The Immune System and the Antiviral Responses in Chinese Giant Salamander, *Andrias davidianus*

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The Chinese giant salamander, belonging to an ancient amphibian lineage, is the largest amphibian existing in the world, and is also an important animal for artificial cultivation in China. However, some aspects of the innate and adaptive immune system of the Chinese giant salamander are still unknown. The Chinese giant salamander iridovirus (GSIV), a member of the Ranavirus genus (family *Iridoviridae*), is a prominent pathogen causing high mortality and severe economic losses in Chinese giant salamander aquaculture. As a serious threat to amphibians worldwide, the etiology of ranaviruses has been mainly studied in model organisms, such as the *Ambystoma tigrinum* and *Xenopus*. Nevertheless, the immunity to ranavirus in Chinese giant salamander is distinct from other amphibians and less known. We review the unique immune system and antiviral responses of the Chinese giant salamander, in order to establish effective management of virus disease in Chinese giant salamander artificial cultivation.

Keywords: Chinese giant salamander, amphibian, antiviral response, immune system, Ranavirus infection

INTRODUCTION

The Chinese giant salamander (*Andrias davidianus*) is the largest and the most primitive urodele amphibian alive worldwide. The Japanese salamander, the American hellbender, and the Chinese giant salamander are few living members belonging to the most ancient amphibian lineage, the family *Cryptobranchidae*, which is distantly related to the *Salamandridae* that emerged some 350 million years ago (1). The morphology of *A. davidianus* is thought to have varied very little over this long period of time and, as such, is considered as a living fossil. Populations of *A. davidianus* have declined remarkably within the past 50 years due to habitat loss, environmental change and pollution, overharvesting, infection diseases, and exploitation for food. As a result, *A. davidianus* has been included in the national class II protected species in China and in the list of Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2008) (2–5). In parallel to this rapid decline of wild populations, farming of *A. davidianus* has developed rapidly since 2005. In China, approximately 2 million *A. davidianus* are artificially reproduced per year (3).

Due to the development of intensive farming as well as intense international and national trading, emerging infection diseases of *A. davidianus* rapidly increased in prevalence in recent years. The major culprit is a ranavirus pathogen, the Giant Salamander Iridovirus (GSIV), which is also known as *A. davidianus* ranavirus (ADRV). Outbreaks of GSIV disease are threatening both wild and captive bred *A. davidianus*, and mortality rate often reaches 100% in densely populated farms (3, 4, 6–10). GSIV disease first outbroke in 2010 in the Shanxi Province and has rapidly spread to all Chinese giant salamander farming areas, including Hubei, Jiangxi, Hunan, Henan, Sichuan, and Shaanxi provinces (3, 4, 6–8).

As a member of the genus *Ranavirus* (RVs) (family *Iridoviridae*), GSIV is a large icosahedral double-stranded DNA virus. Ranaviruses have emerged as major amphibian pathogens, infecting and causing outbreaks of a wide variety of captive and wild species worldwide (11). The detailed homologous gene comparisons of GSIV with other ranaviruses revealed that GSIV contained 85%, 93%, 94%, 97%, and 99% homologous genes in *Ambystoma tigrinum* virus (ATV), tiger frog virus (TFV), common midwife toad ranavirus (CMTV), frog virus 3 (FV3), and *Rana grylio* virus (RGV) genomes, respectively (8). Moreover, based on the phylogenetic analysis of the concatenated sequences and the Bayesian tree, GSIV is more closely related to CMTV compared to ATV, TFV, FV3, and RGV (12).

With regard to the aquaculture of the Chinese giant salamander and the disease caused by GSIV, besides advances in fundamental comparative immunology, a better understanding of the immune system ontogeny and antiviral immune response is imperative. In contrast to mammals, amphibians have an external development free of maternal influence. As such, their immune system has to develop rapidly within a few days to provide protection to early larval stages against pathogens. In addition, the immune system of anuran amphibians is usually regarded as more efficient than that of urodels. This view is probably due in large part to the disproportionate studies on very few species, the anura *Xenopus laevis* and the urodele *Ambystoma mexicanum* (13, 14). Anurans such as *X. laevis* undergo a remarkable complete metamorphosis that also profoundly remodel their immune system, whereas urodels exhibit a range of developmental transition from partial and incomplete to cryptic metamorphosis (15, 16). Indeed, some salamanders transition through only partial morphological changes such as internalization of the gills, while unlike anurans, they retain their tails in adult stages. In contrast, axolotls exhibit a characteristic called "neoteny" and reach sexual maturity without undergoing metamorphosis (14). *A. davidianus* also undergoes a limited developmental transition during metamorphosis, including the internalization of the gills and the retention of the tail at adult stage.

With regard to the immune system, salamanders such as tiger salamanders and Mexican axolotls are also distinctive compared to frogs, perhaps in part due to their high regeneration capacity (13). Recent advances of genomics and transcriptomics have revealed that many potential orthologues of mammalian innate and adaptive immune response-related genes are present and expressed in urodel species (17, 18). In particular, molecular and functional studies have recently been undertaken in the Chinese giant salamander, which stir misconception of immune capacity of urodel species.

This review presents recent progresses in our knowledge about the organization of immune system and lymphopoiesis of Chinese giant salamander as well as the antiviral responses, which is essential for the protection of wild and artificially cultured Chinese giant salamander.

ORGANIZATION OF THE CHINESE GIANT SALAMANDER IMMUNE SYSTEM

The immune systems of jawed vertebrates are categorized into two interconnected arms: innate and adaptive. Unsurprisingly, the two types of immunity are present in the Chinese giant salamander, as well as genes and pathways that define innate and adaptive immunity. The summary of identified immune genes of *A. davidianus* and *X. laevis* is shown in **Table 1**.

