

Ranavirus Host Immunity and Immune Evasion

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1 Introduction

Infections of ectothermic vertebrates by members of the genus *Ranavirus* (RV; family *Iridoviridae*) and the resulting disease outbreaks and die-offs among wild and farmed populations have escalated at alarming rates recently and raised considerable concerns. While it is apparent that individual teleost, amphibian, and reptile species vary in their susceptibility to these pathogens, the immune and viral determinants of ranaviral diseases are at present unclear. In fact, with the rapid rise in both the prevalence of ranavirus infections and the remarkable capacity of these viruses to infect new hosts, ranaviruses such as *Frog Virus 3* (FV3) are now considered to be a potential global threat to poikilothermic populations (Gray and Miller 2013). There is a pressing need to determine whether the susceptibility of a given ectothermic species reflects its inability to mount a protective antiviral immune response or the capacity of the ranavirus to overcome otherwise intact immune barriers. Indeed, ranaviruses appear to possess an array of immune evasion and host modulation mechanisms. Thus, a more thorough examination of the ranavirus-host immune interface at the molecular and cellular levels is necessary in order to devise potential preventative measures against these viral agents.

Compared to mammals, ectothermic vertebrates possess a complex immune system, but they mount relatively less effective adaptive immune responses. Ectotherms display poorer T lymphocyte expansion, fewer antibody isoforms, and

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generally a less developed immunological memory response than mammals (Robert and Ohta 2009). Thus, these organisms may rely more heavily on innate immune responses to facilitate clearance of pathogens such as ranaviruses. Innate antiviral immune defenses in ectothermic vertebrates are distinct from those described in mammals, although the exact contribution and efficacies of this immune response need to be elucidated in the context of ranavirus infections.

This chapter summarizes recent advances in our understanding of the contributions of innate and adaptive immune responses to the elimination and/or progression of ranaviral infections in poikilotherms as well as an overview of the strategies of these pathogens for evading host immune barriers.

2 Innate Immune Responses to Ranavirus Infections

2.1 Antimicrobial Peptide Responses to Ranaviral Infection

Antimicrobial peptides are an important element of anuran innate immunity that provides protection to skin and mucosal surfaces against a variety of pathogens. These small molecules are synthesized and stored in the dermal granular glands and secreted into mucus in response to stress or injury (Rollins-Smith 2009; Rollins-Smith et al. 2005). Antimicrobial peptides are also involved in defenses against ranaviruses. Esculentin-2P (E2P) and Ranatuerin-2P (R2P), two antimicrobial peptides isolated from *Rana pipiens*, are capable of inactivating both FV3 and channel catfish virus (CCV) within minutes and at temperatures as low as 0 °C. This suggests that direct interaction of these molecules with the viruses rather than inhibition of viral replication is responsible for the drop in infectivity (Chinchar et al. 2001). The ability of antimicrobial peptides to function across a broad range of temperatures presumably reflects the ectothermic nature of the host. Notably, 50 µM of E2P or R2P was sufficient for 99% inactivation of CCV, whereas a ten times greater concentration of either peptide was necessary to achieve 90% inactivation of FV3 (Chinchar et al. 2001). It was postulated that the greater resistance of FV3 to inactivation reflected the difficulty of antimicrobial peptides to target the inner lipid membrane beneath the FV3 capsid. Presumably, this inner membrane requires disruption in order for viral inactivation to occur. Other antimicrobial peptides, including Ranatuerin-2YJ, Dybowskin-YJb, Dybowskin-YJa, Temperin-YJa, and Temperin-YJb have been identified and cloned from the skin of *Rana dybowskii* infected with Rana grylio virus (RGV) (Yang et al. 2012). Interestingly, all of these peptides conferred concentration-dependent inhibition of RGV plaque formation, while viral clearance coincided with increased expression of genes encoding these molecules (Yang et al. 2012).

2.2 Innate and Inflammatory Immune Responses

Ranaviral infections are widely associated with prominent host inflammatory responses. Indeed, akin to mammalian viral infections, ranavirus-elicited inflammatory responses represent a double-edged sword as they are both critical for viral clearance, but may also exacerbate ranavirus-mediated disease and adversely affect host survival. As it stands, there is substantial documentation of innate immune responses and associated inflammation to ranavirus infections across a range of poikilothermic host species (Carey et al. 1999; Chen and Robert 2011; Grayfer et al. 2014; Jancovich and Jacobs 2011; Morales et al. 2010).

2.2.1 Anuran Amphibians

Our research group has adopted and optimized the infection of *Xenopus laevis* by FV3 as a model for ranavirus-ectothermic vertebrate (particularly anuran) antiviral immunity. This model pairs FV3, the best-described ranavirus at the molecular level, with *Xenopus laevis*, which possesses the most-characterized amphibian immune system. Typically, our experimental approach involves the intraperitoneal (i.p.) injection of *X. laevis* adults or tadpoles with FV3 followed by an assessment of the progress of infection, viral replication, and the host immune response. Although similar immune responses may be obtained in *Xenopus* by using i.p. injection or water bath exposure (Robert et al. 2011), the former tends to be more convenient and consistent for immunological studies. By this approach, we have been able to delineate the sequential progression of the innate and adaptive immune responses of adult *X. laevis* throughout the course of FV3 infection (Morales et al. 2010). In *X. laevis* adults, histochemical and flow cytometric analyses revealed that activated mononuclear and polymorphonuclear phagocytes are recruited to, and heavily represented within peritoneal exudates as early as 1 day following i.p. infection (Morales et al. 2010). We subsequently observed the peritoneal recruitment and accumulation of natural killer (NK) cells by 3 days after infection, whereas lymphocyte recruitment, including the increased presence of T cells, was not observed until 6 days post-FV3 challenge (Morales et al. 2010). Notably, the rapid accumulation of peritoneal leukocytes was concomitant with substantially elevated inflammatory gene expression. Among the hallmark inflammatory genes examined, we observed significant increases in the expression of *X. laevis* tumor necrosis factor- α (TNF- α) as early as 1 day postinfection and persisting to 3 days after FV3 exposure (Morales et al. 2010). Expression of the interleukin-1-beta (IL-1 β) gene, encoding an early proinflammatory cytokine produced by macrophages, was elevated at days 1 through 6 of FV3 challenge, while the anti-inflammatory arginase-1 (Arg-1), a marker of alternatively polarized (M2) macrophages (Joerink et al. 2006b, c), was elevated at day 1 post-viral challenge and subsequently decreased (Morales et al. 2010). Together these findings show an effective and well-coordinated antiviral immune response,

