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Fighting Virus and Parasites with Fish Cytotoxic Cells

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1. Introduction

Cell-mediated cytotoxic (CMC) activity is the main cellular immunological response to kill tumor cells, virus-infected cells and parasites (Groscurth, 1989). In mammalian species this is carried out by several leucocyte populations depending on the non-specific/innate and specific/adaptive immune response. Among the last ones, the CMC activity is carried out by cytotoxic T lymphocytes (CTLs), expressing the co-receptor CD8, after repeated antigen contact and restricted to major histocompatibility complex (MHC) I. Among the innate cytotoxic cells, acting without previous neither sensitization nor MHC I restriction, the most important are the natural killer (NK) cells, which consist on large granular lymphocytes (markers: $CD16/56^{+}CD8^{-}$). However, other cell types such as the lymphokine-activated killer cells (LAK), adherent lymphokine-activated killer cells (ALAK), antibody-dependent cytotoxic cells (ADCC), macrophages, neutrophils and acidophils are also responsible for innate CMC activity (Groscurth, 1989). This CMC activity has been described in all the vertebrate animals with substantial differences. In the case of fish, the first vertebrate group showing both innate and adaptive immune system, they are not an exception. However, deeper studies are needed to clearly understand the appearance and evolution of the fish cytotoxic cells and their activity from an evolutionary point of view. Furthermore, the great potential of aquaculture industry and lack of commercial antiviral and antiparasitic vaccines for fish make necessary to increase the knowledge on the CMC activity of fish.

2. Cell-mediated cytotoxic activity in fish

In all the fish studied so far, different populations of leucocytes from head-kidney (the main haematopoietic tissue in fish), peripheral blood, spleen, thymus, peritoneal exudates or gut display variable cytotoxic activity. The fish innate CMC, not restricted to the MHC, is mainly carried out by the named non-specific cytotoxic cells (NCC), which show great differences at morphological and functional levels between fish species (Carlson et al., 1985; Evans et al., 1984a-d, 1987; Graves et al., 1984). The adaptive cytotoxic activity is restricted to the MHC, shows memory and is formed by CTLs (Fischer et al., 2006; Nakanishi et al., 2002, in press; Somamoto et al., 2000; Verlhac et al., 1990). Most of the data from fish CMC come from the activity against xenogeneic tumor cells but recently the interest to evaluate their

potential against viral infections and the generation of proper tools is increasing. One of the main problems associated with the study of the fish immune system, and the CMC in particular, is the lack of proper tools. Most of the studies are based on morpho-functional data but the lack of commercial antibodies is a serious task to definitely identify the leucocyte-types involved. Furthermore, most data obtained in mammalian CMC come from very few species such as human, rat and mouse which show some differences at molecular and cellular levels. However, in fish, the number of evaluated species is larger, including nurse shark (*Ginglymostoma cirratum*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus* or *Tilapia mossambica*), channel catfish (*Ictalurus punctatus*), bicolour damselfish (*Stegastes partitus*), Atlantic salmon (*Salmo salar*), Japanese flounder (*Paralichthys olivaceus*), orange-spotted grouper (*Epinephelus coioides*), zebrafish (*Danio rerio*), European sea bass (*Dicentrarchus labrax*) or gilthead seabream (*Sparus aurata*), what greatly increases the variability and difficult the interpretation and correlation between species. However, most of the knowledge comes from the cytotoxic activity against xenogeneic or allogeneic cells and there is few information regarding the role and importance in combating fish virus and parasites.

2.1 Fish innate cytotoxic cells

First evidences showed that head-kidney leucocytes from several freshwater fish (common carp; crucian carp, *Carassius cuvieri*; grass carp, *Ctenopharyngodon idella*; pond loach, *Misgurnus anguillicandatus*; and northern snakehead, *Channa argus*) were cytotoxic towards mammalian cell lines (Hinuma et al., 1980). Afterwards, a series of reports on channel catfish widely described the morphology, biochemical and physical requirements, killing mechanisms, etc. of these leucocytes (Carlson et al., 1985; Evans et al., 1984a-d, 1987; Graves et al., 1984). They showed for the first time that head-kidney catfish have small non-adherent, non-phagocytic and agranular cells displaying the cytotoxic activity, which were catalogued as monocyte-like but also resembled to lymphocytes (Evans et al., 1988). Obviously, they showed different morphological features than the mammalian NK cells, but very similar functional properties. These leucocytes were called non-specific cytotoxic cells (NCC) and are considered phylogenetical precursors of the mammalian NK cells. However, studies since then, including more fish species, have shown that there are many different leucocyte-types displaying the innate CMC activity but sharing the NK cell functions. Thus, the term of fish NCC population should be renamed as NCC activity more than a subpopulation since it is not a discrete and concrete leucocyte type. After that, fish NCCs have been characterized as single or heterogeneous population of leucocytes (Table 1) including lymphocytes, monocyte-macrophages and/or granulocytes (neutrophils and/or acidophils) (Bielek, 1988, 1991; Cammarata et al., 2000; Cuesta et al., 1999; Greenlee et al., 1991; Kurata et al., 1995; McKinney et al., 1986; Meseguer et al., 1994, 1996; Mulero et al., 1994; Ordás et al., 2011; Pettey & McKinney, 1983; Sasaki et al., 2002; Seeley & Weeks-Perkins, 1993).