Innate Immune System

Innate immunity provides an effective initial defense and is critical to activate adaptive immune responses in amphibians as in mammals (53, 54). Typical innate molecules and pathways include pattern recognition receptors (PRRs), complement system, antimicrobial peptides (AMPs), interferons (IFNs), lectins and inflammatory cytokines, signaling factors, and chemokines (19, 20). Notably, PRR-mediated pathogen detection and recognition is central to the activation of innate and adaptive immune system (20). The molecular interaction of PRRs with pathogen-associated molecular patterns (PAMPs) stimulates immune effector cells, such as dendritic cells, macrophages, and natural killer (NK) cells, and induces related pathways and cytokines, such as Toll-like receptor pathway and the downstream ILs and IFNs, which contributes to the eradication of the pathogens (19, 20, 53, 54).

To date, five classical cell-associated PRRs have been identified and characterized in several vertebrate species. These include three transmembrane PRRs—Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and Class A scavenger receptors (cA-SRs)—and two cytoplasmic PRRs—RIG-I like receptors (RLRs) and NOD-like receptors (NLRs) (17, 20).

The TLR is a crucial class of PRRs; these transmembrane receptors play important roles in inflammation activation, IFN production, and shaping of adaptive immunity (20, 55). In mammals, the TLR family consists of up to 13 gene members. Ten TLRs were identified in human and 12 TLRs were identified in mouse, whereas at least 17 TLRs were detected in some fish species (20, 21, 56–61) and 11 to more than 20 TLRs in amphibians. For example, in the anuran *Bombina maxima*, a total of 11 *TLR* genes encoding 32 different transcripts have been reported, including gene orthologs of *TLR1-4, 6-8* and *Toll/IL-1 receptor* (62). Twenty functional different *TLR* genes and several adaptor proteins were characterized in *Xenopus tropicalis*

TABLE 1 | Identified immune genes of *A. davidianus* and *X. laevis*.

		A. davidianus	*X. laevis*	Ref.
Innate immunity	TLRs	TLR (1–3, 5–8, 10, 13, 19, 20)	TLR (1–9, 12–14, 19, 20)	(17, 21, 22)
	NLRs	NAIP, IPAF, APAF1, NOD proteins (NOD1, NOD2, NOD3, NOD5, CIITA)	Nod 1 and 2, Nod3, NLRX1, CIITA, IPAF, NAIP	(17)
	RLRs	RIG-I, MDA5, LGP2	RIG-I/DDX58	(17, 23)
	AMPs	Cathelicidin-like precursor	Magainin, xenopsin, caerulein, peptide glycine-leucine-amide	(19, 24, 25)
	Complement	C1-9, MASP, CR1, CR2	C1-9, MASP, Bf	(17, 22, 26)
	Signaling adaptor	MyD88,	MyD88, NFKB, IKBB, DAP10, DAP12, TCRgamma	(4, 17, 19)
	Cytokines	IL-1β, TNFα, IL-6, IL-18, I-IFN, IFNγ, IFNβ, IRF3	IL-1β, LTα, LTβ, TNFα, IL6, IFNα	(4, 17, 19, 27, 28)
Adaptive immunity	Antigen presentation	MHC I, MHC II, β2M	MHC I, MHC II, DM, β2M, Psm8-10, Tap1, Tap2, Cathepsin L, Tapasin, calreticulin, calnexin	(29–34)
	Antigen receptors	IgH (D, M, Y), IgL (lambda, kappa, type III), TCR β, Rag 1, 2	IgH (M, X, D, F, Y), IgH (λ, κ, σ), TCR α, β, γ, δ, Rag1, Rag 2, AID, TdT	(35–48)
	Accessory molecules	CD8a, CD8b	CD2, CD4, CD8 α, β, BTLA, CD274, CD276, JAM, VTCN1, CD28, CTLA4, HVEM, CD40LG, CD40, MCAM, ICAM, IL-2, 3, 4, 5, 7, IFNγ	(19, 26, 49, 50)
	Signaling molecules	CD3E	LCK, CD3ε, CD3ζ, Fyn, Igα	(51, 52)

tadpoles and adults (22, 63, 64). While all the mammalian *TLR* gene orthologs exist in both *X. laevis* and *X. tropicalis*, some TLR family members such as TLR14 have been expanded in *Xenopus* (13). In the Chinese giant salamander, 11 *AdTLRs* (*TLR 1, 2, 3, 5, 6, 7, 8, 10, 13, 21, 22*) have been identified. *AdTLR3, AdTLR5*, and *AdTLR7* share clear orthology with fish and mammalian counterparts. The bony fish-specific *TLR21* and *22* genes and the mouse-specific *TLR13* gene orthologs are present in *A. davidianus*, while *AdTLR2, AdTLR5*, and *AdTLR21* have duplicated gene copies (17). Genes encoding the downstream molecules of TLR signaling pathway including the adapter MyD88 and the pro-inflammatory cytokines such as TNFα, IL-1β, IFNγ, IL-6, and IL-18, have also been identified in *A. davidianus* (4, 17, 27). AdTLRs are less diversified than fish and *X. laevis* although some TLRs and the downstream pathways are conserved.