with sequential recruitment of innate and adaptive immune cell effectors and corresponding immune gene activation. The elevated level of Arg-1 gene expression at 1 day postinfection may be reflective of resident, rather than recruited inflammatory myeloid populations. Indeed, subsequent to FV3 peritoneal inoculation, we have consistently observed elevated mRNA transcripts for macrophage and granulocyte colony-stimulating factor receptors (M-CSFR and G-CSFR, respectively), indicative of accumulating myeloid infiltrates (L. Grayfer and J. Robert, University of Rochester, unpublished data). Notably, the elevated expression of M-CSFR (and G-CSFR) within peritoneal leukocytes (PLs) is typically accompanied by significantly increased expression of the M1 macrophage marker, inducible nitric oxide synthase (iNOS), which catalyzes the production of the antimicrobial nitric oxide by inflammatory macrophages (L. Grayfer, F. De Jesús Andino and J. Robert, University of Rochester, unpublished data). This supports the observation of decreased Arg-1 expression with the onset of an inflammatory state within the peritoneum and indicates that Arg-1 and iNOS functions are opposite across multiple groups of vertebrates (Joerink et al. 2006a, b, c; Wiegertjes and Forlenza 2010).

It is important to emphasize that amphibian susceptibility to ranaviruses varies considerably among species, their respective stages of development, and even between different populations of the same species (Miller et al. 2011). These differences likely result from multiple complex determinants including host and ranavirus genetic variability as well as the respective host immune status. Extensive immunological studies of *Xenopus* suggest that tadpoles possess a distinct immune system from that of adults. The larval system is typically more immature, particularly with regard to adaptive immunity (e.g., poor T cell and antibody responses). In this regard, it is altogether not surprising that tadpoles are typically unable to fully control ranavirus infections and succumb to these pathogens (Bayley et al. 2013; Grayfer et al. 2014; Hoverman et al. 2010; Landsberg et al. 2013; Reeve et al. 2013). However, it is clear that in some amphibian species, adult frogs also die from ranavirus infections (Sutton et al. 2014). Indeed, several reports indicate that compared to larvae of given amphibian species, metamorphic (Brunner et al. 2004; Haislip et al. 2011; Reeve et al. 2013) and adult (Duffus et al. 2013) animals may be more susceptible to ranaviruses. Given the rapid development of tadpoles and their drastic metamorphic remodeling, species-specific immune development pathways may explain differences in ranavirus susceptibility. Extensive immune remodeling may render metamorphs more susceptible than larvae at critical developmental stages. Moreover, the fact that ranavirus-infected *Xenopus* tadpoles bear lower viral loads than adults, yet succumb more readily to FV3 infection (Grayfer et al. 2014) suggests that it may be more difficult to detect this virus in tadpoles than adults or that lower viral loads trigger markedly greater disease in tadpoles.

To delineate possible inefficiencies in innate immune responses of anuran tadpoles that may account for ranavirus susceptibility, we performed an extensive comparison of immune gene expression patterns between FV3-infected *X. laevis* tadpoles and adults (De Jesús Andino et al. 2012). In contrast to infected adult frogs, tadpoles exhibited poor and considerably delayed anti-FV3 inflammatory gene responses (De Jesús Andino et al. 2012). TNF α , IL-1 β , and IFN γ gene expression

in tadpole PLs, splenocytes, and kidneys did not significantly increase until 6 days post FV3 challenge, which is in contrast with the robust and quick (1 dpi) upregulation of these genes in infected adults (De Jesús Andino et al. 2012). Notably, stimulation of tadpoles with heat-killed *Escherichia coli* readily elicits rapid induction of the above genes within 24 h, suggesting that the immune delays are FV3 specific (De Jesús Andino et al. 2012). These inefficiencies in the tadpole innate immune response to FV3 may reflect multiple nonexclusive issues including viral immune evasion, defect(s) in the tadpole pathogen sentinel receptor system, or physiological tradeoffs to forego energetically costly inflammatory responses in favor of growth and development. Thus, these modest and delayed immune responses are likely contributing factors for the characteristically higher susceptibility of anuran tadpoles to FV3 infection and severe disease.

2.2.2 Urodel Amphibians

Consistent with the notion that hosts mount broad inflammatory responses to ranaviruses, a comprehensive microarray analysis of axolotls (*Ambystoma mexicanum*) infected with the *Ambystoma tigrinum virus* (ATV) revealed the upregulation of numerous hallmark pro-inflammatory and innate immune gene components in the spleens and lungs of these animals (Cotter et al. 2008). These genes included (but were not limited to) phagocytic receptors and intracellular components, cytokine signaling molecules, complement components, NADPH oxidase subunits (myeloid enzyme catalyzing the reactive oxygen antimicrobial response), and myeloperoxidase (granulocyte enzyme catalyzing the production of hydrogen peroxide) (Cotter et al. 2008). In contrast to what has been observed in *X. laevis* infected with FV3 (Morales et al. 2010; Morales and Robert 2007), no lymphocyte proliferation genes were upregulated in response to ATV infections (Cotter et al. 2008). This lack of an efficient adaptive response in this species may explain why ATV is so lethal to urodels. Alternatively these observations may reflect a different infection strategy by ATV.

