express CD8 molecules in fish (e.g. subpopulations of dendritic-like cells) [50], CD8 can be utilized as a main CTL marker [51]. The presence of $CD8^+$ lymphocytes in teleost fish has been demonstrated by using antibodies recognizing the corresponding species specific CD8α molecules [52], and in ginbuna crucian carp, $CD8^+$ T cells have been reported to execute antigen-specific cytotoxicity [53–57]. Likewise, rainbow trout (*Oncorhynchus mykiss*) $CD8\alpha^+$ T cells have been reported to show a CTL phenotype [28,58]. CD8β+ cells in Japanese flounder were identified as $CD3^+$ T cells [59]. Recently, grass carp (*Ctenopharyngodon idella*) CD8αα homodimers were reported to bind specifically to the pMHC-I complex [60]. However, further studies on cell types expressing CD8 homodimers and/or heterodimers are needed to dissect their roles in CTL functions in fish.

In regard to the other TCR co-receptor, CD4, two different CD4 molecules have been identified in teleost fish, CD4-1 and CD4-2 [61–63], which have been associated with helper and Treg functions [64–70]. As in most mammals, only in fish thymocytes co-expression of CD4 and CD8 molecules is a normal observation [65,68].

2.1.3. MHC class I

Classical MHC class I is the key molecule to present antigenic peptides generated through the intracellular pathway to $CD8^+$ lymphocytes [7]. The MHC class I genes in teleost fish have been categorized into five lineages thus far [16]. Of those lineages, the only one with polymorphic classical type sequences is lineage U [71,72]. Teleost fish classical MHC class I genes and molecules are expressed by similar cell types and are enhanced by similar types of stimulation as in mammals [73–75]. Furthermore, similar to mammals, teleost classical MHC class I heavy chains can form complexes with β_2-microglobulin and peptides of ~9 amino acids, which suggests a similar function in antigen presentation [76,77], and the observed allelic variation is expected to cause differences in the population of presented peptide antigens [71,77]. However, although in salmonid fish the allelic classical MHC class I variation shows extreme levels of diversification [72,78], the single classical gene locus has not been convincingly linked with differences in disease resistance [79]. On the other hand, teleost fish allelic variation in classical MHC class I was readily found linked with allograft rejection [80,81], which may help to inhibit the potential grafting of cancer (discussed in Ref. [79]). Interestingly, within the U lineage, nonclassical MHC class I (aka "Ib") genes can also be found in rainbow trout, where they are located on different chromosomes than the single classical gene [82]. In the genomic region where most of the U lineage Ib genes are situated, named the "IB region", rainbow trout individuals show variations regarding the presence/absence of intact Ib genes ("haplotype variation") [83,84]. In mammals, knockout of nonclassical MHC class I genes is known to result in the absence of certain T cell populations (e.g. Ref. [85], and an interesting observation is that the IB regions in rainbow trout and Atlantic salmon were found to be linked with resistance against viral diseases in quantitative trait loci (QTL) investigations [86,87].

CTLs can only kill virus-infected cells when they share identical MHC class I molecules (MHC restriction), but the availability of experimental MHC class I matched effector/target cell systems for evaluating CTL function is limited in teleost fish. Thus far, convenient systems have been established only in ginbuna carp and in rainbow trout where clonal fish and MHC class I-matched target cell cultures are available [88,89]. Moreover, in these species CTLs can directly be recognized due to introduction of anti-CD8 antibodies [48,51]. Another approach to utilize MHC class I matched target cells has been applied in catfish (*Ictalurus punctatus*) and grouper (*Epinephelus coioides*). Here autologous cells were used as virus-infected target cells to evaluate CMC [90,91]. For example, in grouper, a cytolytic activity of $CD8\alpha^+$ cells isolated from primary cultured fin cells of nervous necrosis virus (NNV)-infected fish against autologous NNV-infected cells was shown while NNV-infected allogeneic or red seabream iridovirus (RSIV)-infected autologous cells were not killed [91]. Thus, even in fish species where no MHC class I matched cell culture cells are available as targets, antigen-specific CMC assays can be utilized. Details on CMC mediated by CTLs are further discussed later in this review.

It has been demonstrated that antigen-specific CTLs are present in fish and that they share functional and phenotypical similarities with their mammalian counterparts [52]. MHC class I multimer reagents are powerful tools to identify antigen-specific CTLs [92] and this technique was successfully applied in virus-infected grass carp using MHC class I tetramers with virus-specific peptide [76]. Notably, using X-ray crystallography analysis, Chen et al. (2017) reported the conservation of the overall structure of classical MHC class I^+ β_2m^+ peptide (pMHC-I) complexes between grass carp and higher vertebrates [77]. However, the glutamine residue in the α3 domain, which is critical for CD8 binding in chicken and in mammals, is replaced by alternative residues in teleost fish [77]. However, grass carp CD8α molecules can still specifically bind to pMHC-I complexes [60]. Although the way of interaction between CD8 molecules and the pMHC-I complex in fish is different from that in mammals, it still may be concluded that the interaction itself is evolutionary conserved [60].

2.2. Signal 2 (co-stimulatory interaction)

Interactions between CD28 family and B7 family molecules are critical for the regulation of activation and tolerance of T cells following TCR-mediated signal transduction. In mammals, T cells can express five molecules belonging to the CD28 family at their surface: 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) [93]. APCs express binding molecules for those CD28 family members at their surface, namely B7s and B7-homologous (B7-H) family members [94]. As for the CD28 family, teleost fish possess CD28 and CTLA-4 while likely no ICOS and PD-1 [95,96]. Both CD28 and CTLA-4 are structurally conserved in teleost fish and retain specific proline-rich amino acid motifs in their V-like domains, suggesting interaction with their respective B7 ligands. Like in mammals, zebrafish CTLA-4 shows a high constitutive expression in Treg cells [64]. The cytoplasmic tail of teleost CD28 possesses a motif typical for PI3K signalling in mammals, which can induce interleukin (IL)-2 production. Moreover, Hu et al. (2012) reported that antibody ligation of CD28 from tongue sole (*Cynoglossus semilaevis*) induced significant levels of cellular proliferation [97]. Therefore teleost CD28 and CTLA-4

Table 2
Signal 2-related molecules (CD28 family).

