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Review article

Fish Immunity Against Nervous Necrosis Virus (NNV) Infection: A Review

R.Irfan-Hakimi[1], Azila Abdullah[2], M.Nur-Nazifah[1*], M. Firdaus-Nawi[1], Amalin-Sofea AR[1*], MK. Hazreen-Nita[3]

ABSTRACT
Fish are unique organisms fitted with a certain degree of immune system that are similar to mammals; consists of innate and adaptive immunity. Innate immunity comprised of non-specific cellular and the nonspecific humoral components while adaptive immunity consists of specialized systemic cells and mechanisms that are categorized into two main groups: the humoral and cellular components. In response to nervous necrosis virus (NNV) infection, both innate and adaptive immunity mechanisms play crucial roles. NNV invasion in fish triggers phagocytosis by antigen-presenting cells (APCs). These cells process viral proteins into peptide fragments, presenting them on major histocompatibility complex (MHC) class II molecules, a crucial protein component that initiate specific adaptive immune response against NNV infection. This will eventually activate adaptive immunity pathway to stimulate the proliferation and maturation of the B cells. As B cells matured, antibodies will be produced to suppress the pathogen through series of process known as opsonization and neutralization before phagocytosis takes place. Following secondary exposure with the same antigen, B cells will rapidly proliferate, releasing significant number of antibodies to fight the antigen. Overall, this review elucidates the complex interplay between innate and adaptive immune responses in fish, highlighting their crucial roles in combating NNV infection through mechanisms such as phagocytosis, antigen presentation, and antibody production.

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Introduction

Aquaculture in Malaysia

Malaysian aquaculture production for freshwater species were dominated by red tilapia (*Oreochromis* spp.), freshwater catfish (*Clarias gariepienus*) and striped catfish (*Pangasionodon hypophthalmus*) with recorded production at 30,022 tonnes, 31,987 tonnes and 21,144 tonnes and wholesale value of RM 322 million, RM 159 million and RM 165 million respectively [1]. While for marine species, the most cultured species were seabass (*Lates calcarifer*), snapper (*Lutjanus* spp.) and grouper (*Epinephelus* spp.) whose production stood at 34,186 tonnes, 5417 tonnes and 2584 tonnes with wholesale value worth RM 480 million, RM 277 million and RM 96 million respectively 1AFS., Annual Fisheries Statistic. Department of Fisheries, Ministry of Agriculture & Agro-Based Industry, Malaysia. However, expansion of aquaculture industry in Malaysia was hampered by the amount of good quality fish seeds [2], leading to the fish fries and fingerlings importation from neighboring Asian countries such as Thailand, Taiwan, Indonesia and Vietnam. Nevertheless, fish importation without improper biosecurity practices and measures may lead to the introduction of diseases causing agents [3]. Hastein (2001) stressed out that importation of live aquatic animals possessed higher risks of pathogen transfer which has led to the transmission of aquatic animals' diseases worldwide. This has been documented [5] where infectious diseases caused by bacteria, virus, fungi and parasites were found to be introduced at imported countries within 1978 to 2005. In 1996, importation of common carp (*Cyprinus carpio*) as ornamental pet trade has led to the outbreak of contagious viral disease, Koi herpesvirus disease (KHD) with major outbreaks observed in farms culturing common and Koi carp in Israel next two years [6].

In aquaculture, diseases are caused by a diverse array of pathogenic agents, including parasites, bacteria, fungi, protozoa, and viruses [7]. These infectious organisms can significantly impact fish health, growth, and survival

[1] Kulliyah of Science, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia
[2] National Fish Health Research Centre, Batu Maung, 11960 Penang, Malaysia
[3] Faculty of Agro-Based Industry, University Malaysia Kelantan, Jeli Campus, 17600 Jeli, Kelantan, Malaysia
*Corresponding Author: M. Nur-Nazifah, e-mail: nurnazifah@.iium.edu.my

rates, leading to substantial economic losses in the aquaculture industry. The severity and prevalence of these diseases can vary depending on environmental factors, host susceptibility, and the virulence of the pathogen [8]. Among these pathogens, viral diseases have emerged as a particularly concerning issue over the past two decades. Outbreaks of viral infections have been frequently reported, with consequences that cannot be overlooked. These viral pathogens often result in high mortality rates, sometimes exceeding 90% in susceptible populations, and can lead to significant economic losses due to reduced production, increased treatment costs, and trade restrictions [8,9].

Fish Immune System

Immune system can be characterized as a set of humoral and cellular component to barricade the body against foreign substances including pathogens and toxins following the presence of endogenous and exogenous substances that initiate this system [10].