2.2.3 Teleost Fish

There is a substantial literature documenting innate immune and associated inflammatory responses to ranavirus infections in bony fish. Infection of the Epithelioma papulosum cyprini (EPC) teleost cell line with four distinct ranaviruses, FV3, European catfish virus (ECV), Doctor fish virus (DFV), and *Epizootic haematopoietic necrosis virus* (EHNV), resulted in distinct inflammatory gene expression profiles (Holopainen et al. 2012). Specifically, EHNV and FV3 elicited expression of the hallmark pro-inflammatory genes, TNF α and IL-1 β , whereas ECV and DFV induced the transient expression of a generally immunosuppressive gene, transforming growth factor-beta (TGF β) (Holopainen et al. 2012). Interestingly, all four viruses elicited expression of apoptotic components and β 2-microglobulin, which is critical for surface MHC class I expression and cytotoxic T cell function, suggesting

that at least with respect to FV3 infection of teleosts (as compared to axolotls), the adaptive immune response may be elicited by ranaviral infections.

A recent microarray study examined the transcriptional response of fathead minnow (FHM) cells following infection with either wild type (wt) FV3 or a knockout (KO) mutant lacking the truncated vIF-2 α gene. Infection with wt FV3 resulted in the upregulation of numerous immune related genes by 8 h p.i., including IL-8, IFN, IFN regulatory factor (IRF) -1, -2, and -3, IL-1 β , etc. For the most part, similar genes were upregulated in cells infected with the KO mutant, but the magnitude of the induction was generally lower (Cheng et al. 2014).

2.3 *Determinants of Ranavirus-Induced Pathogenicity and Mortality*

An appropriate, timely resolution of inflammation is just as important as the induction and progression of this response, because a prolonged inflammatory response increases the risk of tissue damage and host death (Fullerton et al. 2013). Although sparse, there is evidence suggesting that ranavirus infections may exacerbate inflammatory responses, accounting for some of the observed ranavirus pathology. For example, in 1997 a novel iridovirus was isolated in Saskatchewan (Canada) from larval tiger salamanders (Bollinger et al. 1999). These animals suffered from exacerbated inflammation, necrosis, and characteristic ranavirus-induced cytoplasmic inclusions within splenic, renal, lymphoid, and hematopoietic tissues (Bollinger et al. 1999). Similarly, whole populations of ranavirus-infected green-striped tree dragons (*Japalura splendida*) exhibited systemic hemorrhaging, necrosis, granulomatous, and necrotic inflammation, as well as severe renal pathology, hyperanemia, and extensive hepatic damage (Behncke et al. 2013), culminating in mass mortality. Ranavirus infection within pythons suggests that inflammation may be a determinant of ranaviral pathology (Hyatt et al. 2002). Mortality of largemouth bass infected intraperitoneally with largemouth bass virus (LMBV) is believed to result from virally induced inflammation and associated necrosis (Zilberg et al. 2000). Consistent with these inflammatory symptoms, juvenile bass inoculated with LMBV exhibited corkscrew swimming and distended abdomens (Zilberg et al. 2000). Notably, the deeper tissues of infected fish were unaffected, bringing into question whether virus-induced damage was due to target cell accessibility or the limitations of LMBV cell tropism. The latter suggests that inflammation and necrotic damage resulting in mortality may be the result of primary injuries at the initial sites of infection. Indeed, the above observations are reminiscent of earlier studies of FV3 infections in rodents (Gut et al. 1981; Kirn et al. 1980, 1982), in which, despite the inability of FV3 to replicate at 37 °C (Aubertin et al. 1973), the initial viral inoculum was responsible for extensive inflammation, necrosis, and liver damage.

Our recent findings in *Xenopus* support the induction of a pro-inflammatory response by FV3 (Grayfer et al. 2014). Mindful of the idea that *X. laevis* adults presumably mount effective anti-ranaviral responses leading to viral clearance, we were intrigued to find that (at least during acute infections) adults possessed significantly greater FV3 loads (1 to 2 orders of magnitude) than tadpoles, which are typically more susceptible to FV3 infections (Grayfer et al. 2014). Also, a possible influence of temperature on viral loads was observed in wood frog tadpoles, where these animals succumbed to infection quicker at 25 °C but at higher viral loads than those maintained at 15 °C (J. Chaney and M. Gray, University of Tennessee, unpublished data). These results suggest that FV3 virulence is not strictly dependent on the magnitude of viral replication. Additionally, immunocompetent tadpoles may be more vulnerable to ranaviral virulence factors and other environmental parameters than adults. In support of this hypothesis, we observed that although tadpoles pre-stimulated with recombinant *X. laevis* type I interferon (r χ IFN) possessed viral loads several logs lower than adults, they nonetheless succumbed to FV3 infection (Grayfer et al. 2014). Furthermore, despite lower FV3 loads, IFN-treated larvae experienced damage to multiple organs, including extensive loss of tissue architecture and cellular organization through necrosis and apoptosis, albeit without extensive leukocyte infiltration (Grayfer et al. 2014). Therefore, even at markedly reduced viral loads, ranaviruses may confer irreversible tissue damage in tadpoles relatively early in infection, resulting primarily from virus-mediated cytopathology rather than from viral replication. Indeed, as seen in rodent models of FV3, ranaviruses may trigger toxic and potentially lethal effects, irrespective of their capacity to replicate within their host cells (Gendrault et al. 1981). This notion has recently been substantiated with Grouper iridovirus (GIV). Replication-deficient UV-inactivated GIV induced apoptosis in two of the three infected cell lines (Pham et al. 2012). Similarly, heat- and UV-inactivated FV3 elicits FHM cell apoptosis and inhibits host RNA and protein synthesis (Chinchar et al. 2003; Raghov and Granoff 1979). Based on these findings, we hypothesize that inoculation of animals with sufficient inactivated virus will induce toxicity in the absence of virus replication. If this hypothesis holds true for other members of the genus and family, we may need to consider that these viruses are more pathogenic than previously thought.