Receptors	In mammals			In teleost fish			Reference
	Expressing cells	Ligands	Main Immune Functions	Orthologous genes	Observations in fish like in mammals	Unique observations in fish	
CD28	Most T cells	B7-1, B7-2, ICOSL (human)	Promote cellular proliferation and survival following T cell activation.	Yes	Cytoplasmic tail of trout CD28 can bind to phosphatidylinositol-3-kinase to induce IL-2 production. Antibody to tongue sole CD28 can induce lymphocyte proliferation.	The consensus SH3 PYAP motif of mammalian CD28 was not present in the fish while the overall structure of fish CD28 is similar to that of mammalian CD28.	[95, 97]
ICOS (Inducible T-cell COStimulator; CD278)	Activated T cells, Treg cells	ICOSL (B7 Homolog 2)	Enhance adaptive T cell responses.	Not identified			[96, 101]
CTLA-4 (CD152)	Activated T cells, Treg cells	B7-1, B7-2	Bind ligands with a higher affinity than CD28, suppress effector T cell responses.	Yes	*CTLA-4* expression within trout leucocytes was quickly up-regulated following PHA stimulation and virus infection.	Trout CTLA-4 has a B7 binding site, but lacks a conserved tyrosine based signalling motif.	[95]
PD-1 (CD279)	Activated T cells, Treg cells	PD-L1, PD-L2	Induction and maintenance of tolerance. Attenuate an immune response.	Not identified			[96, 101]
BTLA (CD272)	Resting γδT cells, activated αβT cells, resting B cells	TNFRSF14	Negative costimulatory receptor.	Yes	Multiple BTLA genes are present in teleost fish.	no	[96]

molecules potentially have similar functions as the respective mammalian molecules although signalling motifs are apparently missing in the cytoplasmic region of fish CTLA-4. In contrast to mammals, which possess separate B7-1 (CD80) and B7-2 (CD86) molecules, a single CD80/86 gene has been identified in teleost species and is believed to be the ancestor from which CD80 and CD86 arose by gene duplication in more evolved species [98–100]. Trout CD80/86 up-regulates the expression of IL-2 in blood leukocytes and is expressed on B cells [98]. Moreover, CD80/86 in zebrafish is essential for adaptive immune responses initiated by B cells [99]. Other members of the B7 family, B7-H1/DC, B7-H3, and B7-H4, have been identified in teleost too [101,102], and interestingly in fugu B7-H1/DC inhibits T cell proliferation in association with increment of both IL-10 and IFNγ expression while both B7-H3 and B7-H4 induced T cell growth through IL-2 induction and the suppression of IL-10 [102]. Overall, teleost fish utilize repertoires of the B7-CD28 system similar but not identical to mammals. Known teleost signal 2 molecules are summarized in Table 2.

2.3. Signal 3 regulation by cytokines

In mammals, to fully activate naïve $CD8^+$ T cells, "inflammatory warning signs" which are delivered in the form of specific cytokines induced by the infection are important [103,104]. At every stage of the response, anti-viral $CD8^+$ T cells are sensitive to cytokines, such as to inform the presence of an infection, to influence the developmental choices of the responding cells, and to support the survival and maintenance of the differentiated anti-viral $CD8^+$ T cells (reviewed by Ref. [105]). According to genomic data, teleost fish appear to be equipped with a similar cytokine network as in mammals, and at least some of these cytokines show bioactivities similar to those of their mammalian counterparts (reviewed by Refs. [11,14]). This chapter summarizes current knowledge in teleosts about homologues of several mammalian cytokines that can be considered as particularly important for CTL activity [105], as well as a few chemokines, although the knowledge on how teleost CTLs are controlled by these cytokines and chemokines is still limited.

2.3.1. IL-12 and interferon gamma (IFNγ)

IL-12 has a major effect on the level of IFNγ production by $CD8^+$ T cells [106], and IFNγ is a major cytokine produced by CTLs [107]. IFNγ is a key inducer of type 1 immunity in which cell-mediated cytotoxicity plays an important role (e.g. Refs. [11,108,109]). IFNγ is also produced by activated Th1 cells [110] and NK cells [111]. Recent studies confirmed a direct importance of IFNγ for CTLs in mammals. For example, Bhat et al. (2017) reported that autocrine production of IFNγ by CTLs enhances their motility and promotes killing of primary target keratinocytes in mammals [112].

In teleosts, genes for homologues of IL-12 subunits were identified [113], and studies were performed in some fish species at the gene expression analysis level (reviewed in Ref. [51]). More recently, recombinant proteins of IL-12 were developed in orange-spotted grouper [114], rainbow trout [115] and amberjack [116]. In these studies, recombinant IL-12 was shown to induce Th1-type immunity and protection against intracellular pathogens such as Nocardia.

Homologues of IFNγ have been identified and studied intensively in many fish species. For instance, Takizawa et al. (2011) showed that trout IFNγ is transcribed in $CD8\alpha^+$ lymphocytes as well as in $CD8\alpha^-$ lymphocytes ($CD4^+$ enriched) and that its expression was upregulated by the T-cell mitogen PHA [58]. In rainbow trout leucocytes, gene expression of IL-15 [117] was upregulated by recombinant IFNγ, suggesting its role in promoting Th1 responses. In addition, recombinant IFNγ enhances respiratory burst activities in trout macrophages [118] as well as in goldfish macrophages and neutrophils [119]. By using recombinant proteins, *in vitro* antiviral activities of IFNγ were demonstrated against crucian carp hematopoietic necrosis virus in ginbuna [120] and against infectious pancreatic necrosis virus in Atlantic

salmon [121]. In both reports, viability of virus-infected cell lines was improved by recombinant IFNγ. Taken together, these studies suggest that teleost homologues of IFNγ possess similar characteristics as mammalian IFNγ (e.g. Nagata et al., 1980 [122]), although direct effects on CTLs still need to be demonstrated. Zebrafish and rainbow trout express two IFNγ genes (*IFNγ1-1* and *IFNγ1-2*) [123,124]. In zebrafish, while IFNγ1-1 expressing cells were found to show neither granzyme nor granulysin expression, thus possibly representing an ILC1-like cell type, in a population transcriptionally resembling NK-like cells, IFNγ1-2 was co-expressed with granzymes or granulysins [125]. In rainbow trout, both genes were highly up-regulated by IHNV infection and after injection of plasmid DNA encoding the glycoprotein of IHNV [124].

Different from mammals, zebrafish [124,126], channel catfish [127], goldfish [128], common carp [129], grass carp [130], Indian major carp [131] and ginbuna crucian carp [120], possess additional, fish-specific, IFNγ homologues, namely IFNγ-related proteins (IFNγrel). IFNγ and IFNγrel are structurally different in their C-terminal region, and IFNγrel lacks a putative nuclear localization signal [128,132]. In ginbuna crucian carp, recombinant proteins of IFNγrel showed high antiviral activities similar to IFNγ [120]. In addition, administration of recombinant IFNγrel resulted in an increase of the numbers of T cells and IgM^+ cells in scale allografts and in an acceleration of allograft rejection [133]. This suggests, that teleost fish possess unique IFNγ-homologues but with similar functions as mammalian IFNγ. Since the knowledge on IFNγ-rel is still limited, studies on producing cell type and major targets should be a topic of future investigations.