Although fish immune system is physiologically comparable to higher vertebrates including mammals, there are few peculiarities between the fish immune system and the immune system of other vertebrates [11]. From birth, fish have a well-developed and robust innate immunity mechanisms, with the presence of crucial innate immune cells to recognize and eliminate pathogen including phagocytes, macrophages and natural killer cells. However, the adaptive immune system, that crucial for the secretion of specific antibodies, takes time to develop and mature. In contrary, innate immune system mechanisms of mammals is not well developed at birth, but becomes more effective over time. For adaptive immunity, both fish and mammals include B and T cells to provoke immune responses. Unlike mammals, fish lack discrete lymph nodes. Instead, their immune system relies on diffuse lymphoid tissues distributed throughout their body. These tissues, which include the thymus, kidney, and spleen, serve analogous functions to mammalian lymphoid organs, supporting immune cell development and facilitating immune responses. This diffuse organization of lymphoid tissues in fish is an evolutionary adaptation to their aquatic environment and provides effective immune surveillance across their body systems.

When immune responses take place, mammals produce higher antibody compared to fish [12]. While mammals produce five main classes of antibodies (IgM, IgG, IgA, IgE, and IgD), fish have a more limited antibody class. Fish primarily produce three classes of immunoglobulins: IgM, which is the predominant systemic antibody; IgD, whose function is not fully understood but is believed to play a role in antigen recognition; and IgT (or IgZ in some species), which is specialized for mucosal immunity. This difference in antibody diversity reflects the evolutionary divergence between fish and mammals and the unique adaptations of the fish immune system to aquatic environments. The presence of IgT in fish and its absence in mammals is a result of evolutionary divergence and adaptation to different environments. IgT, discovered early 20s, is a teleost-specific immunoglobulin that plays a crucial role in mucosal immunity, particularly in the gut and gills of fish [12]. This specialization reflects the unique challenges faced by aquatic organisms in protecting their mucosal surfaces from pathogens. Mammals, evolving in terrestrial environments, developed different strategies for mucosal immunity, primarily relying on IgA [12]. The absence of IgT in mammals and its presence in fish highlight the distinct evolutionary paths of their immune systems, shaped by their respective environmental pressures and physiological needs. While both mammals and fish are equipped with mucosal immunity responses, mucosal immunity in fishes are more noticeable compared to mammals. Mucosal surfaces in fishes include skin, gills and digestive tract incorporated with specialized immune cells that produce antibodies to provide protection against pathogen. On the other hand, although the role of classical fish major histocompatibility complex (MHC) in fish appear to be similar to those in mammals [13]. The fish MHC are clear, precise and easier to distinguish compared to mammals. Researchers defined that fishes typically have less highly polymorphic MHC genes as well as less complex MHC system compared to mammals.

As fishes are classified as ectothermic or cold-blooded animals where its body temperature follows surrounding temperature [14], immune system plays an essential role in maintaining its internal homeostasis. This complex system made up of specific organs to detect and identify any mobilizing pathogen that attack the hosts in the bloodstream. Despite the diversification and variations, fishes' immune system was comparable across the vertebrate linage, comprise of two important mechanisms, innate and adaptive immunity [15]. Across species and environment, freshwater and marine fishes are said to generally have immune system mechanisms that are alike and comparable [16]. In general, freshwater and marine fishes possess similar innate immune mechanisms, including physical barriers like skin and mucous, antimicrobial peptides, phagocytic cells (such as macrophages and neutrophils) and complement proteins. These components initially offer primary protection and help to provokes the immune response against pathogen [15]. Besides, IgM were found to be expressed in both freshwater and marine species [17]. Previous study [18] also reported that IgT or IgZ antibody classes were identified in both marine and freshwater fishes. Hence, those clearly supports that both freshwater and marine species fishes possessed similar adaptive immunity mechanisms. Overall, while

adaptations and modifications might be observed on specific circumstances, freshwater and marine fish species commonly possess similar immune system mechanisms in order to protect themselves from pathogens.

Viral Nervous Necrosis

Viral nervous necrosis (VNN) or also known as Viral encephalopathy and retinopathy (VER) is one of the most devastating viral diseases that leads to significant mortality rate, particularly in larval stages [19,20]. The causative agent for VNN/VER is nervous necrosis virus (NNV), a betanodavirus class of virus that belongs to the *Nodaviridae* family [21]. Betanodavirus are non-enveloped and icosahedral in structure. This small virus (25-20 nm in diameter) was made up of two positive-sense ssRNA molecules labelled as RNA1 and RNA2 (Figure 1). The RNA1 (3.1kb) consists of RNA dependent RNA polymerase (RdRps) that are subjected to viral replication, whilst RNA2 (1.4 kb) encodes the viral capsid protein (42 kDa) [21]. Previously, it was reported that additional segment, designated as RNA3, was sub-genomically synthesized from RNA1 that encodes polypeptide B2 [23]. However, this non-structural protein that inhibits the cell's RNA silencing mechanisms only exists in infected cells as it was not captivated into the viral particles [21].

The common entry of NNV infection is through the eyes and brain organ, [24] before spreading through the blood circulation and rapidly attack the host central nervous system via the peripheral nervous system, causing death to the fish [26]. Following the infection, virus will proliferate, leading to the blockage of the blood circulation and increased capillary pressure [27]. Accumulation of fluid will ultimately increase the force between the capillary membrane and interstitial space, caused edema and ruptured the fish's inner eye lining. This will eventually lead to the dysfunction of the eye's organ from its normal physiological state [25]. Similarly, Yuwanita and Yanuhar (2013) also find out that signs of bleeding (hemorrhage) in eye's organ were common during the time of NNV infection in humpback grouper as a result of burst blood vessels in the eye's lining. In addition, NNV infected fish will usually possess weaker nervous system, hence, fish will lose its nerve control, develop lethargy motion [28] and cause death up to 100% especially in the juvenile stages [29].