3 The Complex Roles of Macrophage-Lineage Cells in Ranaviral Disease

3.1 Inferences from Rodent Models of FV3 Infection

The involvement of macrophage-lineage cells in ranavirus infections may be inferred from initial studies conducted 30 years ago using rodents as models of hepatitis (Gut et al. 1981; Kirn et al. 1980, 1982). These early studies revealed that Kupffer cells (liver macrophages) were the principal targets of FV3 infection and

that their death was linked to the loss of hepatic clearance and culminated in severe hepatitis and mortality (Gut et al. 1981). These studies also implicated inflammation as a contributor to FV3-mediated pathology, including extensive leukotriene release by Kupffer cells (Hagmann et al. 1987). Inhibition of leukotriene synthesis within FV3-infected animals dramatically reduced virally elicited hepatic damage (Hagmann et al. 1987), suggesting that pathology was largely due to inflammatory responses.

Although FV3 is not a mammalian pathogen and, with the exception of expression of select early genes (Lopez et al. 1986) does not replicate at 37 °C (Aubertin et al. 1973), this work nonetheless supports the current hypothesis that, because of their high phagocytic and endocytic activity, macrophage-lineage cells are integral targets for ranavirus infections. In fact, the absence of replication at 37 °C may be viewed as an advantage for investigating the mechanisms of FV3 cell entry. In cultured rat Kupffer cells, viral particles appeared in phagocytic vacuoles and endocytic compartments promptly following FV3 infection (Gendrault et al. 1981). Moreover, a substantial proportion of FV3 virions that attached to cells displayed viral capsid-host membrane fusion and release of viral core contents into cell cytoplasm (Gendrault et al. 1981). This observation suggests that the underlying mechanisms governing ranavirus entry are universal and facilitate entry of cells from organisms as evolutionarily distant as mammals, fish, and amphibians. In line with this reasoning, it is likely that cells of the myeloid lineage serve as ranaviral targets precisely because of their high efficiency of ingestion of extracellular materials, facilitated by an array of endocytic/phagocytic surface receptors, several of which likely recognize and bind ranaviruses. This feature of vertebrate professional phagocytes may have been targeted as a ranaviral infection strategy and may explain why ranaviruses successfully cross host species boundaries. Furthermore, because ranaviruses cannot replicate at mammalian body temperatures, the above-described literature implies that the pathological events seen in FV3-infected rodents are not the result of full virus replication. Instead, cell death is presumably triggered by preformed lytic factors encapsulated within FV3 virions or the expression of early viral gene products (Lopez et al. 1986). Similar to the mRNA present within adenovirus virions (Chung et al. 2003), FV3 early gene expression at nonpermissive temperatures may also result from the release of prepackaged virulence factor-encoding mRNAs rather than from de novo viral transcription. Indeed, FV3 infection of mammalian cells induces rapid cellular RNA, DNA, and protein synthesis arrest (Elharrar et al. 1973). Furthermore, factors solubilized from FV3 virions result in cellular toxicity and inhibit host macromolecular synthesis (Aubertin et al. 1973; Kim et al. 1972).

3.2 Amphibian Vectors of Ranaviral Dissemination and Persistence

Increasing evidence from natural ranavirus infections of amphibians supports the idea that macrophages are important not only for antiviral defense, but also to ranavirus infection strategies. We utilized FV3 infection of *X. laevis* as a platform for

the study of the ranavirus-host immune interface, with converging lines of evidence from our past and current work confirming FV3-*X. laevis* macrophage interactions. We have demonstrated that FV3 persists within amphibian hosts for several months following the resolution of clinically apparent disease (Robert et al. 2007). In addition, FV3 DNA could be detected in healthy animals that were not infected in the laboratory. This suggests that FV3 adopts some form of quiescence as a means of maintenance within immuno-competent hosts. Notably, FV3 effectively infects frog peritoneal leukocytes both *in vitro* and *in vivo*, persists within these cells and undergoes active viral transcription for up to 12 days subsequent to infection (Robert et al. 2007). Since peritoneal leukocytes are comprised predominantly of macrophage-lineage cells, our findings not only corroborate the ranavirus-macrophage tropism, but also suggest that these terminally differentiated, long-lived populations are ideal vectors for viral dissemination, or serve as “within host” reservoirs.

The above hypothesis has been substantiated by our subsequent transmission electron microscopy analysis of FV3-infected *X. laevis* peritoneal leukocytes in which we detected icosahedral virus particles in peritoneal leukocytes bearing macrophage morphology (Morales et al. 2010). These FV3-infected cells exhibited small numbers of intracellular viral particles, implying that FV3 may employ mononuclear phagocytes as a reservoir for dissemination. FV3-macrophage interaction is reminiscent of the HIV-macrophage relationship in which viral particles accumulate within the myeloid cells as a mechanism of dissemination (Coiras et al. 2009; Goodenow et al. 2003; Gousset et al. 2008; Groot et al. 2008). Interestingly, several of these FV3-infected peritoneal macrophages not only contained cytoplasmic virions, they also shed virions into the extracellular milieu (Morales et al. 2010), confirming that these cells likely function as both reservoirs and vectors of viral dissemination within their hosts.