2.3.2. IL-2 and IL-15

IL-2 is a growth factor for antigen-stimulated T cells and controls the clonal expansion of T-cells after antigen recognition. Activated CTLs need IL-2 as a growth factor at later stages of the immune response (reviewed by Ref. [134]). Thus, IL-2 plays a crucial role in the early phase of immune response by promoting the differentiation of naive $CD8^+$ T cells into the corresponding CTL effector cells expressing perforin, granzyme B, and IFN-γ and, later on, into memory cells [135]. IL-2 has also been demonstrated to be an essential cytokine in the formation of immune tolerance [136]. IL-2 secretion by activated T cells forms part of a self-stimulatory loop for these cells, but also provides an important negative feedback loop through the stimulation of Treg cells [137–139]. The constitutive levels of IL-2Rαβγ are highest on Treg cells and the high-affinity of this receptor complex for IL-2 allows these cells to efficiently compete with other cells for IL-2 [140]. In contrast to IL-2, IL-15 is particularly important for the stimulation of natural killer (NK) cells, intra-epithelial lymphocytes (IELs), and CTLs [141,142]. IL-15 has been shown to be superior to IL-2 in promoting the development and maintenance of memory CTLs (reviewed by Ref. [143]). For instance, IL-15 induces expansion and activation of memory CTLs and enhances their effector function in HIV-positive patients [144].

The signalling of IL-2 and IL-15 depends on how IL-2 and IL-15 interact with their “genuine” receptor IL-2Rβγ. IL-2 is expressed by activated T cells as a free cytokine, and binds directly to the IL-2Rαβγ with high-affinity or to the IL-2Rβγ with intermediate affinity [145,146]. Different from IL-2, IL-15 is predominantly expressed together with IL-15Rα by APCs [147]. Complexes of IL-15 with IL-15Rα expressed at the APC surface, or secreted, can bind to IL-2Rβγ of other cells like for example $CD8^+$ lymphocytes [148]. While monomeric IL-15 is relatively poorly secreted and unstable, heterodimers of IL-15 and IL-15Rα are similarly potent as IL-2 [142,149,150]. Co-expression of IL-15 and IL-15Rα allows for their efficient production and surface display, and also cleavage and/or secretion of bioactive IL-15/IL-15Rα heterodimers (e.g. Refs. [148,151]). Hasan et al. (2016) have shown in humans that IL-15/IL-15Rα complexes efficiently mediate proliferation of high-avidity central memory CTLs while single IL-15 molecules did not [152].

In contrast to mammals, teleosts do not possess IL-2Rα and both IL-2 and IL-15 must rely on IL-15Rα [64,153]. This suggests that teleosts possess a different control system for IL-2 and IL-15 signalling. The bioactivity of IL-2 and monomeric IL-15 has been reported in several teleost species. In rainbow trout (*Oncorhynchus mykiss*) and yellow croaker (*Larimichthys crocea*), *E. coli*-derived recombinant IL-2 increases gene expression of IFNγ and has a positive feedback on its own expression *in vitro* [154,155]. In addition, Wang et al. recently reported that recombinant trout IL-2 induced gene expression of CD8α and CD8β in peripheral blood leukocytes *in vitro* [156]. Splenocytes stimulated with recombinant trout IL-15 induced a large increase in IFNγ gene expression [117]. This effect was still apparent 24 h post-stimulation.

Since in mammals the important bioactive form of IL-15 is represented by a heterodimer with IL-15Rα (IL-15/IL-15Rα) as mentioned above, our group has tested and confirmed the impact of rainbow trout IL-15/IL-15Rα heterodimers on trout leukocytes *ex vivo* (Yamaguchi et al. preprint [157]). If the bioactivity of such heterodimers can also be proven in fish *in vivo*, IL-15/IL-15Rα could be a good candidate for novel molecular adjuvants in aquaculture.

2.3.3. IL-21

Another interesting cytokine in terms of CTL control is IL-21. Although in mammals this cytokine is not essential for CTL development, it promotes CTL function by induction of perforin, granzyme B, and IFNγ. In contrast, however, it prevents terminal differentiation of $CD8^+$ lymphocytes maintaining a less differentiated phenotype [158]. IL-21 alone could augment the frequency of antigen-specific CTLs *in vitro* and *in vivo* [158,159]. IL-21 also promotes synergistic effects with other cytokines. While IL-21 alone did not drive a large expansion of $CD8^+$ lymphocytes, a synergistic effect was recorded when applied together with IL-7 or IL-15 [160]. In addition, IL-21 can synergize with IL-7, IL-15, or IL-18 to enhance IFNγ production by $CD8^+$ lymphocytes [161].

In teleosts, recombinant trout IL-21 induces gene expression of several other cytokines including IFNγ, e.g. in head kidney leukocytes isolated from rainbow trout [162]. According to the same work, IL-21 maintained gene expression of CD8α, CD8β, and IgM at a later stage of *in vitro* stimulation. Intraperitoneal injection of recombinant IL-21 into rainbow trout increased gene expression of IFNγ, IL-10, IL-21, IL-22, CD8, and IgM [162]. Since the synergistic effect of IL-21 with other cytokines has not been reported so far in teleosts, this point should be studied in the near future.

2.3.4. IL-10

Cytokine networks not only promote T cell responses following infection but can also suppress their magnitude and quality. IL-10 is the first identified inhibitory cytokine and was first referred to as “cytokine synthesis inhibitory factor” [163]. IL-10 signalling to helper and cytotoxic T cells tends to reduce their proliferation and effector activity, including the production of effector cytokines such as IFNγ. IL-10 can also indirectly inhibit T cell responses by decreasing the antigen presenting capacity of macrophages and DCs and reducing their expression of pro-inflammatory cytokines which amplify effector responses [164,165]. In contrast, although IL-10 generally inhibits inflammatory reactions of the immune system, stimulatory roles for IL-10 were also described [166]. In addition, it was also demonstrated that IL-10 in combination with IL-2 increases the proliferation and the cytotoxic activity of CTLs [167].

As for teleosts, recombinant proteins of IL-10 were produced in several fish species, including cyprinids, rock bream (*Oplegnathus fasciatus*) and amberjack (*Seriola dumerili*) [168–173]. Recombinant goldfish (*Carassius auratus*) IL-10 downregulates IFNγ gene expression in splenocytes [168]. In common carp (*Cyprinus carpio*), IL-10 did not affect survival and proliferation of T cells from naive animals. However, it promoted survival and proliferation of T cells from immunized animals, when used in combination with the immunizing antigen *in vitro* [169]. In addition, gene expression analysis suggests that carp IL-10 stimulates a subset of $CD8^+$ memory T cells, while downregulating

Table 3
Effector genes/molecules of CTL responses published in teleosts.