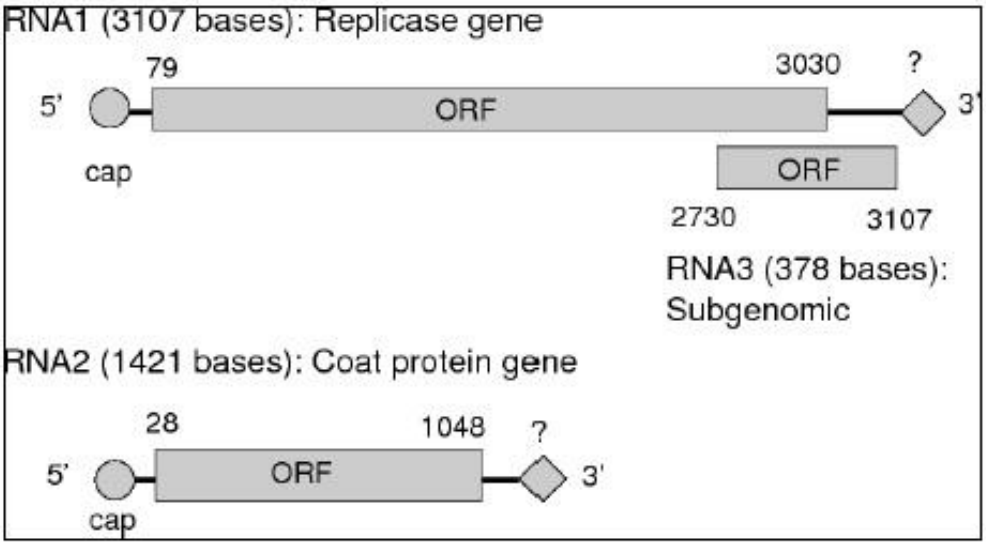

Fig 1 Betanodavirus genome organization. The genome of betanodaviruses consists of two RNA segments: RNA1 and RNA [35]. These two segments encode different viral proteins and play distinct roles in the viral life cycle.

To date, betanodavirus has been reported in more than 120 cultured and wild species [30], becoming a major threat affecting the global aquaculture production. Betanodavirus infections have been reported globally particularly in regions with extensive aquaculture activities [31] including south and east Asia (Japan, Korea, Taiwan, China, Phillipines, Thailand, Singapore, Indonesia, Vietnam, Brunei and Malaysia), Mediterranean (Bosnia, Greece, Portugal, Italy, France and Spain) and Oceania (Australia and Tahiti). Scientists reported through several studies [33] that both marine and freshwater species are vulnerable to betanodavirus infection. Infections in marine species has been reported in several species including Asian seabass (*Lates calcarifer*), European seabass (*Dicentrarchus labrax*), Japanese parrotfish (*Oplegnathus fasciatus*), turbot (*Scophthalmus maximus*), Redspotted grouper (*Epinephelus akaara*), Striped jack (*Pseudocaranx dentex*), Giant grouper (*Epinephelus lanceolatus*), Sevenband grouper (*Epinephelus septemfasciatus*), Atlantic cod (*Gadus morhua*), Golden pompano (*Trachinotus blochii*) and Red snapper (*Lutjanus campechanus*) [34]. Most recently, betanodavirus has also been isolated in freshwater species such as Chinese catfish (*Silurus asotus*), Australia catfish (*Tandanus tandanus),* medaka (*Oryzias latipes*), freshwater tilapia (*Oreochromis* spp.), guppy (*Poicelia reticulata*) and zebrafish (*Danio rerio*) [32,33,37,38,39]. Although researchers [32,33,34] proved that

betanodavirus infections are susceptible for both marine and freshwater species, few experiments suggested that Asian seabass at juveniles' stages are more prone to betanodavirus as early as 10 days post hatching (DPH) onwards with high mortalities rate recorded at 80% 19. Apart from small size fishes that are more vulnerable to viral infections than bigger size fishes, [19] stressed out that this could also be associated with several factors including cannibalistic nature of seabass and stress factors such as overcrowding in the tanks or cages.

Innate Immunity

Innate immunity, or commonly referred as natural immunity is the first line of defense that protect the hosts against any foreign agent [15]. Innate immunity is immensely crucial for several reasons. Firstly, this defense mechanism is non-specific, hence, do not rely on individual molecular structure for detection of the foreign pathogen. Therefore, the protection provided is relatively quick, limiting pathogen ability to survive. Thirdly, innate immunity works regardless surrounding temperature fluctuation, serves as useful tool for ectothermic vertebrates since adaptive immunity takes relatively longer time and rely on the surrounding temperature to mount a response. Before specific response take place, innate immunity will react against invading pathogen, initiating several immunological responses. Verrier et al., (2012) stressed that innate immune response played a crucial part to counter virus infection as quick induction of this non-specific mechanisms is essential to prevent viral replication. Although without any immunological memory [41], innate immune response serves as an early signal that will eventually initiate adaptive immune system to establish its response [42].