In general, i.p. infection of adult *X. laevis* with FV3 leads to extensive recruitment of leukocytes, including a large number of cells with macrophage morphology, to the site of infection (Morales et al. 2010). The myeloid origins of these leukocytes are supported not only by their expression of macrophage inflammatory genes (TNF α , IL-1 β , and Arginase-1; Morales et al. 2010), but also of the macrophage-lineage marker, M-CSFR (L. Grayfer, F. De Jesús Andino and J. Robert, University of Rochester, unpublished data). Interestingly, while we have been able to amplify FV3 DNA from peritoneal leukocytes isolated from virus-infected frogs up to 21 days postinfection, FV3 early and late transcripts were detected at 6, but not 15 or 21 days postinoculation (Morales et al. 2010). This suggests that the viral genome is maintained within macrophage-like cells in a state of dormancy. Possibly, the inability to detect FV3 genomes among peritoneal leukocytes at later times may reflect the dissemination of these cells to distal sites within the *X. laevis* host.

In *Xenopus*, the kidney represents a focal site of FV3 replication. Interestingly, active FV3 gene transcription is seen in some, but not all FV3-infected frogs for up to 9 days after infection, whereas viral genomic DNA may be amplified from some of these animals 2 weeks subsequent to viral challenge (Morales et al. 2010). Notably, this interval is shorter than the 3-week period during which viral persistence is reliably detected within peritoneal leukocyte populations. These differences possibly reflect

the distinct interactions between FV3 and these various cell targets. Presumably, kidney cells are productively infected and serve as sites of active FV3 replication, indicated by high viral titers and extensive tissue damage (Grayfer et al. 2014). Conversely, macrophage-lineage cells are terminally differentiated, nondividing, long-lived cells, which ostensibly serve as reservoirs for dormant (non-replicating) ranavirus.

Collectively, these observations implicate frog macrophage-lineage cells as central targets of FV3 infection, serving as likely cellular targets for persistence, quiescence, and dissemination. Indeed, we have recently observed that FV3 genomic DNA may be amplified from *in vitro*-infected, cultured peritoneal phagocytes without detectable viral gene expression as long as several months after initial infection (L. Grayfer, and J. Robert, University of Rochester, unpublished data). We believe that the key to further delineating ranavirus-macrophage interactions and ranaviral quiescence is contingent on developing *in vitro* myeloid cell cultures and related reagents.

3.3 Macrophage Reservoirs and Ranavirus Reactivation

We recently provided substantial support for the hypothesis that amphibian macrophages serve not only as vehicles of ranavirus dissemination within the host but also as foci of disease reactivation. When peritoneal phagocytes were isolated from 15 *X. laevis* adults 30 days postinfection, only cells from one individual displayed detectable levels of FV3 DNA and expressed transcripts encoding the viral DNA polymerase and major capsid protein (J. Robert, L. Grayfer, and F. De Jesús Andino, University of Rochester, unpublished data). However, after i.p. injection of heat-killed *E. coli*, nine of the same 15 animals exhibited both detectable viral genomic DNA and active viral gene expression. In addition, immunofluorescence microscopy targeting 53R, an FV3 gene product required for replication and assembly, and HAM56, a *X. laevis* macrophage marker (Nishikawa et al. 1998), revealed that peritoneal macrophages displayed productive FV3 replication (Fig. 1). Thus, it appears that mononuclear phagocytes harbor low levels of viral DNA, which can be reactivated by inflammation. Further research into activation states (both classical and alternative; Auffray et al. 2007; Nahrendorf et al. 2007; Zhao et al. 2009; Ziegler-Heitbrock 2007) of mononuclear phagocytes will be critical to devising preventative measures and understanding the precise infection strategies of these complicated pathogens.

3.4 Ranavirus Infections Among Other Poikilothermic Macrophages

Akin to many other pathogens, ranaviruses presumably overcome macrophage antimicrobial and antiviral barriers, at which point these cells become vehicles for both viral dissemination and persistence. However, exploitation of macrophage-lineage

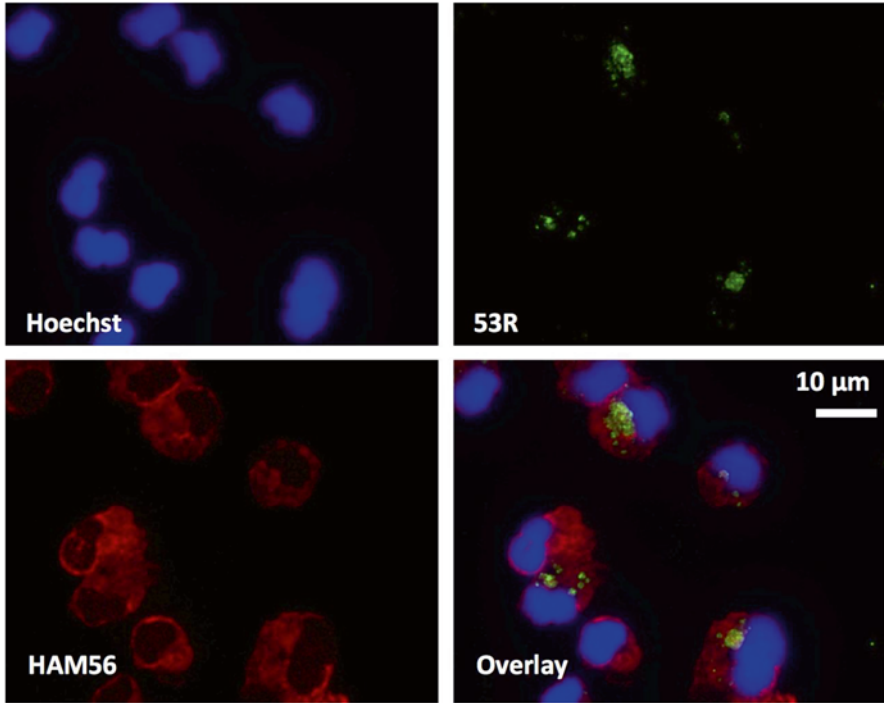


Fig. 1 *Xenopus laevis* HAM 56⁺ peritoneal macrophages infected with Frog Virus 3. *Xenopus laevis* peritoneal leukocytes were infected at a multiplicity of infection (MOI) of 0.3 with Frog Virus 3. Macrophages were stained with an antibody against the macrophage marker HAM56. FV3 was visualized using an antibody against the 53R viral protein. Hoechst was used to visualize the cellular nuclei