CLRs, the other transmembrane PRRs, are able to recognize carbohydrate structures of bacteria, fungi, parasites, and virus through a unique structure known as "C-type carbohydrate recognition domain (CRD)" or "C-type lectin domain" (65, 66). The CLR pathway initiates innate immune responses, including phagocytosis, activation of the complement system, and enhanced NK cell activity, and regulates adaptive immune responses (20, 65). Galectin-1, one important CLRs member, has been identified in *A. davidianus* (66). It contains a highly conserved CRD domain; it exhibits agglutination and binding activity to both Gram-positive and -negative bacteria as well as potential antiviral activity during GSIV infection (66). These limited results suggest diverse functions of CLRs in pathogen defense of *A. davidianus*.

cA-SR is a class of ancient transmembrane PRRs that recognize and bind low-density lipoproteins, bacteria and nucleic acids (61, 67). The cA-SR family includes five members: the scavenger receptor A (SR-A); the macrophage receptor with collagenous structure (MARCO); and the scavenger receptor A member 3 (SCARA3), SCARA4, and SCARA5. A conserved scavenger receptor cysteine-rich (SRCR) domain is present in SR-A, MARCO, and SCARA5, but not in SCARA3 and SCARA4 (61). Some cA-SRs serve as cellular receptors for DNA virus (67). In mammals, herpes simplex virus 1 (HSV-1) and vaccinia virus (VACV) use MARCO for cellular entry, and adenovirus 5 (AdV-5) uses both MARCO and SR-A. In amphibians, it has been reported that FV3 can enter cells *in vitro* by using cA-SRs (67). In *A. davidianus*, further studies are needed to characterize the roles of cA-SRs.

The RLR family members, which recognize viral RNA and induce IPS-1/VISA/Cardif (MAVS) signal and pro-inflammatory cytokines, play crucial roles in intracellular pattern recognition (20, 68). Three important RLR family members are present in *A. davidianus* as in other vertebrates, including retinoic acid inducible gene I (RIG-I), laboratory of genetics and physiology 2 (LGP2), and melanoma differentiation-gene 5 (MDA5). Among them, the AdRIG-I and AdMAD5 amino acid sequences contain three conserved elements: a N-terminal CARD domain, a DExD/H box RNA helicase domain, and a C-terminal RD domain. However, a CARD-containing adapter molecule *IPS-1* gene has not been identified in *A. davidianus* by transcriptomic sequencing and may be missing (17, 23). Although LGP2 lacks CARD domain in other vertebrate (69), the structure signature of AdLGP2 has not been investigated. The RLR activation pathway induces IFN regulatory factor 3 (IRF3) regulated type-I interferon response and subsequently stimulates the production of IFN-stimulated genes (*ISGs*) including *ISG15* and the *Myxovirus resistance* (*Mx*) gene, which has been characterized in the Chinese giant salamander (28, 69–71). Further characterization of the RLR

pathway in *A. davidianus* should now be undertaken and in particular the absence of adapter IPS-1 will have to be confirmed.

NLRs, another important PRRs family, are intracellular sensors of pathogenic products that are able to induce innate pro-inflammatory responses (57). The NLR family is composed of three distinct subfamilies: NODs, NLRPs, and IPAF (72). Genes of these three subfamilies are present in the two *Xenopus* genomes. In *A. davidianus*, genes encoding the canonical NOD proteins (NOD1-3, NOD5, and CIITA), NAIP, IPAF, and apoptotic protease activating factor 1 (APAF1) were identified. Although NLRs did not show a major expansion, multiple isoforms of NOD2, NOD3, NALP3/NALP12, APAF1, IPAF, and CIITA were identified in *A. davidianus* by transcriptomic analysis (17). These isoforms suggest a complex and diversified detection system resulting from alternative splicing and gene duplication (17).

As soluble PRRs, the vertebrate complement system not only plays a critical role in potentiating humoral immune responses and in bridging the host innate and adaptive immune response, but also facilitates the ability of antibodies to clear pathogens. The complement system, including over 30 soluble plasma proteins and receptors, can be activated by three pathways: the classical pathway, the alternative pathway, and the lectin-mediated pathway to initiate defense against multiple pathogens (19, 20, 61). The activation of the classical pathway is triggered by bacterial lipopolysaccharide, pentraxins such as C-reactive protein (CRP), serum amyloid, etc. The alternative pathway is induced spontaneously and primarily depends on recognition of PAMPs. The lectin-mediated pathway is initiated by binding carbohydrate structures such as mannan-binding lectin (MBL), MBL-associated serine proteases (MASPs), and ficolins (53, 61). All these pathways converge to effector function including four sequential steps: (1) opsonization of bacteria for enhanced phagocytosis; (2) recruitment and activation of leukocytes at the site of inflammation; (3) generation of membrane-attack complexes to punch holes in the cell membranes of pathogens; and (4) pathogen destruction (20). Genes encoding proteins of these three pathways, such as C1-9 and MASP, have been identified in *X. laevis*, *X. tropicalis*, and *A. davidianus* (17, 73). Thus, according to recent data, the complement system is likely conserved between anura and urodele.

Another important type of immune effector molecule in amphibian is constituted by antimicrobial peptides (AMPs). The amphibian skin granular glands constitutively secrete AMPs that serve as a nonspecific physical and chemical barrier against a wide range of pathogens (24, 74). AMPs have also been reported active against ranavirus (75), although their protective activity is minimal for Eastern Hellbenders (76). Over 600 AMPs that belong to over 30 families were characterized in amphibians (24, 74, 77). Among those, Cathelicidin is a prominent family of AMPs that acts as multifunctional effector molecules in innate immunity. Cathelicidin is produced as a cathelicidin-like precursor. The critical cleavage step into mature cathelicidin is mediated by an elastase that recognizes the amino acid motive glycine-alanine-serine-threonine-isoleucine-valine.

In the Chinese giant salamander, cathelicidin-like precursor transcripts are abundant in the skin (24).