Effector	Gene	Protein	Species	Associated cell type	Function	Ref.
Perforin	x	-	Carp	n.t.	n.t.	[283]
Perforin	x	-	Channel catfish	NCC	n.t.	[284]
Perforin	x	x	Japanese flounder	n.t.	Ca-depend. haemolysis	[285]
Perforin	x	-	Rainbow trout	n.t.	n.t.	[286]
Perforin	x	-	Rainbow trout	$CD8a^+$ lymphocytes	n.t.	[58]
Perforin	x	x	Zebrafish	Several	Protection in virus inf.	[287]
Granulysin	x	-	Channel catfish	NCC	n.t.	[284]
Granulysin	x	x (peptide)	Japanese flounder		antimicrobial activity	[288]
Granulysin	x	-	Nile tilapia	n.t.	n.t.	[289]
Granulysin	x	x (peptide)	large yellow croaker	n.t.	antimicrobial activity	[290]
Granulysin (various)	x	x	Common carp	n.t.	antimicrobial activity	[291]
Granulysin (various)	x	-	Zebrafish	n.t.	n.t.	[292]
Granulysin	x	x (peptide)	Tongue sole	n.t.	antimicrobial activity	[293,294]
Granulysin	x	x (peptide)	Mudskipper	n.t.	antimicrobial activity	[295]
Granulysin	x	x (peptide)	Turbot	n.t.	antiparasitic activity antiviral activity	[296,297]
Granzyme-like (various)	x	x	Channel catfish	NCC	Protease activity	[284]
Granzyme-1	x	x	Nile tilapia	NCC	Esterolytic activity	[298]
Granzyme-like	x	-	Channel catfish	NCC	n.t.	[299]
Granzyme (various)	x	-	Cod	n.t.	n.t.	[300]
Granzyme-like	x	-	Several	n.t.	n.t.	[300]
Granzyme A/K	x	-	Carp	n.t.	n.t.	[301]
Granzyme-B like	x	x	Ginbuna crucian carp	$CD8a^+$ lymphocytes	Hydrolytic activity	[210]
Granzyme-B like	x	x	Ginbuna crucian carp	$CD8a^+$ lymphocytes	No enzyme activity	[302]
Granzyme-A like	x	x	Ginbuna crucian carp	$CD8a^+$ lymphocytes	Hydrolytic activity	[303]
Granzyme-like I	x	x	Channel catfish	NK-like cells	Caspase 6 activation	[304]
FasL	-	x	Channel catfish, Nile tilapia	NCC	n.t.	[305]
FasL-like	x	-	Gilthead seabream	Leukocytes	n.t.	[306]
FasL-like	-	x	Channel catfish	Lymphoid and fibroblast cell lines	n.t.	[307]
FasL	x	-	Japanese flounder	T-like lymphocytes	n.t.	[308]
FasL	x	x	Nile tilapia	n.t.	Apoptosis induction	[309]
FasL	x	-	Grass carp	n.t.	n.t.	[310]

n.t. = not tested.

$CD4^+$ memory T cells. In amberjack, recombinant IL-10 can likely promote Th2-dominant immunity while inhibiting cell-mediated immunity [170].

2.3.5. Chemokines

Chemokines promote leukocyte mobilization in response to a pathogenic exposure thereby regulating immune responses. They also control the differentiation of recruited immune cells to orchestrate the first steps of both innate and acquired immune responses [174–176]. So far, numerous chemokine genes have been identified in diverse fish species, but only few functional studies related to CTLs have been reported. For example, a homologue to mammalian CCL4 was reported in orange-spotted grouper (*Epinephelus coioides*) [177], and recombinant CCL4 was found to have chemotactic activity for peripheral blood leukocytes, as well as the capacity to up-regulate the transcription of TNF-α1, TNF-α2, IFN-γ, Mx, T-bet and both CD8α and CD8β, suggesting that CCL4 might induce an inflammatory response by skewing lymphocyte differentiation into Th1 direction finally resulting in an activation of cytotoxic cells.

Another chemokine, CCL19, known to control lymphocyte migration and compartmentalization of lymphoid tissues in mammals has been studied in rainbow trout. Its trout homologue CK12 induces a significant chemotactic activity in splenocytes, but not in head kidney or blood cells [178]. In contrast, Sepahi et al. (2017) produced recombinant proteins of trout CK12a (rCK12a) and reported that rCK12a is not chemotactic *in vitro*, but it increases the width of the nasal lamina propria when delivered intranasally [179]. Moreover, rCK12a delivered intranasally or i.p. stimulates the expression of CD8α, granulysin, and IFN-γ in mucosal and systemic compartments and increases the numbers of nasal $CD8\alpha^+$ cell in rainbow trout.

2.4. Transcription factors

Cytokines and chemokines induce signals to cells through downstream signalling pathways that consist of numerous elements including transcription factors. Among them, T-bet and eomesodermin (Eomes) are known to be master regulators of cytotoxic lymphocyte lineages in mammals both required for normal CTL effector differentiation [180–182]. For example, experiments with ectopic expression and loss of function by dominant negative mutation suggest that Eomes induces IFN-γ and cytolytic molecules, such as perforin and granzyme, in cooperation with T-bet [183].

In teleosts, *T-bet* has been identified in many fish species and gene expression analysis has been performed in various studies as the indicator of CTL activation. For example, carp *T-bet* expression was induced together with *IFN*-γ expression after grass carp reovirus infection [184]. In Atlantic salmon, expression of *T-bet* is upregulated by infection with *A. salmonicida* [185]. In ginbuna, strong expression of *T-bet* was observed in T cell subsets [186,187], and after low-dose *E. tarda* infection the expression levels of *IFN*-γ and *T-bet* in vaccinated fish were significantly upregulated [188]. In a ginbuna allogeneic graft transplantation model, expression of *T-bet* (along with *perforin* and *granzyme*) was upregulated in allografts and their surrounding tissues [189]. Eomes has also been cloned in zebrafish [190], Atlantic salmon [191], ginbuna and rainbow trout [187]. Among the various lymphocytes populations such as $CD8\alpha^+$, $CD8\alpha^-$ and IgM^+ leukocytes in rainbow trout, highest expression of Eomes was recorded in $CD8\alpha^+$ cells.

2.5. Effector molecules in cell mediated cytotoxicity

There are several mechanisms by which mammalian CTLs kill their targets.

CTLs program target cells to undergo apoptosis and the principle mechanism is based on a calcium-dependent release of the contents of lytic granules containing cytotoxic proteins. Perforin is one of these proteins. It polymerizes to form transmembrane pores in the membrane of target cells. Granzymes are another class of cytotoxic proteins and belong to the family of serine proteases.

Another granula-independent mechanism is based on the binding of Fas on the membrane of target cell by the Fas ligand present in the membranes of activated CTLs. Interaction between these two molecules leads to the activation of caspases in the target cells and finally in apoptosis (reviewed by Ref. [8]). In addition to proteases, both mammalian NK cells and CTLs store in their cytolytic granules granulysin or its orthologue NK-lysin. Granulysin, in combination with perforin contributes to killing of intracellular pathogens such as *M. tuberculosis* by human CTLs [192] and also porcine NK-lysin has been suggested to contribute to direct killing of bacteria by CTLs [193]. In teleost fish all genes encoding the above listed effector molecules have been found (see Table 3). However, perforin and granzyme genes in teleost fish are diversified because of gene duplication events during evolution and are present as multiple genes [194–196]. This also concerns NK-lysin/granulysin in several fish species (see Table 3). In rainbow trout, $CD8^+$ lymphocytes express both *perforin* and *granulysin* [58]. The functional differences among the respective genes still remain to be elucidated.