cells as vectors of viral dissemination and persistence does not appear confined to FV3, as other members of the genus *Ranavirus* and family *Iridoviridae* have also adopted this mechanism of host infiltration and immune evasion. For example, an iridovirus-like pathogen infects sheatfish kidney macrophages and is capable of down-regulating phorbol myristate acetate-elicited reactive oxygen production by these cells *in vitro* (Siwicki et al. 1999). Likewise, following infection with the Taiwan Grouper Iridovirus (TGIV), elevated numbers of phosphatase-positive, highly phagocytic basophilic and eosinophilic mononuclear leukocytes were detected (Chao et al. 2004). Interestingly, TGIV genomic DNA was found only within the nuclei of mononuclear phagocytes at early times after infection, whereas at later times it was seen in both nuclear and cytosolic compartments and these cells lost their phagocytic capacity (Chao et al. 2004). Clearly, TGIV has evolved intricate and temporally regulated strategies for overcoming and utilizing the very immune cells that would presumably be coordinating the antiviral immune response. It is probable that the strategy of invading mononuclear phagocytes as a means of immune evasion and dissemination is a distinct feature of all vertebrate iridoviruses.

Further development of *in vitro* primary macrophage cultures derived from relevant host species and infection models will provide additional insight into these infection strategies.

4 Antiviral Immune Responses to Ranavirus Infections

4.1 Antiviral Interferons of Ectothermic Vertebrates

The interferon (IFN) response provides a significant contribution to antiviral immunity. IFN responses generally arise as the result of recognition of viral products through an array of host pathogen recognition receptors (PRRs), including toll-like receptors (TLRs), retinoic acid-inducible gene 1-(RIG-I)-like receptors (RLRs), and cytosolic DNA sensors (Baum and Garcia-Sastre 2010; Sadler and Williams 2008). This branch of antiviral immunity consists of three classes of cytokines, type I, II, and III IFNs (Sadler and Williams 2008). IFN γ , the only type II IFN of mammals (n.b., bony fish possess multiple type II IFNs; Grayfer et al. 2010) plays multiple immune and antiviral roles, whereas IFN-I and -III function predominantly as antiviral molecules. Mammalian IFN-I possesses broad cellular specificities, whereas IFN-III targets specific cell subsets (Levraud et al. 2007; Zou et al. 2007). Interestingly, while the distinct receptor systems utilized by IFN-I and -III dictate cell specificity, both cytokine families activate the same downstream Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathways, culminating in similar antiviral outcomes (Sadler and Williams 2008) including the induction of antiviral genes such as protein kinase R (PKR) and Myxovirus resistance (Mx) molecules.

While these IFN responses are well studied among warm-blooded vertebrates, the cold-blooded hosts that are subject to ranavirus infection possess unique and much less understood IFN systems. The mammalian type I IFNs are encoded by intronless genes, comprising the multigene IFN α family (13 in humans) and a single IFN β gene (Hervas-Stubbs et al. 2011). While reptiles and birds also express single exon-encoded type I IFNs (Robertsen 2006; Zou and Secombes 2011), lower vertebrate species including cartilaginous and bony fish as well as amphibians possess type I IFNs encoded by five exon/four intron transcripts and displaying marked sequence divergence from their mammalian counterparts (Chang et al. 2009; Qi et al. 2010; Robertsen 2006; Zou and Secombes 2011; Zou et al. 2007).

Presently, only the type I IFN systems of bony fish have been explored in detail. These IFNs are subdivided into two groups (group I: 2C; group II: 4C) based on cysteine patterns (Sun et al. 2009; Zou et al. 2007), and further classified into four groups (IFN α -d) according to phylogeny (Chang et al. 2009; Sun et al. 2009). Importantly, while multiple distinct mammalian IFNs confer their biological roles through the same receptor complex (Li et al. 2008; Samuel 2001), fish group I and

II IFNs signal through unique receptor complexes (Aggad et al. 2009). Functional studies have been performed predominantly on group I type I fish IFNs (Aggad et al. 2009; Altmann et al. 2003; Long et al. 2004; Lopez-Munoz et al. 2009; Robertsen et al. 2003; Zou et al. 2007), and it has been demonstrated that these IFNs differ in their capacities to establish cellular antiviral states (Aggad et al. 2009; Levraud et al. 2007; Li et al. 2010; Lopez-Munoz et al. 2009). For example, salmonid IFNs a–d possess different transcriptional regulation patterns and distinct antiviral functions, as some of these cytokines are capable of eliciting potent antiviral responses while others are believed not to have antiviral functions at all (Svingerud et al. 2012). The type II IFN systems of amphibians and reptiles remain largely uncharacterized, whereas those of bony fish appear to be much more complex than that of mammals (Zou and Secombes 2011), and will not be addressed further here.