Though very different in terms of origin and morphology, fish NCCs share the cytotoxic activity and showed the same mechanism as the mammalian NK cells (Groscurth, 1989; Lancki, 1998; Roitt et al., 1996): target recognition and binding, activation and delivery of the lethal hit and finally the target death. In the first step, some membrane molecules have been identified playing a role in the fish CMC. Vimentin-like proteins were identified in the

Fish species	Tissues	Effector cells	References
Ictalurus punctatus	HK, Sp, PBL	Small agranular, non-adherent leucocytes (NCC)	Evans et al., 1984c Evans et al., 1988 Graves et al., 1984
Oncorhynchus mykiss	HK, Th, PBL, Sp	Small agranular monocuclear leucocytes RTS11 cell line	Greenlee et al., 1991 Hayden & Laux, 1985 Moody et al., 1985 Ordás et al., 2011
Salmo salar	HK, PBL, Sp	Small agranular monocuclear leucocytes	Moody et al., 1985
Ginglymostoma cirratum	PBL	Macrophages	McKinney et al., 1986 Pettey & McKinney, 1983
Notemigonus crysoleucas	HK, PBL, Sp	ND	Moody et al., 1985
Stegastes partitus	HK, Sp	ND	McKinney & Schmale, 1994a
Oreochromis sp.	HK, PBL, Sp, PE	Lymphocytes Monocyte-macrophages Granulocytes	Faisal et al., 1989 Jaso-Friedmann & Evans, 1999
Fundulus heteroclitus	HK, Sp	ND	Faisal et al., 1991
Opsanus tau	HK, PBL, Sp, PE	Lymphocytes?	Seeley & Weeks-Perkins, 1993
Cyprinus carpio	HK, Sp, PBL, Th	Lymphocytes Monocyte-macrophages Neutrophils	Bielek 1988, 1991 Kurata et al., 1995
Sparus aurata	HK, PBL, Sp, PE, Th	Lymphocytes Monocyte-macrophages Acidophils	Cuesta et al., 1999 Meseguer et al., 1994, 1996 Mulero et al., 1994
Diecentrarchus labrax	HK, PBL, Sp, PE, Th	Lymphocytes Monocyte-macrophages Neutrophils	Cammarata et al., 2000 Meseguer et al., 1994, 1996 Mulero et al., 1994
Danio rerio	PE	ND	Moss et al., 2009

HK, head-kidney; PBL, peripheral blood leucocytes; Th, thymus; Sp, spleen; PE, peritoneal exudate; ND, not determined.

Table 1. Characteristics of representative fish NCCs.

catfish NCCs and inferred to be important in the recognition and binding to the target cells (Jaso-Friedmann et al., 1993). However, the best characterization of this first step was achieved by the finding of the non-specific cytotoxic cell receptor protein-1 (NCCRP-1) by the generation and selection of a monoclonal antibody (5C6) that completely blocked catfish NCC activity (Evans et al., 1988; Jaso-Friedmann et al., 1988, 2001). This receptor showed important features: 1) the 5C6 antibody recognizes the NCCs of most studied fish and even

the mammalian NK and LAK cells, demonstrating its conservation (Cuesta et al., 2005a; Evans et al., 1988; Jaso-Friedmann & Evans, 1999; McKinney & Schmale, 1997); 2) the NCC activity is blocked by the 5C6 antibody (Evans et al., 1988; Iwanowicz et al., 2004; Jaso-Friedmann et al., 1988, 2001); 3) the NCCRP-1 is a 32-34 kDa protein found in the membrane of NCCs and binds to a 42 and 46 kDa from the tumor targets and protozoan that they kill, respectively (Evans et al., 1996; Jaso-Friedmann et al., 1997a, 1997b, 2001; Lester et al., 1994); and 4) it is a type-III membrane protein and its activation led to tyrosine and serine phosphorylation and uses the Jak-STAT signalling pathway (Evans et al., 1999; Jaso-Friedmann et al., 1995, 2001). After binding to the target cell, mammalian NKs and fish NCCs share the same killing mechanisms including granule-dependent (release of perforin and granzymes) and granule-independent (Fas/FasL system) (Cuesta et al., 2003a; Hogan et al., 1999; Jaso-Friedmann et al., 2000; Shen et al., 2002). The release of perforin and granzyme contained in the granules is calcium-dependent and the NCC activity is greatly inhibited or completely abrogated by Ca^{2+}-chelators demonstrating their involvement in the NCC-mediated cytotoxic activity (Carlson et al., 1985; Hogan et al., 1999). In the last decade, fish perforin (Athanasopoulou et al., 2009; Hwang et al., 2004; Toda et al., 2011a) and granzyme (Huang et al., 2010; Praveen et al., 2004, 2006; Wernersson et al., 2006) sequences have been obtained but their gene expression or function has been scarcely related to the innate cytotoxic activity (Ordás et al., 2011; Praveen et al., 2006). The granule-independent killing mechanism has also been identified in fish NCCs by the use of commercial antibodies or functional studies (Ca-chelators) leading to the identification of the Fas/FasL system in fish NCCs (Bishop et al., 2002; Cuesta et al., 2003a; Evans et al., 200, 2001; Jaso-Friedmann et al., 2000; Kaur et al., 2004; Long et al., 2004). After the delivery of the lethal hit, the killing of the target cells occurs by two conserved pathways: necrosis and apoptosis (Cuesta et al., 1999; Meseguer et al., 1994, 1996; Mulero et al., 1994). At the end of the cytotoxic reaction, while NK cells are able to recycle, inactivate or dye (Leibson, 1997) the very few data available in fish NCCs demonstrate that they are unable to recycle and dye by apoptosis after encounter the target cells and kill them (Bishop et al., 2000; Evans et al., 1984a). Finally, it is important to note that in most studies the ratios between fish NCCs and targets is usually higher than when using mammalian NK cells, a fact demonstrated by the very low fish NCC kinetic parameters (V_{max} or K_M) observed (Cuesta et al., 2002a; Evans et al., 1984a). Further characterization of the fish NCCs at molecular and cellular levels will help to elucidate their role in the immune response against virus-infected cells and parasites and the mechanisms involved.