Innate Response Against NNV Infection

Interferons (IFNs)

Following infection, fishes will establish a quick antiviral response through their innate immune system [43] before complex response in form of antibody and memory cells secretion takes place [44].

Following NNV infection, quick induction of innate immune response by teleost fishes lead to the production of Interferons (IFNs). IFN can be characterized as a pH resistant cytokine, with a low molecular weight (20-23- KDa), produced prior to the viral infection [45]. Deonarain et al., (2004) stated that IFN acts as an indicator that determine the stage and development of the viral infection, hence, serves as a crucial element in the innate immune system. Although the reasons for persistence of VNN in adult stage is still in doubt, the awful effect during larval stage infection comes out with a solid justification that innate immune system have a huge role to play in persistent infection [41]. Generally, IFN works by binding to receptors located at the cell membrane, initiating several processes that lead to the stimulation of interferon-stimulated genes (ISGs) [47] .Then, IFN produced will makes its way out of the cell, and directly generate the production of protein including 2',5'-oligoadenylate synthetase, protein kinase P1 and Mx proteins that inhibit viral replication as well as degenerating viral RNA [48]. Normally, production of IFN happened rapidly as early as two days post infection as reported by [49] where IFN were presence in virus infected teleost fish 48 hours post-injection.

In teleost fish weighing less than 200 grams, the production of interferons (IFNs) plays a pivotal role in the innate immune response. These small yet potent signalling molecules serve as a critical first line of defense, offering robust protection to the host during early developmental stages when the adaptive immune system is not yet fully matured. IFNs are rapidly produced in response to viral infections and other pathogenic stimuli, initiating a cascade of antiviral responses within the fish's body. This was justified by [48] as organs in NNV infected groupers showed relatively higher percentage value of IFN in brain (0.55%), eyes (17.73%) and kidney (16.08%) compared to healthy fishes whereby the IFN percentage recorded in healthy fishes are 0.21%, 14.05% and 8.54% in brain, eyes and kidney respectively. Other than that, previous study by [43] through in-vivo and in vitro test suggested that IFN-like cytokines and Mx gene have a major role in assisting the persistence infection in Seabass and Grouper. Indeed, for seabass, within six to twelve hours post infection, published data displayed that infected seabass exhibit intense IFN and Mx responses in kidney and brain organs [50,51]. This may indicate that the capsid protein of nodaviruses is capable enough to mount innate immune genes transcription ahead of NNV infection [52].

Complement system

The involvement of complement system in combating viral infection has been reported previously [53]. The complement can be activated or initiated via a combination of three pathways: the alternative, the lectin and the classic pathways. In vertebrates including fishes, complement system, composed of 35 soluble proteins mostly reported to play its part in innate immunity by directly bind to the viral surfaces [54]. Whilst most researchers are focusing on classical complement pathway, alternative and lectin complement pathway has also been reported to engage against viral infection [55]. Fish complement system has been reviewed by [56] and was shown to be involved in opsonization, phagocytosis and inflammation to combat viral infection.