Adaptive Immune System

As a specific defense system, the adaptive immune system includes humoral immune responses with secreted antibodies and cellular immune responses with activated effector lymphocytes to eliminate specific pathogens after infection. B and T cells are the major cellular components in humoral immune responses and cellular immune responses, respectively. B and T cells expressed surface Ag-specific receptors that have undergone recombination-activating genes-dependent somatic recombination during their development (19).

T-cell receptors (TCRs) are essential for activation of cellular immune responses. After presented and complexed with the major histocompatibility complex (MHC) molecules, the pathogen-derived peptides are recognized by TCRs (19). MHC molecules, including MHC class I and MHC class II, are cell surface receptors. These two classes of receptors have different tertiary structures and expression patterns. Moreover, they interact with TCRs expressed by different types of T lymphocytes. The majority of cells of an organism express MHC class I molecules, which are heterodimers consisting of two subunits: the α subunit encoded in the MHC locus as a polymorphic MHC gene and the β subunit (β-2 microglobulin, β-2m) encoded by a non-polymorphic gene outside the MHC locus. The transmembrane α subunit binds to antigenic peptides that it presents at the cell surface to CD8 cytotoxic T cells. MHC class II molecules are heterodimeric α and β receptors encoded by polymorphic genes that are expressed by antigen-presenting cells (APCs) and that present antigenic peptides to CD4 T helper cells (19, 20).

Between mammals and amphibians, the structure and complexity of MHC molecules are similar. Moreover, the close correlation of the diversity of MHC alleles to disease resistance has been revealed (29, 53, 78). In anurans, depending on species, from one (*Xenopus*) to 2 or 3 highly polymorphic MHC class I genes per haplotype have been found (30, 79). In addition, in *Xenopus*, an extended family of oligomorphic *MHC class I-like* genes has been characterized outside the MHC locus (80). The existence of such *class I-like* genes in other anuran species remains to be determined. The number of *MHC class II a* and *β* genes varies between 1 and 2 among anuran species (19, 30–32). In the Chinese giant salamander, although the precise number of *MHC class I* and *class II* genes per genome is still unknown, a previous report has identified 26 *class IA*, 27 *class IIA*, and 17 *class IIB AdMHC* alleles and most variations between alleles have been detected in putative peptide-binding region (PBR) (29). Following GSIV infection, the transcription of *MHC* isoforms was increased, consistent with their roles in the immune response (29).

B-cell receptors (BCRs) are pivotal elements in humoral immune response that are expressed in two forms: (1) membrane receptors on B cell surface that recognize antigen, and (2) secreted immunoglobulins (Igs) present in the blood circulation and are produced in large amounts by plasma cells (20). As in mammals, genes encoding the BCRs and Igs in amphibians share conserved structure with a constant and

variable domain in both light and heavy chains. Five Ig heavy chains have been characterized in *Xenopus*: IgM, IgY, IgX, IgD, and IgF (13, 19, 35–37, 81). IgM-positive cells are located in most host tissues, including spleen, liver, kidney, and gut. IgX-positive cells are mainly distributed at the epithelial layer of the gut and skin, and are less frequent in the spleen and liver, which suggests that IgX may be involved in mucosal immunity (82). *Xenopus* IgY-positive cells are located in the blood circulation, spleen, and liver but generally absent in the gut. IgY is the functional analog to mammalian IgG. The switch from IgM to IgY is thymus-dependent and temperature-dependent, and requires the collaboration of T cells (81). *Xenopus* IgD and IgF were not identified until 1996, and both of them are expressed mainly in the spleen. *Xenopus* IgD, which is orthologous to the isotype IgW of lungfish and IgW/X of cartilaginous fish, is considered as ancient as IgM. IgF consisting of two constant domains and a hinge region is the shortest Igs in *Xenopus*. The two constant domains are similar to those of fish and the fourth constant domains of IgY, which suggests that IgF is produced by tandem duplication of IgY followed by a loss of internal constant domains (19, 38, 82, 83).

From recent reports, genes encoding three IgH chains (AdIgD, AdIgM, and AdIgY) have been detected and characterized in *A. davidianus* (39). AdIgD displays four constant domains (C_H1–C_H4) and a hinge region, which is distinct from other amphibians. Furthermore, AdIgY△Fc, a unique IgY form that has not been described in other amphibians, was detected in serum of adult Chinese giant salamanders. Further diversification of AdIgH appears to occur by alternative splicing (39). Furthermore, *AdIgY* transcripts were detected by *in situ* hybridization in the thymus, kidney, liver, and spleen of *A. davidianus* (40). More recently, we have identified some Ig light chain transcripts by transcriptomic analysis, including *lambda light chain*, *light chain type III*, and *kappa light chain*. Their antiviral functions remain to be further studied.

LYMPHOCYTE DEVELOPMENT AND HOMING

It is well known that mammalian equivalent of lymph nodes are absent in *Xenopus*. In addition, the bone marrow has only a minimal role in hematopoiesis, which occurs mainly in the liver and the spleen. Thus, the spleen serves as both a primary lymphoid organ especially for B-cell differentiation, and a secondary lymphoid organ where B and T cells accumulate. As in mammals, the thymus is the site of T-cell differentiation and maturation in *Xenopus*. The liver and kidney are also sites of lymphocytes and other leukocyte accumulation (19, 53, 81, 84, 85). In *A. davidianus*, in addition to the thymus, spleen, and liver, the kidney also appears to serve as a lymphopoietic organ (**Figure 1A**) (40).