Apart from fish NCCs, other innate cytotoxic cells resembling the mammalian NK cells have been discovered. In catfish peripheral blood leucocytes (PBL), two populations of NK-like cells have been identified: one able to kill allogeneic, but not autologous, cells and the other able to kill virus-infected catfish cells (Hogan et al., 1996, 1999; Shen et al., 2002, 2004; Stuge et al., 1997, 2000; Yoshida et al., 1995). These NK-like cells were able to proliferate after weak alloantigen stimulation and presence of specific growth factors giving to clonal NK-like cells, what has greatly allowed further characterization. First, they morphologically resembled the mammalian NK cells and resulted in large granular lymphocytes, similarly to those previously identified in carp (Bielek, 1988, 1991; Shen et al., 2002, 2004). Second, they were negative for 5C6 antibody and this NCC-marker failed to block the NK-like cells-mediated cytotoxic activity (Shen et al., 2002, 2004). Moreover, they express neither T (T cell receptor –TCR- α, β or γ chains) nor B (immunoglobulin -Ig- chains) lymphocyte markers

(Shen et al., 2002, 2004). Clonal catfish NK cells induced apoptosis in their target cells by means of the calcium-dependent perforin/granzyme-mediated secretory lytic pathway since Ca-chelators completely abolished their cytotoxic activity (Hogan et al., 1999). Moreover, an antibody against leucocyte-function-associated antigen (LFA)-1, which is an adhesion molecule, inhibited the clonal catfish NK-like cell activity (Yoshida et al., 1995). Finally, clonal catfish NK-like cells bound to IgM through an FcμR and exerted an ADCC activity (Shen et al., 2002, 2003, 2004), which has been related to the presence of a similar antibody receptor (CD16) in mammalian NK cells.

Unfortunately, very little is still known about the fish innate receptors involved in the proper recognition of target cells. In mammals, it is widely known the presence of activating and inhibitory NK receptors that mediate the recognition and differentiate between self, normal and altered cells (Bakker et al., 2000). In humans, they belong to the killer immunoglobulin (KIR) or C-type lectin membrane (NKG2/CD94) receptors with either activating (ITAM) or inhibitory (ITIM) intracellular motifs. In fish, orthologs to human KIR and NKG2/CD94 gene receptors have been identified and named novel immune-type receptor (NITR) and KLR, respectively (Litman et al., 2001; Yoder 2004). Functional characterization of these receptors will help to elucidate the innate cytotoxic populations in fish, their regulation and role in disease.

2.2 Specific cytotoxic cells or CTLs

First evidences of the existence of specific cytotoxic cells in fish come from *in vivo* studies of allograft rejection (skin and scales), graft-versus-host reaction or delayed hypersensitivity reaction (DTH) (Manning & Nakanishi 1996; Nakanishi et al., 2002, in press). These experiments showed a great infiltration of lymphocytes and macrophages to the graft site, thymectomy greatly reduced these responses and the second exposure greatly reduced the time of response and increased the fish survival. All together clearly demonstrated the necessity of repeated sensitization and suggested the role of T lymphocytes. Afterwards, with the use of specific antibodies, it has been clearly demonstrated that the infiltrated lymphocytes were T-type, and very recently that were $CD4^+$ (T helper) and $CD8^+$ (T cytotoxic or CTL) (Abelli et al., 1999; Nakanishi et al., in press). However, these aspects are not reviewed here in depth since these concepts do not apply to aquaculture industry.

In vitro studies with PBLs from channel catfish, rainbow trout and ginbuna crucian carp (*Carassius auratus langsdorfii*) have been used as models for fish CTL characterization and have also demonstrated the restriction to the MHC class I (Fischer et al., 2006; Manning & Nakanishi 1996; Nakanishi et al., 2002, in press; Shen et al., 2002; Somamoto et al., 2002). These have demonstrated that immunized fish are able to kill hapten-modified autologous cells, allogeneic cell lines and allogeneic erythrocytes (Fischer et al., 1998; Nakanishi et al., 2002; Verlhac et al., 1990). In the case of channel catfish, the use of mixed leucocyte reactions (MLR) from PBLs and cloning of the cytotoxic effectors resulted in five types of clones (Stuge et al., 1997, 2000). The first type of clones (I) was related to catfish CTLs since they showed the following characteristics: exerted specific cytotoxic activity to the allogeneic cells used for immunization, expressed TCRαβ genes but not the Ig, were large granular lymphocytes and killed their targets by the calcium-dependent perforin/granzyme-mediated secretory lytic pathway (Shen et al., 2002; Stuge et al., 1997,