3. Cell-mediated cytotoxicity

3.1. CTL functions

Although this chapter reviews our current knowledge on antiviral CMC in teleost fish, it needs to be mentioned that the field of CMC has been pioneered by basic investigations using methods of transplantation immunology. In retrospective, this approach was not surprising since central molecules in CMC, namely MHC molecules, were not discovered by scientists working in the field of infectious diseases, but in transplantation medicine. In the seventies of the last century, the so called histocompatibility ("tissue matched") antigens have been discovered as one of the main players in graft rejection [197], for which Dausset together with Snell and Benacerraf were awarded the Nobel prize in physiology in 1980 [198]. Based on these fundamental discoveries it were Zinkernagel and Doherty (1974) who found another, even more important physiological role of MHC molecules namely in the CTL-mediated cytotoxicity against viruses for which they received the Nobel prize in 1996 [199]. Thus, studies on CTL-mediated cytotoxicity in teleosts, were also initiated by investigations in allospecific rejection [200] cumulating in the establishment of TCR αβ+ allospecific cytotoxic cells, TCR αβ+ nonspecific cytotoxic cells, allospecific TCR αβ+ noncytotoxic cells, TCR αβ− nonspecific cytotoxic cells, and TCR αβ− allospecific cytotoxic cells [201]. Not much later investigators from the same group of the University of Mississippi Medical Center, Jackson, USA were the first to show antiviral CMC in a channel catfish model (*Ictalurus punctatus*) [90]. However, due to the lack of MHC class I matched effector and target cell systems the nature of antiviral cytotoxic cells has not finally been shown so far in this fish species. Also in ginbuna crucian carp, CTL activities were first suggested by allospecific CMC [202], and such activities were later also found for rainbow trout [203].

Since antiviral CTL activity is MHC class I restricted, corresponding investigations became only possible with the use of clonal fish, either naturally occurring (e.g. ginbuna crucian carp) or artificially produced (e.g. rainbow trout), and the availability of MHC class I matched cell lines to the respective clonal fish. Strong suggestions for MHC class I restricted CMC of CTLs against virus-infected cells has been shown first in a system of clonal ginbunas and syngeneic target cells (derived from the respective clone) infected with carp hematopoietic necrosis virus (CHNV) [88,204–207] (see Table 4). While virus infected syngeneic cells were killed by effector cells isolated from infected fish, infected allogeneic (MHC class I mismatched) target cells were not.

More evidence on the existence of bona-fide CTLs in fish has been concluded from experiments in which effector cells were characterized on their expression profiles. Viral antigen-driven expansion of putative ginbuna CTLs (expressing TCRβ and CD8α mRNA) by putative CHNV-infected antigen presenting cells has been shown in ginbuna crucian carp [208]. Moreover, in the same species $CD8\alpha^+$ cells purified from CHNV-infected fish were identified to induce virus-specific cytolysis indicating CTLs as anti-viral effector cells [54]. However, cells other than CTLs ($CD8\alpha^-$ cells) were shown to be the dominant effectors in the same experiments suggesting that natural killer (NK)-like cells and monocytes are the main effector cells. Nevertheless, adoptive transfer of $CD8\alpha^+$ lymphocytes, isolated from CHNV-infected donors to naïve syngenic recipients resulted in protection of the latter from virus challenge further underlining the role of fish CTLs in antiviral immunity [54,206]. Most likely ginbuna $CD8\alpha^+$ cells kill virus-infected target cells by utilising perforin and granzyme since this was found to be the mechanism by which allospecific cytotoxic cells eliminated their targets [209,210].

Another MHC class I system of effector and target cells has been developed in rainbow trout. For this system, fish have been cloned in the Nagano Prefectural Experimental Station of Fisheries, Akashina, Nagano, Japan by gynogenesis over two generations by suppression of mitosis and meiosis in the first and second generations, respectively. The homozygous clone expresses the MHC class I allele Onmy-UBA*15010 which is also expressed in RTG (rainbow trout gonade)-2 cells a cell line that is widely used in fish virology. CMC assays for virus-specific CTL activities have been shown against MHC class I matched hematopoietic necrosis (IHNV)- and viral haemorrhagic septicaemia virus (VHSV)-infected RTG-2 cells using effector cells from virus infected fish, respectively [211,212].

These studies on the functional importance of CTLs in fish antiviral defence have further been supported by the regulation of CD8 expression during virus infection (see Table 4). However, in some cases CD8 expression also stayed at the level of naïve fish [213–216] or was even downregulated as shown by Kumari et al. (2013) after infectious pancreatic necrosis virus (IPNV) infection in rainbow trout [191], after nodavirus infection in Atlantic halibut [35] and after infectious salmon anaemia virus (ISAV) infection in Atlantic Salmon [217,218]. However, loss of regulation or even downregulation of CD8 might be a secondary effect after inhibition of the MHC class I pathway, a strategy by which certain mammalian viruses, such as herpesviruses (reviewed by Ref. [219]), cow pox virus and human immunodeficiency virus (reviewed by Ref. [220]) escape from being recognized and eliminated by the immune system. As a result of this immune evasion mechanism $CD8^+$ lymphocytes cannot be triggered anymore by MHC class I expressed viral peptides. Other more direct mechanisms such as infection of $CD8^+$ lymphocytes followed by virus-induced cell death might also be a possible scenario. Hetland et al. (2011) suggested that an increase of type I interferon expression at 6 days after ISAV infection [221] could have resulted in an attrition of $CD8^+$ T cells since similar observations have been made regarding memory T cells in viral infections of mice [222–224].

There is also certain evidence on antiviral CTL memory in fish, since effector cells isolated from re-infected ginbuna crucian carp showed an even stronger killing activity against syngeneic cells infected with the respective homologous viruses IPNV [88], or CHNV [206], when compared to the killing activity against cells infected with a heterologous virus (eel virus from America - EVA). Further evidence on secondary CTL responses was shown by adoptive transfer experiments with $CD4^+$ and $CD4^-$ cells isolated from CHNV-infected ginbuna [66]. When transferred to naïve syngeneic ginbuna both subpopulations induced a cell-mediated antiviral immune response in the recipients. Recently, it has been shown that the fish rhabdovirus IHNV can interact with olfactory sensory neurons in the nasal cavity of rainbow trout resulting in a recruitment of $CD8^+$ T cells to the olfactory mucosa [222–225].

Table 4
Published studies on the generation of virus-specific CTL in fish.