T-Cell Differentiation

In jawed vertebrates, the differentiation and maturation of T lymphocyte primarily occur in the thymus (86). In gnathostomes, histogenesis of thymus has been described in four consecutive steps: (1) appearance of the thymus primordium; (2) colonization of the primordium by lymphocyte precursors, (3)
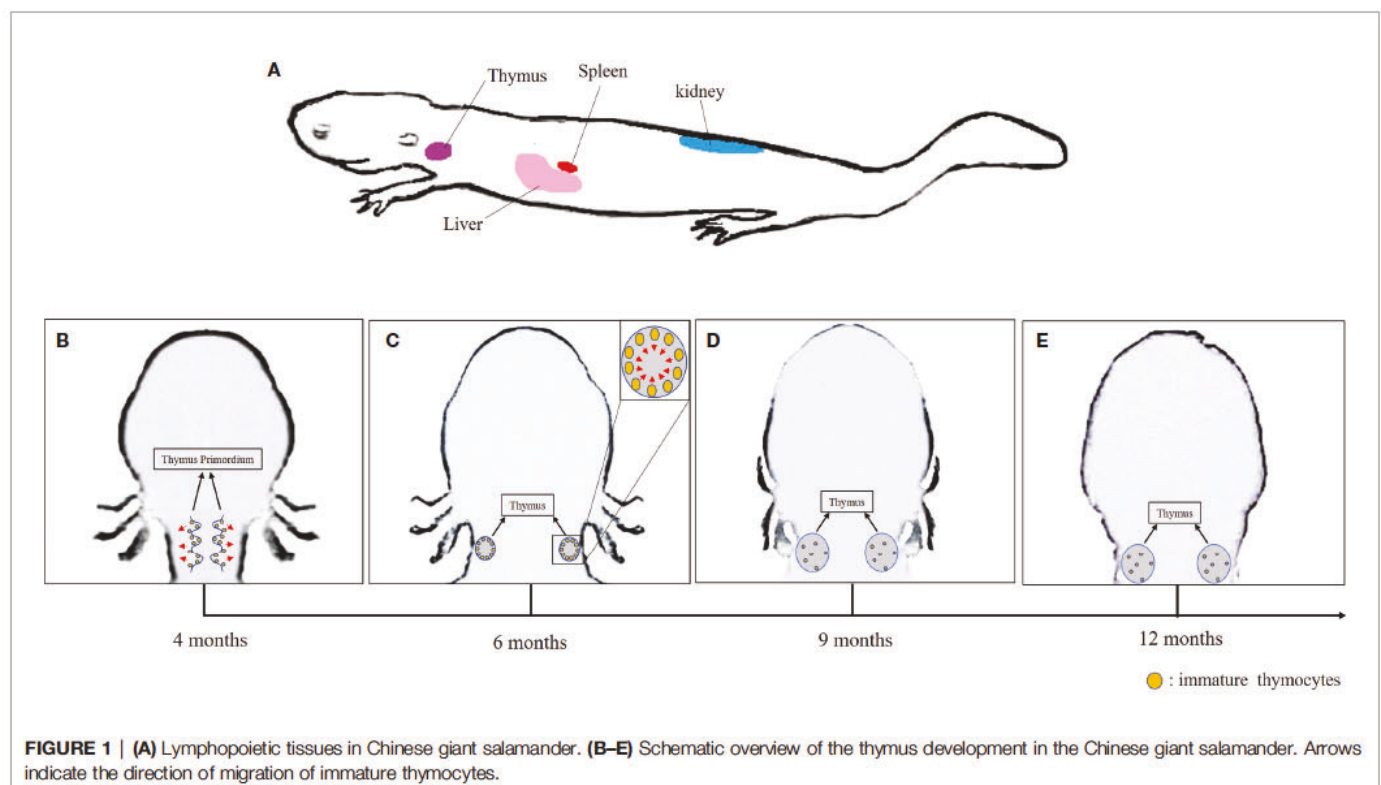

FIGURE 1 | (A) Lymphopoietic tissues in Chinese giant salamander. **(B–E)** Schematic overview of the thymus development in the Chinese giant salamander. Arrows indicate the direction of migration of immature thymocytes.

initiation of thymocytes differentiation and expansion; and (4) formation of cortex and medulla architecture (87). In *Xenopus*, the thymus primordium emerges at 3 days post-fertilization (dpf), discernable cortex and medulla regions appear at 6–8 dpf, while lymphocyte precursors colonization followed by thymocytes differentiation occurs at 5–7 dpf onward (88–90). During metamorphosis, the majority of tadpole thymocytes die, and the thymus is colonized by new stem cells that differentiate into adult type thymocytes and mature T cells distinct from larval T cells (15, 88). In urodeles that undergo incomplete metamorphosis, the lymphoid precursor cells colonize the thymic pharyngeal buds at 3–4 weeks; they then spread and distribute in the adjacent mesenchyme before migrating to the thymus epithelial primordium. The immature thymocytes accumulate under the capsula and develop in a centripetal manner (16).

As expected, the thymus is the main site for T-cell differentiation in the Chinese giant salamander as in other jawed vertebrates. Significant expression of *rag1* and *rag2* indicative of TCR rearrangements in immature thymocytes were detected in thymus primordium in 4-month-old salamanders, which is similar to other salamanders, whose B- and T-cell differentiate from 2 months to 8 months after fertilization (14, 40). In *Xenopus*, a transitory pause of *rag* gene expression occurs during metamorphosis, which might be correlated with tadpole thymocyte deletion to make room for new adult thymocyte generation (90). More research is required to determine whether any thymocyte deletion might happen in *A. davidianus* from hatching stage approximately 42 dpf to 4 months of age (91). Interestingly, along with the thymocyte differentiation, the spatial distribution of *rag1*, *rag2*, and *tcrβ* signals changes over time in *A. davidianus*. Both *rag* and *tcrβ* transcripts were observed in the marginal area of the thymus primordium of 4-month-old salamanders. These transcripts became predominantly distributed in the sub-capsular region over the medulla in 6-month-old salamanders, and disseminated over the cortex and medulla of thymus after 9 months of age (**Figures 1B–E**). Although the expressions of *rag1* and *tcrβ* were definitely detected in the kidney, liver, and spleen, the roles of these organs in T-cell differentiation and homing remain to be determined (40). It is known, for example, that mature thymic-derived T cells migrate to *Xenopus* spleen and zebrafish kidney through blood circulation where they remain stored (19, 92). Therefore, we speculate that immature T cells differentiate in thymus and then mature T cells immigrate and remain stored in the kidney, liver, and spleen of the *A. davidianus* (40).