2000). Unfortunately, it is not known whether they express the CD8αβ co-receptor that will definitely demonstrate that they are CTLs. This approach also produced other type of clones: group II) clones consisting on TCRαβ$^+$ and CD4$^+$ lymphocytes showing non-specific cytotoxic cells and killing the targets by both the perforin/granzyme and Fas/FasL system pathways (Edholm et al., 2007; Stuge et al., 2000; Zhou et al., 2001); group III) alloantigen-specific TCRαβ$^+$ non-cytotoxic cells presumed to be T helper lymphocytes; group IV) TCRαβ$^-$ non-specific cytotoxic cells defined as NK-like cells and described above (Shen et al., 2002, 2004); and group V) TCRαβ$^-$ alloantigen-specific cytotoxic cells presumed to be γδT cells (Zhou et al., 2001). In the model using rainbow trout PBLs, the presence and function of CTLs has been documented thanks to the use of clonal trout effectors and MHC I-matching RTG-2 cell line targets, both sharing the same allele (Dijkstra et al., 2003). Sensitized-rainbow trout showed that only sorted IgM negative (sIgM$^-$) PBLs were able to kill the targets in a specific manner (Fischer et al., 2003, 2006). These data suggested the involvement of trout CTLs that was further evidenced by the up-regulation of TCRα and CD8α genes in these sIgM$^-$ cells after allogeneic cell immunization. The generation of monoclonal antibodies for rainbow trout CD8α has allowed further characterization of this population (Takizawa et al., 2011). Sorted trout CD8α$^+$ lymphocytes showed great expression of perforin or NK-lysin genes (related to the cytotoxic activity, either specific or not), as well as their up-regulation upon stimulation with the T-lymphocyte-mitogen PHA-L. However, further studies are still needed to clearly identify them as the trout specific cytotoxic cells or effectors since tissue distribution and gene expression pattern in CD8α$^+$ cells show some contradictory results and deserve deeper analysis. In the last model, the use of clonal ginbuna crucian carp has been very productive. They firstly proved the existence of specific cytotoxic response against syngeneic virus-infected cells (Somamoto et al., 2000, 2002, 2006). Afterwards, they have purified CD8α$^+$, CD4$^+$, IgM$^+$ and CD8α$^-$CD4$^-$IgM$^-$ leucocytes by means of house-produced antibodies and found that only the CD8α$^+$ population was able to kill the allotargets in a specific manner, what definitely demonstrates the specific cytotoxic activity of fish CTLs (Toda et al., 2009). Moreover, they have also showed that these CTLs mediate the target cell killing by the perforin-mediated pathway since perforin and granzyme B inhibitors abolished almost completely the cytotoxic activity (Toda et al., 2011a, 2011b).

Further studies in other fish species have documented the presence of TCR and CD8 genes indicating presence of CTLs, but functional characterization of the CTL-mediated CMC activity is still lacking. Thus, CD8 genes, alpha or beta chains, have also been sequenced in fugu (*Takifugu rubripes*) (Suetake et al., 2007), Atlantic salmon (*Salmo salar*) (Moore et al., 2005), European sea bass (Buonocore et al., 2006), gilthead seabream (Randelli et al., 2006), Atlantic halibut (*Hippoglossus hippoglossus*) (Patel et al., 2008), common carp (Sun et al., 2007) or orange-spotted grouper (Xu et al., 2011). Unfortunately, CD8α gene might not be the definite CTL marker. In mammals, CTLs are characterized by the presence of the CD8αβ while the expression of the homodimer CD8αα is detected in NK cells, γδT cells and intestinal intraepithelial lymphocytes (Bonneville & Lang, 2002). Thus, unexpected data obtained in the functional characterization of CD8α$^+$-purified lymphocytes could reside in the potential purification of other cells different to CTLs with non-specific activity. However, further studies are needed to clearly ascertain the CTL presence, distribution and role in these fish species, some of them with aquaculture interest.

3. Cytotoxic response against fish tumors

Fish tumors are quite rare in the wild. However, aquaculture management, intensive culture conditions and environmental contamination may increase the incidence of fish tumors. Although some aspects, such as tumour structure and nature, metastasis or lethal effects have been studied, little information exists concerning the involvement of the immune system in protection against tumours (Campbell et al., 2001; McKinney & Schmale, 1994a, 1994b, 1997; Romano & Marozzi, 2004; Schmale et al., 1994, 2004; Thompson & Kostiala, 1990; Vicha & Schmale, 1994). Thus, most of the information regarding fish cytotoxic activity comes from the use of hapten-modified autologous cells or xenogeneic/allogeneic cell lines (Evans et al., 1984a-d, 1987; Fischer et al., 2006; Graves et al., 1984; Manning & Nakanishi, 1996; Nakanishi et al., 2002; Shen et al., 2002; Verlhac et al., 1990). So far, fish immune response against tumors has been slightly evaluated. In the bicolour damselfish naturally suffering of neurofibromatosis (DNF) (caused by a retrovirus), study of the immune response has provided information with respect to CMC activity, morphology and distribution, degranulation of eosinophilic granular cells (EGCs) and lymphocyte proliferation (Vicha & Schmale, 1994; McKinney and Schmale, 1994a, 1994b; Campbell et al. 2001; Schmale et al. 2004). Most of the cytotoxic activity of damselfish leucocytes against DNF-derived target cell lines resided in the spleen whilst in the head-kidney it was quite low. Interestingly, specificity suggested that this activity was likely carried out by CTLs in the spleen and by NCCs in the pronephros (McKinney & Schmale, 1994a). Later, they demonstrated that the $5C6^-$ lymphocytes showed all the cytotoxic activity against the retrovirus-infected DNF tumor cell lines, suggesting the presence and role of damselfish CTLs, whilst the $5C6^+$ leucocytes were only able to kill xenogeneic erythrocytes (McKinney & Schmale, 1997). Unfortunately, deeper characterization of this CMC model has been abandoned.