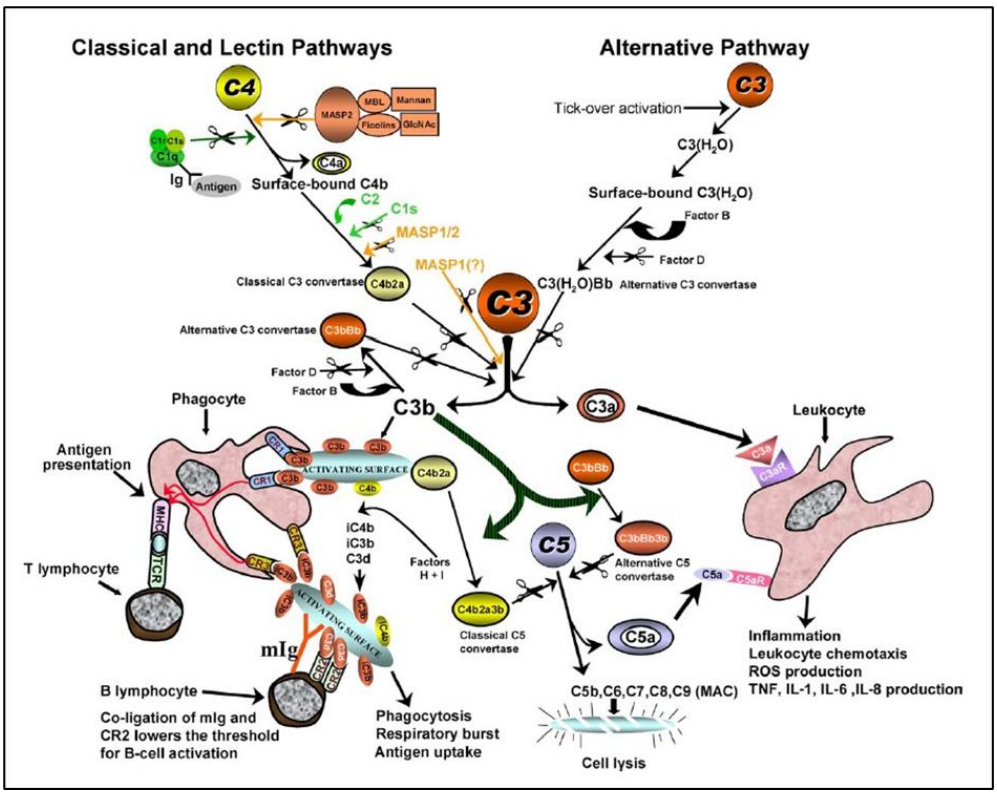

Fig 2 Complement pathway mechanism in teleost fish. Complement system in teleost fish plays a crucial role in their immune defense, contributing their ability to combat infections in aquatic environments [56].

The classical pathway, predominantly studied in mammals, is initiated by the interaction between antigen-antibody complexes and the C1q component of the C1 complex. Specifically, the Fc portion of IgG or IgM binds to C1q, triggering the activation of C1r and C1s proteases. The alternative pathway, more prevalent in fish serum compared to mammals, is characterized by spontaneous activation or "tick-over" mechanism. This pathway is initiated by the hydrolysis of C3 to C3(H_2O), which can then interact with factor B and factor D to form the C3 convertase (C3bBb). This convertase can cleave more C3, amplifying the response. The lectin pathway is activated when pattern recognition molecules, such as mannose-binding lectin (MBL) or ficolins, recognize carbohydrate moieties on microbial surfaces. This recognition leads to the activation of MBL-associated serine proteases (MASPs), which then cleave C4 and C2 to form the C3 convertase (C4b2a), similar to the classical pathway. All three pathways converge at the formation of C3 convertases, leading to the generation of C5 convertases and ultimately the assembly of the MAC. This system provides rapid and effective defense against a wide range of pathogens, including viruses playing a critical role in fish immunity against NNV infection.

Overall, activation of complement pathway assists the innate immunity especially during viral infection through phagocytosis and cytolysis of pathogens, solubilization of immune complexes, and inflammation [57] In fact, scientists reported that apart from its crucial role in activating innate immune response, complement helps to enhance and intensify humoral immunity [58]. Specifically, it has been demonstrated that bounding of C3 and C4 molecules to antigen during the activation process boost the intake and production of antigen presenting cells (APCs), hence, in turn, induce and results to more effective primary and secondary immune responses during viral infection [59].

Natural killer cells (NC) and non-specific cytotoxic cells (NCC)

Natural killer cells (NKC) are crucial element that ensure innate immunity works against viral infection [41]. NKC carry out lysis and apoptosis, as well as secreting cytokines (group of signaling proteins produce to regulate humoral responses) to destroy impaired cells attacked by virus during antiviral response [60]. NKC are proved to have positive link with macrophages as NKC are responsible to initiate macrophages by triggering them to produce type I IFN. In fact, those induced cytokines acted as a key bridge between innate and adaptive immunity.

Similar to mammals, teleost fishes have non-specific cytotoxic cells (NCC) that are engaged to trigger immune response during viral infections [61]. Basically, NCC will be able to recognize specific size protein (40 KD) and in turn directly produce cytotoxic cells when NCC are in contact with susceptible target cell membrane [62]. As in mammals, perforin also have its own role to barricade viral infections in teleost fishes. Perforin is

the most common signaling proteins associated with natural killer cells (NKC) and NCC during viral infections [63]. They act by forming pores, allowing cytotoxic molecules such as granzymes to enter the target cells and directly initiate programmed cell death (apoptosis) to remove the infected cells [62] (Figure 3).