B-Cell Differentiation

In contrast to mammals, the bone marrow is not the site for B-cell lymphopoiesis in most lower vertebrates. Instead, B-cell differentiation occurs in different tissues across jawed vertebrate species. The anterior kidney is the site for B-cell differentiation in most bony fish, whereas this is the spleen in urodels (90, 93, 94). In addition, the liver appears to serve as a transitory site of lymphopoietis in the Mexican axolotl (94). Both the liver and the spleen are considered to serve as sites of B-cell differentiation in *Xenopus* (19).

In the Chinese giant salamander, the spleen, kidney, and liver were identified as likely sites of B-cell differentiation. Notably, apparent lymphocyte differentiation was localized by *in situ* hybridization in adjacent zone of the blood vessel and the hematopoietic layer of the liver, and also at the periphery layer of the kidney (40). It is possible that the Chinese giant salamanders exhibit more sites of B-cell differentiation due to their evolutionary status between aquatic and terrestrial animals. As such, virus induces B-cell responses in Chinese giant salamanders may occur in multiple tissues (95). As an organized lymphoid structure, the *Xenopus* spleen is well delimited into a white pulp and red pulp (96). The white pulp has a lymphoid structure that is organized by concentric layers of lymphocytes surrounding a central artery (85). The white pulp is surrounded by red pulp that contains leukocytes and erythrocytes (97). In contrast to anuran amphibians, the red pulp and the white pulp are not well delimited in *A. davidianus* spleen (40).

ANTIVIRAL RESPONSE TO GSIV

GSIV infection of the Chinese giant salamander results in extremely high mortality (close to 100%) in both juvenile and adult salamanders. Infected salamanders show swollen head and neck, necrotic limbs, and hemorrhages on the skin (3, 6). Similar high mortality has been reported for ATV infection of larvae and adult tiger salamanders as well as for FV3 infection of *Xenopus* tadpoles (98, 99). During infection, transcript and protein levels of the GSIV major capsid protein (MCP) mainly increase in the kidney and spleen, which suggests that these organs are the major targets for GSIV replication (4). Similarly, the kidney is the main site of FV3 replication in adult frogs (13, 98). To determine whether the high susceptibility of *A. davidianus* to GSIV infection is due to an inefficient immune system, innate and adaptive immune responses need to be examined in more detail. We review below what has been undertaken to date.

Innate Immune Response to GSIV

To assess the innate immune response of *A. davidianus* against GSIV, changes in relative expression of several innate immune genes in adult animals were monitored during infection by quantitative real-time PCR. As a critical element in intracellular pathogen recognition, *TLR7* gene expression was induced in the kidney and liver as early as 6 h following infection with GSIV and at 48 h post-infection (hpi) in the spleen (21). Other PRRs, such as the CLRs and RLRs, were also induced following GSIV infection. Indeed, expression of *AdGalectin-1* was increased at 1 day post-infection (dpi) in the kidney and both *RIG-1* and *MDA5* transcript levels were increased as early as 12 hpi in the kidney and spleen (23, 66). Thus, the *A. davidianus* host innate immune system is able to detect and recognize viral infection. Changes in expression of downstream genes of the RLR pathway, such as *IRF3*, *type I IFN*, and *ISGs* genes during GSIV infection were also monitored. *IRF3* expression was increased in the spleen at 3 hpi (28). *Type I IFN* transcript levels were upregulated in peripheral blood leukocytes at 12 hpi,

while *Mx* transcripts levels were upregulated in the spleen at 6 hpi and in the kidney at 12 hpi (70, 71). Inflammatory genes such as *TNF-α* and *IL-1β* were significantly induced in the spleen at 3 dpi and in the kidney at 7 dpi, while the macrophage migration inhibitor (*mif*) gene was induced in the spleen at 3 dpi and in the kidney at 12 dpi. Collectively, these results suggest a faster and stronger inflammatory response in the spleen compared to the kidney following GSIV infection in the Chinese giant salamander (4). In adult *Xenopus*, FV3 infection also induces a rapid response (as early as 1dpi) of *type I IFN* and *Mx1* and *2*, as well as pro-inflammatory and inflammatory-related genes (*IFNγ*, *IL-1β*, and *TNF-α*) in the kidney (100, 101). In contrast, delayed and lower transcription of *IL-1β*, *TNFα*, and *IFNγ* genes was observed in FV3-infected tadpoles compared to adult frogs, and *type I* and *type III IFN* gene responses of tadpoles were distinct from adult frogs (100, 102–104). Overall, these results suggest that the innate immune response is efficiently triggered upon RV infection in the giant salamander as in amphibians and that there is no obvious defect of detection and defense by the innate immune system.

The genomic structure of *type I IFN* genes is different between higher and lower vertebrates. In mammals, birds and reptiles, *type I IFNs* genes are intron-less and are clustered in the same chromosome, whereas bony fish *type I IFNs* genes typically contain introns. *Xenopus* present an interesting intermediary state in the evolution of *IFN* genes by having expended families of both intron-less and intron-containing type I *IFN* genes (105). The Chinese giant salamander *type I IFNs* appear to consist of only intron-containing genes, which would be consistent with their ancient evolutionary origin antecedent to that of anuran amphibians such as *Xenopus*.