The use of zebrafish as a model for human cancer would also help to understand the fish immune response against tumors, and concretely the role played by cytotoxic cells. As mentioned above, zebrafish showed NCCs in the peritoneal cavity that were positive for the 5C6 antibody and exerted cytotoxic activity against xenogeneic tumor cells (Moss et al., 2009). Moreover, the complete genome sequence allow to identify major molecules involved in the cytotoxic activity such as NCCRP-1, TCR, CD8, perforin, granzymes, Fas/FasL, etc. The easy generation of transgenic zebrafish and mutants would also be a very valuable tool to study the fish CMC activity against tumors. Further studies should focus on the leucocyte infiltration to the tumor site and identification of the potential molecules involved in the activity of the cytotoxic cells.

4. Cytotoxic response against parasites

Fish parasites represent a serious problem in the aquaculture since there are no available vaccines or effective treatments. Whilst some aspects of the fish immune response against parasites have been studied very little is known about the role of the cell-mediated cytotoxic activity (Buchmann et al., 2001; Jones, 2001). First study evaluated the NCC activity in catfish parasitized with *Ichthyophthirius multifiliis* (Graves et al., 1985a). They found that moribund *Ichthyophthirius multifiliis*-infected fish showed decreased NCC activity in the head-kidney against xenogeneic cells when compared to control specimens. Strikingly, this activity was increased in the PBLs of the same fish as consequence of an activation of the

NCC killing capacity and affinity (Graves et a., 1985a). A second study determined that catfish NCC were able to bind and kill 50-60% of *Tetrahymena pyriformis* after 10 h of co-incubation (Graves et al., 1985b). Furthermore, NCC binding to xenogeneic tumor cells and *Ichthyophthirius multifiliis* or *Tetrahymena pyriformis* parasites shared the same antigen, that in the case of parasites, consist on a 46 kDa (Evans et al., 1998a, 1996; Graves et al., 1985a; Jaso-Friedmann et al., 1997b; Lester et al., 1994). In another study, gilthead seabream specimens were parasitized with the enteric *Enteromyxum leei* parasite (Cuesta et al., 2006). This parasitation increased head-kidney NCC activity against tumor cells indicating that parasitized fish posses enhanced cytotoxic cells activity. Moreover, parasite-exposed fish either parasitized or not, showed increased NCC activity. However, no other study has evaluated the role of the cell-mediated cytotoxicity against fish parasites and deserves further evaluation due to the interest for aquaculture industry.

5. Cytotoxic response against viral infections

Viral diseases are responsible for most of the economic losses suffered in modern aquaculture since they produce high levels of mortality and no effective antiviral treatments are available. Moreover, fish farming practices such as growth under very high densities, introduction of species in new areas, continuous transport between hatcheries, nurseries and growing plants are increasing the spread of pathogens and the number of susceptible and reservoir species. However, while most available information focuses on the mechanisms involved in pathogen susceptible fish immune responses, further knowledge is also important in pathogen-reservoir fish systems. Among the major immune mechanisms to kill virus, the interferon (IFN) and the CMC are the most important, but most efforts have only focused on the IFN pathway (Ellis, 2001; Robertsen, 2006). Regarding the CMC activity against virus, this can be mediated by innate or specific cytotoxic cells (Table 2). Regarding the innate CMC activity against virus-infected cells, first studies demonstrated that salmonid kidney, spleen and PBL leucocytes were able to kill infectious pancreatic necrosis virus (IPNV)-infected cells much more than to non-infected cells (Moody et al., 1985; Yoshinaga et al., 1994), and similarly in catfish against channel catfish virus (CCV)-infected cells (Hogan et al., 1996), demonstrating the antiviral activity of fish NCC and NK-like cells, respectively. In the orange-spotted grouper, $CD8^+$ PBLs also showed non-specific cytotoxic activity against nodavirus (nervous necrosis virus or NNV)-infected cells suggesting a role for NK-like or γδT cells (Chang et al., 2011). Fish exposure to virus also increases the fish innate cytotoxic activity. Thus, gilthead seabream injected with viral hemorrhagic septicemia virus (VHSV), which did not replicate at the assayed conditions, increased the NCC activity, demonstrating the importance of studying the antiviral immune response in reservoir fish species (Esteban et al., 2008). Moreover, NNV-infection increased the NCC activity of head-kidney leucocytes from 1 to 15 days post-injection in both gilthead seabream and European sea bass (unpublished data). Recently, we have also demonstrated that trout RTS11 (monocyte-macrophage cell line) cells exposed to VHSV increased their cytotoxic activity against xenogeneic tumor cells and up-regulated the NKEF (natural killer enhancing factor), granzyme and perforin gene expression whilst trout head-kidney leucocyte infection with the VHSV increased the innate cytotoxic activity but failed to significantly change the expression of these genes (Ordás et al., 2011).