Mammalian IFN-III is comprised of interferon lambda (IFN λ) -1, -2, and -3 (also designated as IL-28A, IL-28B, and IL-29). These molecules are encoded by five exon/four intron gene transcripts and signal through a receptor system composed of the interferon lambda receptor-1 (IFN λ R1) and interleukin-10 receptor-2 (IL-10R2; reviewed in reference Kotenko 2011). Intriguingly, while *bona fide* type III IFNs either do not exist, or have not yet been identified in bony fish, amphibians are now known to possess both type I IFNs with the same five exon/four intron gene organization as their fish counterparts, as well as true type III IFNs (Qi et al. 2010). This is especially relevant when considering that amphibians are key evolutionary intermediates between fish and mammals and inhabit both aquatic and terrestrial habitats. In fact, a hallmark characteristic of fish and amphibian type I IFNs is the five exon/four intron genomic organization, which is distinct from the reptile, avian and mammalian intronless type I IFNs (Robertsen 2006; Robertsen et al. 2003; Sun et al. 2009). Hitherto, there has been substantial debate regarding the precise phylogenetic relationship of fish IFN-I to higher vertebrate IFN-I and -III. Fish cytokines exhibit exon/intron gene organization similar to that of mammalian type III IFNs, yet possess hallmarks of higher vertebrate type I IFNs such as conserved cysteine positioning and (with the exception of the catfish IFN-I) a C-terminal CAWE motif, a conserved sequence motif found within nearly all IFNs (Lutfalla et al. 2003; Qi et al. 2010; Robertsen 2006; Zou et al. 2007). It will be interesting to determine the respective roles of these molecules in fish and amphibian antiviral immunity to RVs, particularly considering that fish appear to only have type I IFNs, while frogs possess both IFN types I and III (Qi et al. 2010).

4.2 Interferon Response to Ranavirus Infection

As described above, an important antiviral gene product synthesized during the interferon response is the Myxovirus resistance (Mx) protein (Samuel 2001). Mx proteins are believed to be pivotal to the establishment of the antiviral state conferred by IFN (Samuel 2001). Mx proteins are high molecular weight GTPases

belonging to the dynamin superfamily and are known to facilitate intracellular membrane remodeling as well as intracellular trafficking (Kochs et al. 2005). As in mammals, teleost Mx proteins function as antiviral mediators, with distinct Mx isoforms from different species conferring somewhat unique antiviral effects. To date the Mx of most, but not all, fish species have proven ineffective in preventing infection by various members of the family *Iridoviridae*. For example, Japanese flounder Mx is capable of inhibiting the replication of two species of rhabdovirus, but is incapable of inhibiting replication of Red seabream iridovirus (RSIV, genus *Megalocyttivirus*; family *Iridoviridae*) (Caipang et al. 2003). Similarly, Barramundi Mx inhibits replication of the nodavirus viral nervous necrosis virus (VNNV) and of Infectious pancreatic necrosis virus (IPNV) but fails to show antiviral effects against Taiwan grouper iridovirus (TGIV) (Wu et al. 2012; Wu and Chi 2007). Likewise, Senegalese sole Mx confers antiviral effects against the IPNV and Viral hemorrhagic septicemia virus (VHSV, family *Rhabdoviridae*), but not against the ranavirus European sheatfish virus (ESV) (Alvarez-Torres et al. 2013). Finally, rainbow trout Mx1 is antiviral towards IPNV, Salmonid alpha virus (SAV, *Togaviridae*), and infectious hematopoietic necrosis virus (IHNV, *Rhabdoviridae*), but is not effective at blocking replication of EHNV (Lester et al. 2012; Trobridge et al. 1997). Possibly, the host antiviral responses coevolved with local ranaviral isolates. Thus, the inadequacy of antiviral components such as Mx1 in dealing with foreign ranaviral isolates may culminate in a global threat represented by geographically distant ranavirus strains introduced by subclinically infected migratory hosts or imported due to international trade.

Perhaps the most commercially and aquaculturally important fish species in southern Europe is the gilthead seabream, at least in part because of its natural resistance to most viral pathogens (Cano et al. 2006, 2009). In fact, the only viral disease affecting commercial seabream populations is Lymphocystis disease virus (LCDV, genus *Lymphocystivirus*, family *Iridoviridae*) (Cano et al. 2013). Interestingly, seabream possess at least three Mx proteins. One Mx isoform effectively inhibits replication of VHSV and LCDV, a second Mx molecule effectively inhibits replication of European sheatfish virus and LCDV, and the third is protective against VHSV (Alvarez-Torres et al. 2013; Fernandez-Trujillo et al. 2013). This represents the first example of a teleost Mx molecule effectively inhibiting DNA virus infection. This is interesting, considering that LCDV nonetheless plagues this species. It is noteworthy that in contrast to the mortality caused by many members of the family *Iridoviridae*, seabream effectively clear LCDV infections, although it is believed that they may harbor the virus non-symptomatically. Thus, the efficacy of the teleost IFN/Mx response may well dictate the susceptibility of individual fish species to highly virulent pathogens such as iridoviruses. Notably, many fish species are infected by, and clear LCDV. Since these infections involve fish skin (Leibovitz 1980), systemic antiviral responses such as Mx may be less important to the resolution of LCDV.

In another example, Japanese flounder IFN-inducible transmembrane (IFITM) protein is upregulated in response to *Rana grylio* virus (RGV) infections (Zhu et al. 2013). Furthermore, through overexpression and siRNA knockdown studies, flounder IFITM1 was shown to play an important role in the cellular antiviral response to

RGV (Zhu et al. 2013). IFITM1 functions by suppressing viral-host cell entry and targeting the Golgi apparatus (Zhu et al. 2013).