To further determine the ability of type I IFN to provide resistance to GSIV infection, recombinant Chinese giant salamander type I IFN and Mx1 were assayed *in vitro*. Transfected overexpressions of *type I IFN* and *Mx* genes in Chinese giant salamander cell lines led to inhibition of GSIV replication, which demonstrates their antiviral activity against GSIV (70, 71). Additionally, *IFNβ* and *ISGs* (*ISG15* and *Mx* genes) transcript levels were all decreased in *AdIRF3*-silenced leukocytes, which implicated AdIRF3 in the regulation of IFN signaling and ISG production (28). Using *in situ* hybridization, significantly less *MCP* transcripts were detected in the spleen from 7 dpi to 15 dpi, which suggested that high expressions of *TNF-α*, *IL-1β*, and *mif* genes might help to reduce GSIV replication (4). In adult *Xenopus*, FV3 clearance typically occurs within 2 weeks post-infection (106). Macrophages and NK cells are critical innate cell effectors in *Xenopus* host response during early stages of FV3 infection, and increased expression of *IL-1β*, *TNF-α*, *Mx1*, and *IFN-γ* genes was detected in infected tissues and leukocytes of adult frogs (13, 100, 107). These data reveal that the innate immune system functionally and critically contributes to reduce RVs replication in amphibians. However, RVs are able to overcome this initial innate host defense both in the Chinese giant salamander and *X. laevis* tadpoles.

Another innate effector system that may play a significant role in RVs defense is AMP secretion. In *Rana catesbeiana* and *Rana pipiens*, some AMPs can inactivate FV3 by direct contact or increase resistance to infection by enhancing immune responses (24, 108). In tiger salamanders, ATV-induced viral plaques in cell culture were reduced by some mixtures of AMPs, but the effects were different among these preparations (109). Skin AMPs from Hellbender also have detectable *in vitro* antiviral activity, albeit insufficient to provide effective protection to RVs (76). Five putative mature cathelicidins, which belong to an important family of AMPs, are predicted from transcriptomic data in the Chinese giant salamander skin, but their antiviral role remains to be investigated (24).

Adaptive Immune Response to GSIV

The critical roles of MHC class I antigen presentation and activation of antiviral cytotoxic CD8 T cells have been rigorously demonstrated in *X. laevis* (13, 110). Likewise, the suboptimal function of classical MHC class I presentation in tadpoles until metamorphosis may explain in part the higher susceptibility of *X. laevis* tadpoles to FV3 (88, 100).

As mentioned above, 70 MHC alleles and multiple novel splice variants have been characterized in *A. davidianus*. Notably, full-length transcripts and individual splice variant isoforms of MHC class IA, class IIA, and class IIB have been found to be upregulated after GSIV infection, consistent with crucial roles of both MHC class I and class II molecules in host antiviral responses (29).

T- and B-lymphocyte responses correlate with viral clearance in adult *X. laevis* (13). The virus susceptibility of adult *X. laevis* was significantly increased after depletion of CD8 T cells, demonstrating the crucial role of CD8 T cells in anti-FV3 response (110). Upon primary ranaviral infection, the proliferation of CD8 T cells in the spleen and accumulation in the kidney appeared from day 6 onward, which was associated with FV3 clearance. During a secondary FV3 infection, earlier but lower proliferation and infiltration of CD8 T cell in parallel with faster clearance of FV3 was detected compared to the primary infection. These data provide evidence that CD8 T-cell memory and protective response are both involved in *X. laevis* immune defenses against FV3 (13, 110). According to microarray technology, ATV-infected axolotl showed upregulation of innate immune-related genes rather than adaptive immune-related genes (111). Consistent with the study in axolotl, expression levels of *CD8alpha* and *CD8beta* genes were downregulated in GSIV-infected Chinese giant salamander, suggesting a weak or inhibited CD8 T-cell response (26). However, more evidence is needed to establish whether the CD8 response is overcome, counteracted by GSIV, or inherently weak. Moreover, in *X. laevis*, an invariant T (iT) cell subset (iVα6), which is restricted by the MHC class I-like molecule XNC10 and expresses the invariant rearrangement Vα6-Ja1.43, is also critically involved in antiviral responses (101, 112, 113). In tadpoles, abrogation of Vα6 iT cell development significantly increased viral replication and host mortality at early stage of FV3 infection (101, 112). In adult *Xenopus*, the lack of Vα6 iT cells resulted in a less effective antiviral response, such as delayed viral clearance and more

FV3-induce kidney damage (113). In addition, transitory depletion of Vα6 iT cells resulted in delayed interferon and cytokine genes responses and long-lasting negative inability to control FV3 infection in tadpoles, which further supported the important role of Vα6 iT cells in *Xenopus* immune surveillance system (114). Although iT cells have been demonstrated in *Xenopus* and mammals (112, 115), they are still unknown in *A. davidianus*.

While viral clearance may not be as efficient as *Ambystoma mexicanum* (4, 39), studies to date have not revealed an obvious lack of antiviral innate and adaptive immune response in *A. davidianus*. As such, it would be premature to conclude that the salamander immune system is deficient. Indeed, elicitation of effective and durable protective immunity has been obtained by vaccination in *A. davidianus* (93, 116, 117). Priming giant salamanders with self-assembly of the major capsid produced in the yeast expression system into virus-like particles induced long-lasting neutralizing antibodies and resulted in more than 50% protection of these animals to GSIV infection. These important findings imply that the high susceptibility of the Chinese giant salamander to GSIV is not due to intrinsic weakness of its immune system but perhaps rather the result of the recent spill out and transmission of a virulent ranavirus, that is able to overcome the host immune responses.