Fish	CMC activity	References
Channel catfish	NK-like activity against CCV-infected cells	Hogan et al., 1996
Atlantic salmon	CMC activity against IPNV-infected cells	Moody et al., 1985
Rainbow trout	CMC activity against IPNV-infected cells	Moody et al., 1985 Yoshinaga et al., 1994
	VHSV infection induced innate CMC activity, up-regulated NKEF, CD8α, perforin and granzyme genes	Cuesta & Tafalla, 2009 Utke et al., 2007 Unpublished data
	VHSV infection elicited specific CMC activity, up-regulated CD8 α gene	Fischer et al., 2006 Utke et al., 2007
	VHSV DNA vaccine elicited specific CMC activity	Utke et al., 2008
	VHSV and IPNV DNA vaccines up-regulated NKEF, perforin and granzyme genes	Cuesta & Tafalla, 2009 Cuesta et al., 2010 Unpublished data
	VHSV infection of RTS11 cells increased the CMC activity, up-regulated NKEF, granzyme and perforin genes	Ordás et al., 2011
Ginbuna crucian carp	IPNV or EVA infection elicited specific CMC activity	Somamoto et al., 2000
	CHNV infection elicited specific CMC activity, up-regulated TCRβ and CD8α genes	Somamoto et al., 2002 Somamoto et al., 2006
	Generation of *in vitro* virus-specific CTLs and up-regulation of TCRβ and CD8 α genes	Somamoto et al., 2009
	Anal immunization with CHNV-infected cells elicited specific CMC activity	Sato & Okamoto, 2010
Common carp	SVCV infection up-regulated, granzyme A/K or CD8α genes	Forlenza et al., 2008 Huang et al., 2010
Gilthead seabream	NCC activity induced by VHSV injection	Esteban et al., 2008
	NCC activity induced by NNV infection	Unpublished data
Sea bass	NNV infection no affected TCRβ and CD8α genes	Scapigliati et al., 2010
Atlantic halibut	NNV infection no affected CD8α and CD8β genes	Patel et al., 2008
Orange-spotted grouper	CMC activity against NNV- or RSIV-infected cells	Chang et al., 2011
	NNV infection elicited specific CMC activity, increased $CD8\alpha^+$ cells and CD8α gene	Chang et al., 2011
Japanese flounder	VHSV infection up-regulated CD8 gene	Byon et al., 2005
	VHSV DNA vaccine up-regulated CD8 gene	Byon et al., 2006

CMC, cell-mediated cytotoxicity; CCV, channel catfish virus; IPNV, infectious pancreatic necrosis virus; VHSV, viral hemorrhagic septicaemia virus; EVA, eel virus from America; CHNV, crucian carp haematopoietic virus; RSIV, red seabream iridovirus; SVCV, spring viremia carp virus; NNV, nervous necrosis virus; CTL, cytotoxic T lymphocytes; TCR, T cell receptor; NKEF, natural killer enhancing factor.

Table 2. Major studies evaluating the fish CMC activity against virus.

Viral infections also elicited the specific immune response by inducing antibody production and CTL activity (Table 2) (Nakanishi et al., in press). First studies demonstrated that isogeneic ginbuna crucian carp elicited CTL activity against virus. Thus, ginbuna crucian carps immunized with hematopoietic necrosis virus (CHNV) specifically killed CHNV-infected syngeneic cells in a viral antigen and MHC I-restricted manner (Somamoto et al., 2000, 2002), increased the TCRβ and CD8α gene expression (Somamoto et al., 2006) and helped to establish virus-dependent CTL clones *in vitro* (Somamoto et al., 2009). In rainbow trout, infection with VHSV greatly elicited specific- and MHC I-matched cytotoxic cells but a non-specific and MHC I-mismatched cytotoxic activity was also found (Fischer et al., 2006; Utke et al., 2007). Surprisingly, they found that specific CMC activity mediated by CTLs was produced much earlier than the innate activity, in sharp contrast to all the information at this respect. Strikingly, the NKEF gene expression followed the same time-profile than the CTL activity but in the case of CD8α was opposite, adding more controversy to these data (Utke et al., 2007). Furthermore, trout vaccination with VHSV DNA vaccines also elicited CMC activity against MHC I-matched infected cells, suggesting a role for CTLs (Utke et al., 2008). However, they also found a bit lower CMC activity against non-matching-infected cells or cells infected with a different virus, suggesting a role for NCCs or even the ADCC activity since these fish showed high antibody levels, but this has not been confirmed. In other studies, VHSV infection increased the trout NKEF and CD8α gene *in vivo* but failed to modulate the NKEF, perforin and granzyme genes *in vitro* (Cuesta & Tafalla, 2009; Ordás et al., 2011). VHSV and IPNV DNA vaccination also up-regulated the trout CD8α, perforin and granzyme gene expression (Cuesta et al., 2010; unpublished data), giving more consistency to the involvement of CMC activity against viral infections and its activation by DNA vaccines. Moreover, oral vaccination with inactivated CHNV elicited specific CMC activity that resulted viral antigen-specific and restricted to the MHC I (Sato & Okamoto, 2010). In the orange-spotted grouper, nodavirus infection also elicited a CTL response when viral antigens were properly presented by MHC I receptors, as well as increased the CD8α expression at gene and CTL surfaces (Chang et al., 2011). This study represents the first one demonstrating the CTL role against viral infection in marine fish species with great interest for aquaculture industry. Further studies would help to understand the CMC activity against viral infections and to design and probe viral vaccines.