Fish species	Virus	Immunogen	Route of infection	Ref.
Induction of virus-specific CTL activity				
Ginbuna crucian carp (*Carassius auratus langsdorfii*)	CHNV (rhabdovirus)	Live virus	i.p.	[54,57,66,206–208]
	EVA (eel rhabdovirus), IPNV (birnavirus)	Infected syngeneic cells	i.v.	[88]
Rainbow trout (*Oncorhynchus mykiss*)	VHSV (rhabdovirus)	Live virus	i.p./anal	[212]
	IHNV (rhabdovirus)	Peptide/live virus	i.p.	[247]
Orange-spotted grouper (*Epinephelus coioides*)	NNV (nodavirus)	Live virus	i.m.	[91]
Antigen specific CTL bind MHC class I bound viral peptides				
Rainbow trout	IHNV (Rhabdovirus)	Peptide/live virus	i.p.	[247]
Grass carp	GCHV (reoviruses)	Peptide/attenuated virus		[76]
Regulation of CD8 mRNA expression in viral infection				
Ginbuna crucian carp	CHNV (rhabdovirus)	Live virus	i.p.	[207]
Common carp (*Cyprinus carpio*)	KHV (herpesvirus)	Live virus	Bath	[311]
	SVCV (rhabdovirus)	Live virus	Bath	[312]
Atlantic halibut (*Hippoglossus hippoglossus*)	Nodavirus	Live virus	i.m.	[35]
			i.p.	[313]
Olive flounder (*Paralichthys olivaceus*)	VHSV (rhabdovirus)	Live virus	i.p.	[314]
Rainbow trout	VHSV (rhabdovirus)	Live virus	i.p./anal	[212]
	VHSV (rhabdovirus)	Live virus	Bath	[214]
	VHSV (rhabdovirus)	Live virus	Bath	[28]
Atlantic salmon (*Salmo salar*)	SAV (alphavirus)	Live virus	Cohab./bath	[315]
			Cohab./bath	[316]
	ASRV (reovirus)	Live virus	i.m.	[317]
	IPNV (birnavirus)	Live virus	Bath i.p. and cohab./bath	[318] [319]
	ISAV (orthomyxovirus)	Live virus	i.m.	[215]
	IPNV (birnavirus)	Live virus	Bath cohab.	[191]
Orange-spotted grouper	NNV (nodavirus)	Live virus	i.m.	[91]
Rock bream (*Oplegnathus fasciatus*)	Rock bream iridovirus (RBIV)	Live virus	i.p.	[216]
Induction of virus-specific CTL activity				
Ginbuna crucian carp	CHNV (rhabdovirus)	Formalin inactivated virus	Oral	[57,230]
Rainbow trout	VHSV (rhabdovirus)	DNA vaccine	i.m.	[236]
	IHNV (rhabdovirus)	Peptide, live virus	i.p.	[247]
Antigen specific CTL bind MHC class I bound viral peptides				
Grass carp (*Cyenopharyngodon idellus*)	Grass carp reovirus (GCRV)	Live attenuated virus	i.p.	[76]
Rainbow trout	IHNV (Rhabdovirus)	Peptide/Live virus i.p.	i.p.	[247]
Regulation of CD8 mRNA expression in vaccination				
Grass carp	GCHV (reoviruses)	DNA vaccine (ORF S6)	i.m.	[320]
Rainbow trout	VHSV (rhabdovirus)	Live attenuated virus	Oral	[321]
	VHSV (rhabdovirus)	DNA vaccine (G, M)	i.m.	[236]
	IPNV (birnavirus)	DNA vaccine (VP2)	i.m.	[322]
	IHNV (rhabdovirus)	Yeast G display vaccine	Oral	[323]
	IHNV (rhabdovirus)	DNA vaccine G (IRF1A promotor)	Oral	[324]
	IHNV (rhabdovirus)	DNA vaccine G	i.m.	[237]
	IPNV (birnavirus)	DNA vaccine (VP2)	Oral	[325]
	VHSV (rhabdovirus)	DNA vaccine (G)	i.m.	[326]
	IPNV (birnavirus)	DNA vaccine (PP-polyprotein)	i.m.	[238]
Atlantic salmon	IPNV (birnavirus)	Inactivated whole virus	i.p. vacc./cohab. inf.	[233]

3.2. CTL responses to vaccination

Most vaccines are still being developed by the trial-and-error method and many have limited efficiencies, while the reasons for this remain unclear. Due to the fact that viruses are expected to provoke MHC class I-restricted antigen presentation and consequently CTL responses, it might be concluded that antiviral vaccines should induce strong cell-mediated immune responses to be effective. However, this assumption cannot explain the protective mechanisms for all anti-viral vaccines. For example, in humans, protection against Japanese encephalitis virus (JEV), human papilloma virus (HPV), Hepatitis B virus (HBV), Hepatitis A virus (HAV) (reviewed by Ref. [226]), and against rabies virus (RABV) infection correlates with virus-specific serum antibody levels [227]. Also in salmonids, IgM levels were suggested to be the predominant factor in the protection against IPNV [228]. Adoptive transfer of sera from immunized to naïve fish can protect the latter from homologous viral challenge further suggesting the protective effect of antibodies in teleosts (reviewed by Ref. [229]). Thus, one has to bear in mind that specific protection against infection can be based either on humoral or cell-mediated mechanisms or a combination of both. Further research is needed to uncover the

relevance and interrelationship of these mechanisms.

Mucosal vaccines, either delivered by bath or orally, are the preferable type of vaccine in aquaculture because they require fewer work forces and do not cause stress to vaccinated fish when compared to injection vaccines. Many experimental mucosal vaccines do confer protection, but the contribution of CTLs in that protection is a matter of speculation.

Most vaccines for which CTL responses (either functionally or indirectly by CD8 regulation) are reported were DNA vaccines or attenuated live virus preparations (see Table 4). Only one vaccine (against CHNV) was an inactivated vaccine [57,230]. Although administered into the intestinal bulb where antigens are easily degraded, the latter vaccine surprisingly provoked protection and both the generation of CTLs and antibodies. Possibly, the inactivated vaccine was not only administered into the intestinal bulb but also a bit farther which may have prevented degradation of the viral antigens. CTL activity has also been provoked against allogeneic targets by anal or oral administration of alloantigens [231,232]. The latter work might give the vaccinologist a lesson in terms of mucosal tolerance since repeated anal intubation with allogeneic cells resulted in decreased allospecific cytotoxic activity and immunological tolerance has been suggested as one of the obstacles in mucosal vaccination.

In a vaccination trial with inactivated viral vaccines against IPN in Atlantic salmon high antibody levels corresponded with the reduction of post-challenge mortality while CTL signature gene expressions of Eomes, CD8α and granzyme A inversely correlated with viral loads in challenged fish [233]. This suggests that in certain virus diseases, the antibody levels might be more decisive in disease protection after vaccination than CTLs, and that CTL responses seem to occur only when virus replication cannot be kept under control by humoral responses.

3.2.1. DNA vaccines

The success of antiviral DNA vaccines is not surprizing since the plasmid DNA induces intracellular transcription and finally translation into antigenic proteins thus mimicking an (intracellular) viral infection. There are many examples for excellent DNA vaccines in fish (reviewed by Ref. [234]) and recently, a DNA vaccine against Salmon Pancreas Disease (SPD) has been authorized by the European Medicines Agency (EMA) (http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2016/04/news_detail_002516.jsp). Otherwise, DNA vaccines that have been proven to confer good protection when delivered by i.m. injection did often not work when given orally although antigen production was confirmed in the intestinal mucosa (e.g. Ref. [235]).

DNA vaccination for which virus-specific CTLs were functionally identified have been generated against MHC class I matched viral haemorrhagic septicaemia virus (VHSV)-infected RTG-2 cells after DNA vaccination of rainbow trout against the VHSV glycoprotein [236]. In the same work, antigen specificity of immune cells has further been supported by the observation that inflammatory cells isolated from the DNA vaccine injection site showed antigen-specific homing to the homologous injection site.