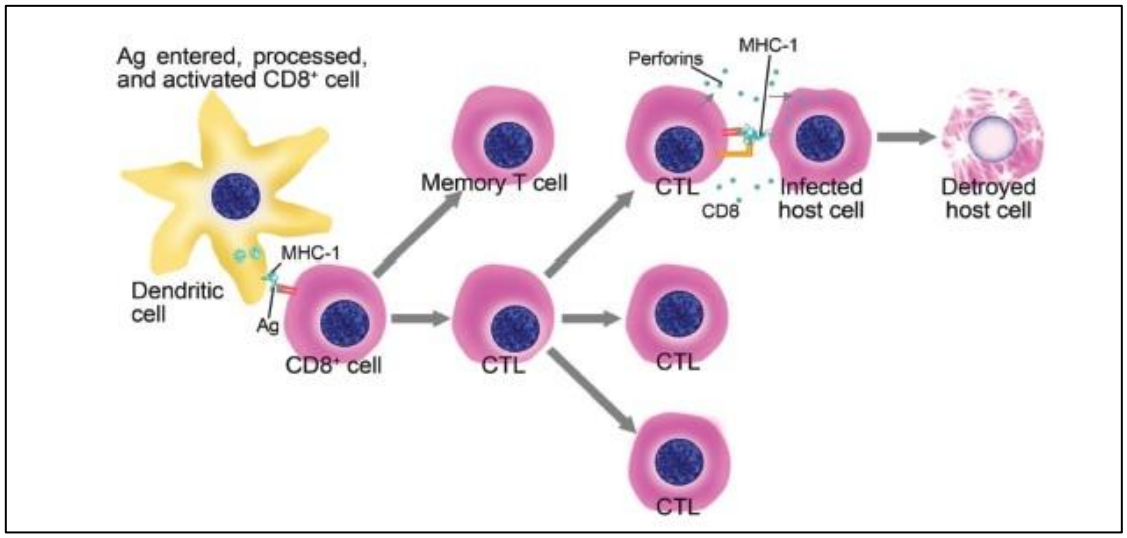

Fig 3 Destruction of infected cells during viral infection. Perforin is found to possess a role regarding the immune defense against virus infections among teleosts fishes [14].

Pathogen recognition receptors (PRRs)

Koyama et al., (2008) mentioned that recognition of viral pathogen is the first crucial early step that serves as a connector between innate and adaptive immunity. Pathogen Recognition Receptors (PRRs) families include the C-type lectins, complement receptors, cytosolic nucleotide-binding domain, LRR proteins and the most commonly identified in fish, Toll-like Receptors (TLRs) [65] . Although TLRs molecules in mammals was reported to be more specific and complex, teleost fish are well conserved with TLRs molecules that are able to at least detect the amino acid sequence level of viral molecules. In fact, TLR3 gene which are responsible for the recognition of dsRNA molecule have been isolated in rainbow trout [65] from leukocytes and tissues following viral infection. Recently, [66] also reported the presence of TLR7 and TLR8 genes specifically to detect ssRNA virus. TLRs operates by detecting the unique conserved molecules of the microbes, labeled as pathogen-associated molecular patterns (PAMPs) before stimulates inflammatory reaction that triggers the innate immunity response [67]. Lee and Kim (2007) explained that PRRs mount the innate immune response through several process including complement pathway, apoptosis, leukocyte activation and cytokine secretion. To date, a total of 13 TLR have been identified in teleost fishes based on research conducted on zebra fish as immunological model [68].

Adaptive Immunity

Compare to innate immunity, adaptive immunity is made up of complex system consist of highly specific systemic cells and processes that interrupt and impede pathogenic growth following an infection. In general, adaptive means the distinction between specific and non-specific and reshaping the immune response against particular foreign pathogen. Rubio-Godoy (2010) mentioned that adaptive immunity was established following early response by non-specific immunity. Although it took longer time to mount a response during the first infection, adaptive immunity is crucial due to its specificity for the particular agent [11]. In fact, subsequent exposure commonly happened in shorter time and higher immensity than the earlier response.

Adaptive immunity mechanisms are incorporated with two major components which are antibodies and lymphocytes [11]. B cells, T cells and lymphocytes are the cells that makes up the adaptive immunity response. B cells, originated from the bone marrow, responsible for the antibody production whilst T cells, found and mature in thymus, differentiate into cells that eventually involved in lymphocyte maturation or in discarding virus-infected cells. To be precise, the secretion of memory cells by B cells is the key feature of adaptive immunity from innate immunity [14].

Adaptive Immune Response Against NNV Infection

B Cells

B cells are necessary type of lymphocyte that established the anti-viral response against infection majorly through humoral immunity [44]. Upon activation, B cells will produce plasma cells and memory cells. In return, plasma cells will generate antibodies that provokes response for the destruction of antigen as well as acting as antigen presenting cells (APCs) [14,45]. In teleost fishes, B cells originated in the head kidney, hence,

labelled as the primary lymphoid tissue [70]. Furthermore, B cells are also found in the spleen of teleost fishes, making spleen as the secondary lymphoid tissues. Bromage (2004) stated that B cells activation takes place in the spleen before differentiated into plasma cells and migrate to the head kidney, hence, justified the presence of antibody secreting cells in the spleen compared to head kidney. Danilova et al., (2005) stressed out that antibodies generate in fin fishes following viral infections are the major key to achieve the long-term adaptive immunity. In addition, immunoglobulin isotypes such as IgD, IgM and IgT were presence in teleost fishes, indicates that IgM expressing B cells responds to antigen stimulation including viral infection [73].