6. Modulation of the fish cytotoxic activity

Fish CMC activity regulation has been widely evaluated and mostly focused on NCC modulation. Fish NCC activity has been shown to be modulated by several chemicals, cytokines, environmental contaminants, stress factors, immunostimulants, etc. First studies dealt with the NCC inhibition by blocking the binding to target in order to characterize the role of NCCRP-1, or inhibiting the killing mechanisms in order to evaluate the perforin- or Fas/FasL-mediated lytic pathway (Bishop et al., 2002; Carlson et al., 1985; Evans et al., 1988, 2000; Hogan et al., 1999; Iwanowicz et al., 2004; Jaso-Friedmann et al., 1988, 2001; Kaur et al., 2004; Shen et al., 2002). Further studies demonstrated that catfish NCC activity is increased by leucocyte treatment with ionophore A23187, A23187 plus phorbol myristate acetate (PMA) or vanadate but no with PMA alone or poly I:C (a mimic for viral infections) (Evans et al., 1984b, 1990, 1998b). Moreover, serum from stressed fish contained cytokine-like factors able to increase the tilapia NCC activity suggesting a role for FasL (Jaso-Friedmann

et al., 2000; Ruiz et al., 2001). Fish NCC activity is also increased by bacterial infections: *Edwardsiella ictaluri* in channel catfish (Evans et al., 1998b), *Aeromonas salmonicida* in brook trout (*Salvelinus fontinalis*) (Dautremepuits et al., 2006) or *Streptococcus iniae* in tilapia (Taylor et al., 2001). In our lab, we have been investigating the immunostimulatory role of many substances and conditions in the gilthead seabream, one of the most important farmed species in the marine aquaculture. This has allowed us to get a lot of information about the regulation of the seabream immune response, and concretely the NCC activity. Thus, we have shown *in vitro* and/or *in vivo* modulation of seabream NCC activity by vitamins C (Cuesta et al., 2002b), E (Cuesta et al., 2001), A (Cuesta et al., 2003b) and D3 (Cerezuela et al., 2009), chitin (Cuesta et al., 2003c; Esteban et al., 2000, 2001), levamisole (Cuesta et al., 2002c), lactoferrin (Esteban et al., 2005), melatonin (Cuesta et al., 2008a), propolis (Cuesta et al., 2005b), inulin (Cerezuela et al., 2008), unmethylated oligodeoxynucleotides (ODNs) containing cytosine-phosphodiester-guanosine (CpG) motifs (Cuesta et al., 2008b, 2008c), probiotic bacteria (Díaz-Rosales et al., 2006; Salinas et al., 2005, 2006, 2008), yeast (Cuesta et al., 2007; Ortuño et al., 2002; Reyes-Becerril et al., 2008; Rodríguez et al., 2003), fungi (Rodríguez et al., 2004), virus (Esteban et al., 2008), environmental contaminants (p,p´-DDE and lindane) (Cuesta et al., 2008d) or stress factors (air exposure, crowding and anaesthetics) (Cuesta et al., 2003d). In general, we have demonstrated great NCC increments after these treatments. Moreover, we have also observed that NCC activity reached the greatest activation, compared to other innate cellular immune responses such as phagocytosis or respiratory burst activity, and did at shorter treatment times and lower dosages. Unfortunately, most of this information has been obtained evaluating the NCC activity against xenogeneic tumor cells and whether this is correlated to the *in vivo* activity against viral infections deserves further investigation. In this sense, few recent studies have correlated the stimulatory role of immunostimulants with an increased viral disease resistance. Thus, probiotic-supplemented diets resulted in reduced mortality of Japanese flounder specimens exposed to lymphocystis disease virus (LCDV) (Harikrishnan et al., 2010) whilst feeding of shrimp with immunostimulant herbs reduced their mortality upon viral disease (Citarasu et al., 2006). Further characterization of the beneficial immunostimulants against viral diseases is needed to control the virus spreading and lethal effects.

Apart from the direct activation of fish cytotoxic activity, the expression of some CMC-related genes (NCCRP-1, CD8, perforin, granzyme, etc.) is also modulated (Table 2), suggesting an increase in the CMC activity. First, the NCCRP-1 gene expression was altered after bacterial infection (Reyes-Becerril et al., 2011; Sakata et al., 2005), administration of immunostimulants (Cuesta et al., 2008b, 2008d; Lazado et al., ; Reyes-Becerril et al., 2008), exposure to contaminants (Cuesta et al., 2008d) or bacterial vaccination (Caipang et al., 2008), depending on the fish species, tissue, time and dose of exposure, and suggests a parallel effect of fish NCC activity. Perforin gene expression is usually up-regulated after immunization of ginbuna crucian carp with tumor cells (Toda et al., 2011a), after PHA-L (*Phaseolus vulgaris* leucoagglutinin) stimulation of trout $CD8\alpha^+$ cells (Takizawa et al., 2011), after VHSV infection of RTS11 cell line (Ordás et al., 2011) and after viral infection or DNA vaccination in rainbow trout (unpublished data), whilst down-regulated after cadmium exposure (Auslander et al., 2008). In a similar fashion, granzyme genes are up-regulated by bacterial vaccination (Caipang et al., 2008), viral infections (VHSV in RTS11 cell line and SVCV in carp) (Huang et al., 2010; Ordás et al., 2011) and viral infection or DNA vaccination