In most reports on antiviral DNA vaccines in fish, CD8 was found to be upregulated. However, expression of CD8 genes seems to be differentially regulated in different organs. While *CD8* expression was increased at the injection site of a DNA vaccine against IHNV, similarly there were corresponding decreased mRNA levels in the anterior kidney [237]. This might be due to efflux of $CD8^+$ lymphocytes from the anterior kidney and influx to the site of DNA injection (and presumably antigen presentation). Generally speaking, DNA vaccines against rhabdoviral glycoproteins seem to provoke a stronger CTL response than DNA vaccines against the IPNV polyprotein [238]. Although CD8 is probably a first-choice marker to predict or confirm CTL responses it cannot be used as the only marker to characterize CTL based vaccine efficiency. Patterns of surrogate markers for vaccine efficiency are complex and sometimes hard to interpret [239]. DNA vaccines do not only provoke immediate antigen processing in the proteasome followed by peptide formation and presentation through MHC class I to $CD8^+$ lymphocytes [240].

After transcription and translation of the coding DNA the respective immunogenic antigens are also released to the extracellular space or displayed at the surface of the corresponding cells. Cell surface antigens can be recognized as a PAMP or by antigen-specific antibodies. This in turn may induce killing of antigen-producing cells by NK cells either through recognition of PAMPs by PRRs or by the interaction of opsonizing antibodies with the Fc receptors on NK cells. APCs such as macrophages and DCs are responsible for the uptake of killed cells and foreign antigens. Since macrophages also display Fc receptors on their surface uptake of antigens and infected cells can be supported by their opsonisation with antibodies. Antigen uptake and processing by APCs is usually followed by antigen degradation and peptide presentation through MHC class II thus provoking T helper cell responses. However, part of the peptides from extracellular origin may also be cross-presented through MHC class I and especially $CD8\alpha^+$ DCs are considered to be very potent cross-presenting APCs in mammals [241]. In fish, mucosal DC-like cells with cross-presenting capacities have been suggested by Granja et al. (2015) and Soleto et al. (2018) [50,242].

3.2.2. Peptide vaccines

Virus infections result in presentation of viral peptides through MHC class I. Information on the amino acid sequences of these peptides offer the possibility to create peptide vaccines. In this approach, chemically synthetized peptides are injected into the organism where they are loaded onto the MHC class I molecules on the surface of nucleated cells. Like in a viral infection this will then provoke binding of antigen-(peptide-) specific CTLs and induction of cell-mediated immune responses. However, there are a number of problems to be solved when developing such vaccines. Due to individual differences in protein processing and presentation in the MHC class I pathway, peptides are somewhat MHC class I allele-specific, meaning that peptides loaded into the peptide binding grove of MHC class I of one individual might not be readily loaded into the corresponding grove of another individual.

However, for a given MHC allele, the bound peptides tend to have some features in common, namely their length and anchor amino acid properties. While MHC class I peptides typically have a length of between 8 and 10 amino acids (aa) MHC class II peptides are between 13 and 25 aa long [243]. One of the early approaches to get information on such peptides is their elution from virus infected cells where they are displayed in the peptide-binding grove of MHC class I followed by their amino acid sequence analysis using mass spectrometry [244]. Peptides can also be predicted *in silico* based on knowledge accumulated on peptide-binding properties of MHC class I alleles. Multiple tools have been developed for the prediction of MHC class I bound peptides in humans. A pan-specific predictor of peptide–MHC-I binding, NetMHCpan, has been finally created to cover the binding specificities of all known human leukocyte antigen (HLA)-I molecules [245]. Later, this database was successfully used to predict MHC class I peptides in other species such as pigs [246]. We have used the latter method to predict peptides from the IHNV glycoprotein loaded into MHC class I of the homozygous clonal rainbow trout C25 expressing the MHC class I allele Onmy-UBA*15010. Peptides with highest scores were able to stabilize recombinant MHC class I and β2m molecules. PBL from rainbow trout injected with one of these peptides and further challenged with a sublethal dosage of IHNV showed a more than three times higher killing activity against IHNV infected MHC class I matched target cells than against IHNV infected MHC class I mismatched target cells [247]. However, further research is needed to show if a single peptide can protect fish from a lethal infection and on which mechanism this protection is based.

Using the ability of properly predicted peptides to stabilize recombinant MHC class I and β2m molecules tools for the recognition of

antigen specific CTLs can be produced [248]. Under natural conditions, cytotoxic T cells specifically recognize MHC class I bound peptides on the surface of virus-infected cells by their TCR resulting in clonal expansion of antigen (peptide)-specific CTLs. We have produced tetramers of refolded recombinant β2m and MHC class I proteins with *in silico* predicted IHNV glycoprotein peptides conjugated with a fluorescent dye. Rainbow trout of the clone C25 have been injected with a high score peptide and boostered with a sublethal IHNV injection. Flow cytometry analysis of leukocytes from these fish revealed a population of lymphocytes that were double positive for the CTL marker CD8 and tetramers of refolded b2m/MHC class/peptide complexes [247]. Together with a similar work of Chen et al. (2010) on grass carp haemorrhagic virus (GCHV) infection those are the only indications that fish possess pMHC-I specific CTLs [76].

3.2.3. Adjuvants that enhance CTL activity

Modern adjuvants are designed to specifically increase the ability of vaccines to trigger those immune responses that are known to be protective in certain infections. There are a number of adjuvants that are supposed to induce CTL responses in mammals. $CD8^+$ lymphocytes can directly or indirectly be triggered by adjuvants. Perez et al. (2013) described indirect induction of CTL responses through stimulation of Th1 cells using the Finlay Adjuvants (AFs) AFPL1 (proteoliposome) [249]. APPL1 is an outer membrane vesicle of N. meningitidis serogroup B and was shown to stimulate antigen-specific $CD4^+$ and $CD8^+$ T cells. Direct stimulation of antigen-specific $CD8^+$ T cells with memory phenotype was shown using an adjuvant based on the pore-forming protein sticholysin II encapsulated into liposomes [250]. The invariant NKT cell agonist α-Galactosylceramide (αGalCer) has been used to enhance the immunogenicity of whole-protein antigens by stimulating robust antigen-specific CTL (and also antibody) responses in mice (reviewed by Ref. [251]). This was described to be initiated by an uptake of protein antigens and αGalCer by professional APCs followed by processing into short antigen peptides and presentation by MHC class I molecules for recognition via the TCR of antigen-specific CTLs and the presentation of lipids by the MHC class I-like CD1d molecules to the TCR of NKT cells, respectively. As a result, activated NKT cells produce cytokine and chemokines, thereby trans-activating DCs. Both activated DCs and NKT cells then stimulate proliferation and differentiation of naïve antigen-specific CTL precursors into effector and memory CTLs [251].