Immune Evasion Strategies of RVs

Although evidence of immune evasion of GSIV is lacking, RV immune evasion strategies have been explored. Immune evasion genes are usually defined as genes that impair host antiviral responses and facilitate virus proliferation. Candidate immune evasion genes in RVs include the α subunit of eukaryotic initiation factor eIF-2 (vIF-2α), the caspase recruitment domain-containing protein (vCARD), hydroxysteroid dehydrogenase homolog (vHSD), the tumor necrosis factor receptor (vTNFR), the RNase III-like protein, and cytosine DNA methyltransferase (vDMT) (13). By analogy to a similar immune evasion in vaccinia virus, vIF-2α can partially inhibit phosphorylation of host eIF-2α by competitive binding to the antiviral protein kinase PKR and thus interfering with the host cell protein synthesis. vHSD is postulated to trigger glucocorticoid synthesis and enhance virus replication through suppression of the immune responses. vCARD may bind RIG-I/MDA5 and/or MAVS/ISP-1 followed by inhibition of interferon induction and/or apoptosis. The RNAse III-like protein has been postulated to block siRNA-mediated interference or to process viral mRNAs. The viral RNAse III can bind and/or degrade dsRNA and block the activation of PKR (118). Disruption of the eIF-2α gene in ATV resulted in increasing pathogenicity and sensitivity to interferon of the recombinant virus, indicating that *vIF-2α* gene is likely immune evasion gene (119). Interestingly, the disruption of the truncated vIF-2α in FV3 also led to reduced viral replication, increased survival and apoptosis, and higher sensitivity to IFN treatment, which suggests that in addition to blocking the activation of PKR, *vIF-2α* contributes to RV immune evasion through other unknown mechanisms (120, 121). Deletion of vCARD from FV3 resulted in low levels of viral replication as well as higher level of apoptosis and cell mortality *in vitro*, which suggested that vCARD might

participate in the regulation of apoptosis. Furthermore, replication of vCARD mutant FV3 was affected in *Xenopus*-IFN treated cells, indicating that vCARD might contribute to interfering IFN response (121). HSD mutant FV3 also resulted in reduced viral replication and mortality but did not lead to a higher level of apoptosis, suggesting that vHSD contributes to viral pathogenesis (121). An additional immediate-early gene, 18K, of unknown function but conserved among RVs, is potentially involved in virulence. Compared to wild type, recombinant FV3 mutants with 18K deletion were found to induce more apoptosis and to replicate less, but also to be more resistant to rXlIFN inhibition, which suggests that 18K that may contribute to virulence and even immune evasion by regulating timely FV3 gene expression and release (121). Only few GSIV viral genes have been investigated to date and their roles of immune evasion are still unclear. Overexpression of GSIV *13R* gene that encodes a viral non-structural protein containing a transmembrane domain (TMD) and a restriction endonuclease-like domain did not affect viral replication (122). In contrast, overexpression of GSIV *1R* gene that encodes virus late transcription factor-3 like (VLTF3 like) domain induced viral replication and promoted cell proliferation (123). Functional studies of GSIV genes and gene products are urgently needed not only for a better characterization of GSIV immune evasion strategies, but also for a broader understanding of *A. davidianus* immune responses.

CONCLUSION AND PERSPECTIVES

Although frogs and salamanders have colonized similar aquatic environment, the morphogenesis of their lymphopoietic tissues is distinct and it is likely that their immune systems exhibit differences too. However, in contrast with earlier studies implying a weak or even deficient immunity in salamanders, recent genomic and transcriptomic studies indicate that all the critical genetic elements of the innate and adaptive immune system are present in salamanders, suggesting that salamanders are not inherently immune defective. This is underscored by host responses to RV pathogens. Although salamanders are highly susceptible to RV infection, the fact that effective vaccination can be obtained in the giant salamanders demonstrates their immune competence, and rather implies that virulence and immune evasion factors allow GSIV pathogens to overcome their immune defenses. Thus, to advance both fundamental and applied immunology of salamanders and especially the giant salamander, it is important in our view, to integrate new genomic and transcriptomic technology with functional studies in the context of current infectious diseases caused by ranaviruses.

To date, major progress of recent studies in Chinese giant salamander immunology have been mainly focused on molecular identifications, while functional exploration has just begun. Useful immune gene sequence information has been obtained by high-throughput deep sequencing technologies, but due to a lack of genome sequences, antibodies, and reverse genetics applied to GSIV and salamanders, gene function often remains speculative compared to other amphibian models such as *Xenopus*. From our perspective, further research on Chinese

giant salamander including better resources and tools must be implemented to increase a full understanding of the variation, adaptation, and plasticity of the immune system in jaw vertebrates as well as to achieve a better control of infectious disease caused be ranavirus worldwide. Among critical gap of knowledge imperative to fill, we have highlighted in this review the following: (1) when and where B lymphocyte and antibody secreting B cell develop and reside; (2) which immune cells present viral antigens; (3) importance of CD8 T-cell responses against ranavirus; (4) whether iT cells represent an important fraction of T cells like in *Xenopus* tadpoles and are critical in host defense against ranavirus; and (5) mechanisms of viral immune evasion.

In conclusion, future research on immune system of the giant salamander will not only contribute to understand the evolution of vertebrate immune systems, but also provide defensive strategy against virus disease in artificial culture.

AUTHOR CONTRIBUTIONS

NJ contributed ideas and drafted the first full version of the manuscript. JR and LZ revised and edited a later version of the manuscript. All authors contributed to the article and approved the submitted version.

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