(unpublished data). The transcript level of CD8 gene is related to the CTL presence, abundance and activity. Thus, fish CD8 transcripts are up-regulated by viral and bacterial infections, viral DNA vaccines, scale grafts, poly I:C or mitogens (Byon et al., 2005 2006; Cuesta et al., 2010; Cuesta & Tafalla, 2009; Forlenza et al., 2008; Overturf & LaPatra, 2006; Somamoto et al., 2005, 2006; Utke et al., 2007; Xu et al., 2011). In some studies, these CD8 gene levels have been correlated with increased CTL activity. Finally, other genes related to the cytotoxic activity have received less attention. In this category, the natural killer enhancing factor (NKEF), which increase the cytotoxic activity in humans but its role is unknown in fish, is up-regulated by viral infections and DNA vaccines (Cuesta & Tafalla, 2009; Ordás et al., 2011; Utke et al., 2007) while granulysin, which is secreted together to granzymes and lyses target cells, gene is up-regulated in $CD8^+$ lymphocytes by mitogen stimulation (Takizawa et al., 2011). Further studies are needed to clearly state the gene expression with either innate or specific cytotoxic activity in fish. Future development of more molecular tools will help to elucidate this fascinating and complex immune response.

7. Future directions

As summarized above, fish posses a wide range of cytotoxic cells with killing activity against tumor cells, virus-infected cells and parasites. Further studies in the future should identify, describe and characterize the cytotoxic cells and mechanisms in the most cultured fish species and those susceptible to be farmed in the future. Another issue is the generation of molecular tools to evaluate the fish CMC and clearly identify the function of NCCs, NK-like and CTLs as well as assay models such as clonal fish, cytotoxic cell clones or MHC I-paired effector and targets (virally infected or not). These tools will also help to design powerful and safe vaccines against problematic virus and parasites for fish aquaculture. Finally, these studies have also to be applied to marine fish, which culture is continuously increasing because of the human demand and high economic value.

8. Glossary

ADCC	Antibody-dependent cytotoxic cells
ALAK	Lymphokine-activated killer cells
CCV	Channel catfish virus
CD4+	T helper lymphocyte
CD8+	T cytotoxic lymphocyte or CTL
CHNV	Crucian carp haematopoietic virus
CMC	Cell-mediated cytotoxicity
CpG	Cytosine-phosphodiester-guanosine
CTLs	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic acid
DNF	Damselfish neurofibromatosis
DTH	Delayed hypersensitivity reaction
EGCs	Eosinophilic granular cells
EVA	Eel virus from America
HK	Head-kidney
IgM	Immunoglobulin M
IPNV	Infectious pancreatic necrosis virus

ITAM	Activating intracellular motifs
ITIM	Inhibitory intracellular motifs
Jak	Janus kinase
KIR	Killer immunoglobulin
LAK	Lymphokine-activated killer cells
LCDV	Lymphocystis disease virus
LFA-1	Leucocyte-function-associated antigen-1
MHC	Major histocompatibility complex
MLR	Mixed leucocyte reaction
NCC	Non-specific cytotoxic cells
NCCRP-1	non-specific cytotoxic cell receptor protein-1
NITR	Novel immune-type receptor
NK	Natural killer
NKEF	Natural killer enhancing factor
NKG2/CD94	C-type lectin membrane receptors
NNV	Nervous necrosis virus
ODNs	Unmethylated oligodeoxynucleotides
PBL	Peripheral blood leucocytes
PE	Peritoneal exudate
PHA-L	Phaseolus vulgaris leucoagglutinin
PMA	Phorbol myristate acetate
RSIV	Red seabream iridovirus
RTG-2	Rainbow trout gonad cell line
Sp	Spleen
STAT	Signal Transducer and Activator of Transcription
SVCV	Spring viremia carp virus
TCR	T cell receptor
Th	Thymus
VHSV	Viral hemorrhagic septicaemia virus

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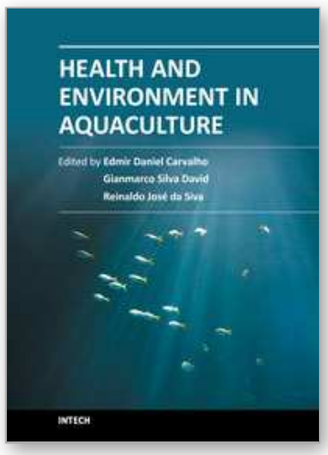

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Aquaculture has been expanding in a fast rate, and further development should rely on the assimilation of scientific knowledge of diverse areas such as molecular and cellular biology, and ecology. Understanding the relation between farmed species and their pathogens and parasites, and this relation to environment is a great challenge. Scientific community is involved in building a model for aquaculture that does not harm ecosystems and provides a reliable source of healthy seafood. This book features contributions from renowned international authors, presenting high quality scientific chapters addressing key issues for effective health management of cultured aquatic animals. Available for open internet access, this book is an effort to reach the broadest diffusion of knowledge useful for both academic and productive sector.

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