Traditional oil adjuvants such as Freund's adjuvant were developed to stimulate antibody based immune responses [252]. However, emulsions of Montanide ISA 50V (incomplete Freund's adjuvant) and Montanide 720 (metabolizable oil with a mannide mono-oleate emulsifier) were shown to induce good CTL and antibody responses in a variety of animal species (reviewed by Ref. [253]). Strong CTL (but also antibody) responses can be induced by ISCOMs (immune stimulating complexes), open cage-like structure of about 40 nm diameter which are formed by interaction of saponins with cholesterol and phospholipids (reviewed by Ref. [254]). Further CTLs have been reported to be induced by Abisco-100 (http://www.isconova.com/products/abisco-research-reagent/abisco-100.aspx)], SPT, Loxoribine, QS-21 (reviewed by Ref. [255]) and CpG DNA [256]. Hioe et al. (1996) have tested different adjuvants on their capacity to support CTL responses to a synthetic peptide representing an MHC class I-restricted *Plasmodium berghei* epitope in mice [257]. When the vaccine was applied s.c. both a lipopeptide and a Montanide ISA 720 formulation elicited CTL responses. However, only the lipopeptide effectively primed for CTLs by the i.p. route of delivery. The latter shows the influence of administration routes on the outcome of immunization. In addition, only the lipopeptide induced memory CTL responses. A database on vaccine adjuvants can be found under http://www.violinet.org/vaxjo/index.php.

A smart approach to support certain immune responses is the use of molecular adjuvants. Molecular adjuvants are stimulatory molecules encoded by a plasmid DNA vector. DNA encoding the secreted protein Ag85B of *Mycobacterium tuberculosis* mixed with a DNA vaccine encoding the HIV gp120 has been shown to induce strong CTL responses through activation of Th1 pathways [258]. In an approach to enhance CTL reactivity, an HIV-1 DNA vaccine was co-injected together with expression vectors encoding CD80 and CD86 resulting in an increase of MHC class I-restricted CTL responses in mice and primates [259].

Other molecular adjuvants with the potential of supporting CTL activities are those encoding the inflammation signalling proteins HSP70 and HMGB1; the transcription factors IRF-1; IRF-3 and IRF-7; as well as the immune regulator of programmed cell death protein 1 (PD-1) (reviewed by Ref. [260]). The transcription factor IRF-1 has also been used as a molecular adjuvant in fish. When applied together with a DNA vaccine encoding the major capsid protein (MCP) gene of red seabream iridovirus (RSIV) antibody titres were only slightly elevated compared to single MCP DNA vaccination, while CD8 or CTL responses were not investigated [261].

To increase vaccine efficiency synergistic approaches to use molecular adjuvants have been applied. It is known in mammals that targeting of antigens on dendritic cells via the DEC205 receptor enhances the presentation of antigenic peptides on MHC class I [262]. While $CD8^+$ T-cell responses to a DEC205 receptor targeted DNA vaccine were only slightly enhanced when separately adding expression plasmids encoding the TLR-9 ligand CpG, the TLR-3 ligand Poly I:C or CD40, a combination of the three molecular adjuvants significantly improved $CD8^+$ T-cell activities [263]. Also in Atlantic salmon, three plasmids encoding three Atlantic salmon type I IFNs (IFNa1, IFNb or IFNc) did only confer enhanced protection against ISAV-mediated mortality when delivered at the same time together with a DNA vaccine encoding the ISAV hemagglutinin esterase [264]. In the same work, elevated mRNA expression levels for IgM and CD8 genes were recorded at the injection site suggesting the influx of B cells and CTLs. An influx of CTLs was further supported by the recorded *perforin 1-2* and *Granzyme A* expressions.

In higher vertebrates, IL-15 has been reported as a potential adjuvant to enhance both cellular and/or humoral immune responses against infectious pathogens using different methods of delivery (reviewed by Ref. [265]). In mice, IL-15 was found to induce long-lived, antigen-specific $CD8^+$ T cells when delivered together with a HIV gp160 vector vaccine, as well as a strong and long-lasting antibody response [266]. It also provoked long-lived antigen-specific memory $CD4^+$ and $CD8^+$ T cells in rhesus macaques vaccinated with tetanus toxoid or influenza vaccines [267] and in chronically Simian immunodeficiency virus (SIV) infected macaques booster-vaccinated against the corresponding disease [268]. When IL-15 was combined with a DNA-vaccine against *Brucella abortus* a protective $CD8^+$ T cell response, but also a humoral response has been reported in mice [269]. Also in mice, co-administration of a plasmid DNA encoding IL-15 improved long-term protection of a vaccine against *Trypanosoma cruzi* by inducing antigen-specific $CD8^+$ memory T cells [270]. Using vaccinia virus vectors encoding IL-15 as a molecular adjuvant, Merkel et al. (2010) have induced long-lasting humoral and T lymphocyte immune responses against tuberculosis, malaria, H5N1 avian influenza, and in a dual vaccine against smallpox and anthrax [271]. When used as a molecular adjuvant, IL-15 provoked an increase in the numbers of blood $CD8^+$ lymphocytes in chicken vaccinated against avian influenza [272], in pigs vaccinated against porcine circovirus type 2 (PCV2) [273], and in mice immunized against porcine reproductive and respiratory syndrome virus (PRRSV) [274]. A recombinant PRRSV vaccine expressing membrane-bound IL-15 as an adjuvant was found to enhance NK and γδ T cell responses in the porcine lung showing the potential of IL-15 to also induce effective mucosal responses [275]. The power of IL-15 as a molecular adjuvant has also been shown in a trial with a multivalent vaccinia vector-based H5N1 influenza vaccine where protective immunity was achieved independently from neutralizing antibodies [276]. In a trial with rhesus macaques displaying chronic

Simian/Human Immunodeficiency Virus (SHIV) viremia, treatment with native heterodimeric IL-15 revealed increased numbers of granzyme B^+ T cells in lymph nodes as well as decreased virus levels in blood plasma and in lymph nodes suggesting that IL-15 can help to uncover and eliminate persistent virus reservoirs [277].

Cao et al. (2017) have injected rainbow trout with a DNA vaccine encoding the IHNV glycoprotein G together with plasmid DNAs encoding seven rainbow trout cytokine genes: IL-2, IL-8, IL-15, IL-17, IL-1β, intracellular interferon (iIFN) 1a or IFN-γ2, respectively [278]. The efficiency of induction for the IL-15 plasmid of immune responses regarding CD8 mRNA expression, IHNV-specific IgM levels and protection upon challenge was rather low when compared to other cytokines. However, significant proliferative responses of leukocytes isolated from vaccinated fish were found, while the phenotype of proliferating cells was not determined.

4. Concluding remarks

According to the present review the existence of teleost cytotoxic T cells has clearly been shown although MHC class I restriction still awaits full approval. More importantly, our knowledge has reached a level where targeted attempts can be undertaken to drive the immune system of teleosts into a Th1 skewed response if this makes sense in terms of disease protection. However, in many fish diseases it remains to be demonstrated if cell-mediated and/or humoral immune responses confer protection. This in turn is of utmost importance for making conclusive decision on how to design vaccines with respect to the type of vaccination (e.g. DNA vaccines or inactivated vaccines; mucosal or parenteral delivery; live or inactivated vaccines) or to the kind of adjuvantation.

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