The role of B cells to induce antiviral immunity is undoubted since B cells are responsible to secrete antibodies, handing over protection against viral diseases [74]. In response to viral infection, teleost fishes have the capability to produce IgMs serum that able to identify viral antigens and this been demonstrated in wide range of fish species. For instance, in the course of viral infection, the presence of fully functional serum IgMs that are able to neutralize virus were recognized in rainbow trout. Although IgM is the common isotypes studied in teleost fishes, several studies have also highlighted the production of IgD and IgT following viral infections. As scientists stated that teleost fishes have three different classes of immunoglobulin (IgM, IgD and IgT), those classes of antibodies that are crucial to identify and counteract pathogen during viral infections can be distinguished through several characteristics [18]. IgM in general was the primary antibody classes that can be found in most species particularly in the earlier stages of the immune response and is the first class of antibodies to be produce upon infection. In fact, IgM was observed to be involved in both systemic and mucosal immunity mechanism in fishes [75]. IgD on the other hand, was observed only in certain species. For instance, IgD transcription was expressed during viral infections as [76] reported that IgD mRNA levels in freshwater carp (*Catla catla*) and rohu (*Labeo rohita*) gradually increase following infection against inactivated rabies virus via intramuscular injection. Several studies reported that IgD is usually observed in low concentrations, hence, its role in fish immune response still remain elusive, but it may have a role in initiating innate immune response [77]. Next, IgT or also labbeled as IgZ, is another class of unique antibody found in teleost fishes such as zebrafish and rainbow trout. Junirahma et al., (2021) found out that grouper (*Epinephelus coioides*) generates specific levels of IgT in specific organs, following vaccination with nodavirus inactivated vaccine. Previous study [18] reported that IgT is important for mucosal immunity, triggering immune response at specific sites as it was primarily exposed at mucosal surfaces including gut and, skin and gills.

T Cells

T cells are another crucial component in adaptive immune mechanisms, provoke its reaction through cell-mediated immunity response **[**78]. Similar as B cells, T cells are also type of lymphocytes that originated and matured in thymus, hence, labelled as T cells or also known as thymocytes. T cells are able to be differentiated from other lymphocytes by the presence of T-cell receptor or also known as antigen-specific receptor on its surface.

T cells families include T helper cells (Th cell), cytotoxic T cells, memory T cells, regulatory T cells and gamma delta T cells. Exposure to NNV infection will lead to the proliferation and differentiation of dendritic cells into Th1 cells and Th2 cells, generating cellular immune responses against the viral infection [25]. In fact, the proliferation of immune cells expressed on the surface of Th cells, known as CD4 plays a vital role in coordinating the adaptive immune response as T cells receptors only distinguish antigen represented by MHC class II molecules [28]. Saito et al., (2010) explained that Th cells expressing CD4, labelled as $CD4^+$ Th cells play a part to provoke the immune response as effector cells or memory cells. Naive $CD4^+$ T cells are able to differentiate into five different effector cells (Th1, Th2, Th17, Th9 and Th22), three subclass of regulatory T cells (Treg, Th3 and Tr-1) and memory T cells. Hence, unique abilities of this immune cells give grounds for their crucial role in synchronizing the immune system, immune pathogenesis and host defense mechanism [80]. In fact, [61] described $CD4^+$ T cells as flexible mainly due to their multiformity as recent finding figured out that T cells related genes such as TCR, CD4 and CD8 as well as MHC class I and II genes are detected in several teleost fish species. Study by [28] demonstrated that proliferation of CD4 and CD8 cells immune cells are detected in Humpback groupers (*Cromileptes altivelis*), following NNV infection. The presence of CD4 and CD8 cells in tissues and organ of groupers explained that the exposure to foreign antigen is able to mount cellular immune response, produced by the differentiation of cytotoxic T cells [81].

CD4 and CD8 cells were formed as a result of the proliferation and differentiation process, initiate from the entry of the antigen into the cells and tissues of the infected fishes. During NNV infection, the virus, acting as an intracellular antigen, undergoes proteolytic degradation within infected cells. The resulting peptide fragments are then transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP). This process is crucial for the subsequent presentation of viral antigens on MHC class I molecules, facilitating recognition by cytotoxic T lymphocytes and initiating a specific immune response against NNV-infected cells. Following that, the peptide will bound with MHC class I molecules, before being

presented on the surface by T cells $CD8^+$ (Cytotoxic T cells (Tc)). Then, cell destruction process (cytolysis), will be carried out by Tc cells with two different mechanism which are exocytic pathway and interaction between Fas ligand (FasL) and Fas expressed on target cells. In addition, Fas ligand is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family and interaction with its receptor induces apoptosis. After the binding of Tc cells with target cells, perforin and granzyme will be secreted in the cytosol of the host cells. Next, perforin spread through the cells and form pores, allowing granzyme to enter the host cells. Granzyme will eventually induce the process of apoptosis, hence, ending the programmed cell death. According to [82], perforin/granzyme-induced apoptosis is the main pathway used by cytotoxic lymphocytes to kill virus-infected and transformed cells.

Overall Mechanisms of Fish Immune System Against NNV Infection

Fish viruses most likely have the same capacity with mammalian viruses, where they developed few approaches to withstand the immune system [41]. Previously, [83] described that IFN suppression is the most common strategy for innate immune evasion following viral infection. Several studies have reported the occurrence of IFN related gene suppression following viral infection in salmon and rainbow trout fishes [84]. Other than IFN suppressions, restriction of apoptosis is also one of the viruses' mechanisms to escape the innate immunity response. By inhibiting apoptosis, infected cells are alive until all the particles were released [85]. Hence, innate immune evasion apparently justified the importance of both innate and adaptive immunities to work in complement [14].

In attempt to mount antiviral response against an infection, both innate and adaptive immunity mechanisms play their own parts. Following NNV infection, innate immunity directly works through TLRs. Once NNV particles successfully pass the physical barrier, invasion starts and this initiate innate cellular and humoral immunities, as well as the specific adaptive immunity. Innate immunity discards the invading pathogen through phagocytosis and involved several components including granulocytes, phagocytes and non-specific cytotoxic cells. During the same course of infection, humoral immunity mechanisms consist of protein and glycoproteins, works together with cellular innate immunity to suppress the growth of microorganisms [86]. Following phagocytosis, small protein fragments were presented by macrophages on MHC class II molecules (Figure 4). Upon activation of adaptive immunity, antigen-presenting cells (APCs) such as macrophages present processed antigens on MHC class II molecules. T helper (Th) cells recognize these antigens via their T cell receptors (TCRs), forming an APC-Th cell complex. This interaction triggers the secretion of cytokines by activated Th cells. These cytokines, including interleukins and interferons, stimulate B cell proliferation and differentiation. As B cells mature, they differentiate into two distinct cell types: plasma cells, which secrete antibodies, and memory B cells, which provide long-term immunological memory. This process is crucial for mounting an effective and specific immune response against pathogens like NNV [87]. Antibodies produced by the plasma cells are responsible to suppress the pathogen through opsonization and neutralization before phagocytosis takes place to destroy and remove the disabled pathogen [14,87].

Intracellular antigen including virus undergo another functional mechanism before being eliminated from the fish body. In case of NNV infection, NNV pathogen undergo phagocytosis and being displayed on MHC Class I by APC. This attracts CD8+ cells to bind with the MHC I of APC through CD8+ receptors. Then, activated CD8+ cells will start to proliferate and produce memory T cells and cytotoxic T Lymphocytes (CTL) that discard the infected cells via cell apoptosis. Following subsequent exposure with the same antigen, as memory cells meet the specific antigen again, b cells will rapidly proliferate and differentiate into plasma cells. This will result in plasma cells releasing significant number of antibodies to clear the antigen.

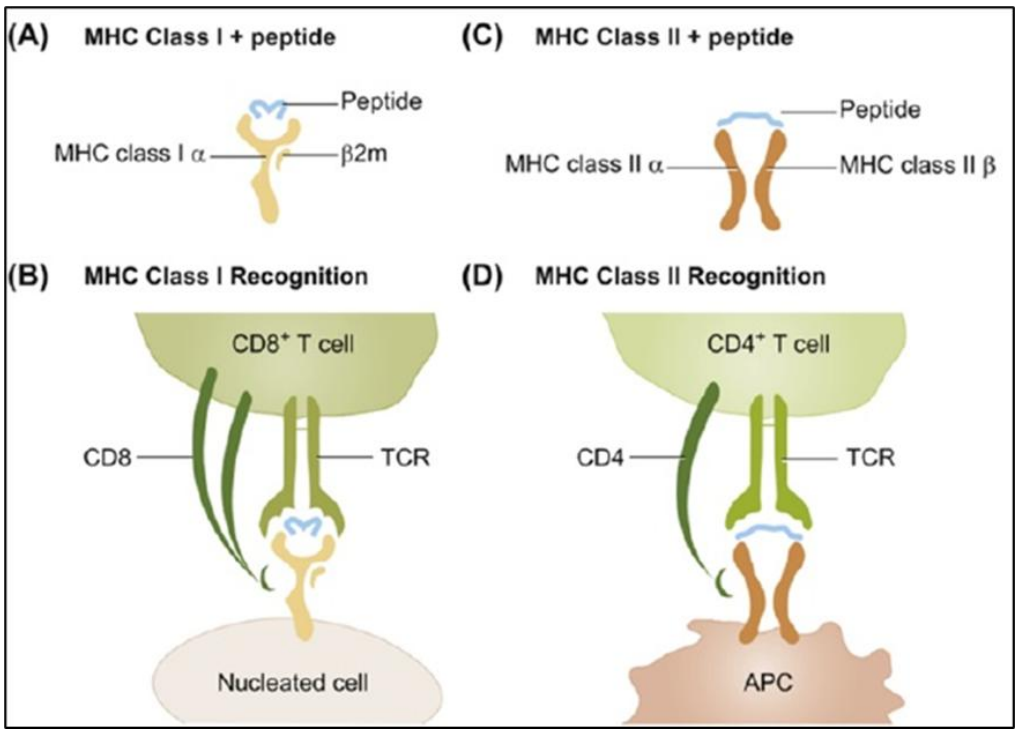

Fig 4 Major Histocompatibility Complex (MHC) Class I and Class II [88]. MHC system is crucial for the adaptive immune response in fish, enabling them to recognize and combat a wide array of pathogens.

Conclusion

Viral nervous necrosis is a serious disease, leading to significant economic loss in aquaculture. Better understanding on key factors including fish immune system mechanisms, the pathogens itself and its entry mechanisms can leave a significant impact to the farming industry. While vaccination seems to be the most effective strategy to prevent the emergence of various diseases in the future, findings on how the fish's immune system works serves as a strong basis as well as lay the foundations for further research, particularly on vaccination. Therefore, the necessary knowledge is crucial and helps in efforts to boost the health and disease protection of fish.

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