As more research is conducted on these individual antiviral components, it is becoming more apparent that there are key, previously unknown factors that participate in the antiviral response of not just poikilotherms, but all vertebrate species. We propose that these individual IFN-elicited antiviral components are most likely interdependent on entire networks of other IFN-regulated molecules. Thus, the relative potency of the antiviral IFN response, at both cellular and whole organism levels, relies on the balance of numerous cellular and molecular components. Since different fish and amphibian species are now known to possess very distinct repertoires of antiviral effector molecules, it is not surprising that these disparate organisms display very different susceptibilities to similar pathogens.

Microarray analysis of axolotls infected with ATV revealed that in addition to a multifaceted inflammatory gene response, these animals also upregulate expression of multiple antiviral interferon responsive genes (Cotter et al. 2008). Among the numerous genes elicited by ranavirus infection were Mx1 genes, antiviral helicases, interferon regulatory factors, an IFITM, and a ribonuclease (Cotter et al. 2008). However, the genes encoding axolotl type I and type III IFN remain to be identified. It will be important to delineate the precise repertoire(s) of antiviral IFNs present within the axolotl genome and examine the transcriptional regulation, as well as functional roles of these moieties during immune responses against ranaviruses such as ATV.

As described above, frogs are now known to possess both type I and type III IFN genes that are transcriptionally upregulated following virus infections (Qi et al. 2010). While there have been no reported functional studies of amphibian type III IFNs, we recently identified a *X. laevis* type I IFN, produced it in recombinant (rX/IFN) form and characterized this molecule in the context of FV3 infections (Grayfer et al. 2014). Pretreatment of *X. laevis* A6 kidney-derived epithelial cell cultures with rX/IFN significantly protected these cells against the cytolytic effects of FV3 (Grayfer et al. 2014). Although control cultures were almost entirely decimated 3 days following FV3 infection and exhibited extensive viral replication, rX/IFN-treated cultures were virtually FV3-free and thriving (Grayfer et al. 2014). Furthermore, treatment of A6 cells with rX/IFN significantly upregulated the expression of Mx1 indicating that stimulation with this cytokine elicits a cellular antiviral state (Grayfer et al. 2014). Following FV3 challenge, the type I IFN response was more robust in *X. laevis* adults than tadpoles (Grayfer et al. 2014). Nevertheless, tadpoles injected i.p. with rX/IFN exhibited significant increases of Mx1 gene expression in the spleen and peritoneal leukocytes (Grayfer et al. 2014). Moreover, upon FV3 challenge, rX/IFN-treated tadpoles showed decreased viral replication and transcriptional activity (Grayfer et al. 2014). Therefore, in addition to delayed innate and inflammatory-associated immune gene responses, it appears that an inadequate type I IFN response also contributes to the higher susceptibility of *X. laevis* tadpoles to FV3.

However, adding to the complexity of the interaction between FV3 and tadpoles is the fact that although rX/IFN-treated tadpoles exhibited prolonged mean survival times following FV3 inoculation and viral loads that were several logs lower, these animal nonetheless incurred extensive organ damage and succumbed to infection

(Grayfer et al. 2014). This is consistent with the notion (as described above) that depending on the species and/or developmental stage, ranaviruses may exhibit virulence factors independent of viral replication. It is intriguing that despite the antiviral potency of *X. laevis* IFN, FV3 ultimately results in tadpole mortality. It will be invaluable to elucidate the respective roles of amphibian type I and III IFNs, their cognate receptor systems, and the respective roles (and possibly defects) of these components during ranavirus infections of tadpole and adult frogs.

5 Adaptive Immune Responses to Ranavirus Infections

Anti-ranavirus immune responses of lower vertebrates are multifaceted, complex, and poorly understood. However, it is becoming evident that clearance of ranaviruses is heavily contingent on successful adaptive immune responses, which have been investigated to date almost exclusively in *X. laevis*.

5.1 Antibody Responses to Ranavirus Infection

The amphibian organization and usage of the immunoglobulin (Ig) heavy and light chain loci are reminiscent of their mammalian counterparts, including V-(D)-J rearrangements, class-switch recombination, somatic hypermutation, and affinity maturation (Du Pasquier et al. 1989, 2000; Hsu 1998). As in mammals, the *Xenopus* Ig class-switch from IgM to IgY (IgG analog) is thymus-dependent and requires T cell-B cell collaboration (Blomberg et al. 1980; Turner and Manning 1974). Although affinity maturation of amphibian IgY results in only a tenfold increase, as compared to the 10,000-fold increase seen with for mammalian IgGs, it has been clearly demonstrated that *Xenopus* humoral immunity is a significant contributing factor to anti-ranaviral immune responses, particularly of adult frogs (Maniero et al. 2006).

Following secondary FV3 infection of *X. laevis* adults, animals produce substantial amounts of virus-specific IgY, first detectable 1 week after infection and peaking around 3 weeks after challenge (Gantress et al. 2003). Indeed, frogs re-infected (in the absence of adjuvant) with FV3 up to 15 months post primary infection, develop anti-FV3 specific IgY antibodies in a thymus-dependent manner that are detectable from 10 days up to 8 weeks post re-immunization (Maniero et al. 2006). Notably, FV3 is effectively neutralized by exposure to this sera *in vitro* (Maniero et al. 2006). In addition, administration of immune sera to naturally susceptible *X. laevis* tadpoles immediately preceding FV3 infection confers partial, but significant passive protection against the virus (Maniero et al. 2006). Clearly, the amphibian antibody response is integral to the clearance of ranavirus infections, while the extent to which this particular immune mechanism contributes to the ultimate anti-ranaviral immunity seen in *Xenopus* adults remains to be determined. These results are consistent with findings

with Red seabream iridovirus (RSIV, genus *Megalocytivirus*) in which vaccination with inactivated virions protected fish from subsequent viral challenge (Caipang et al. 2006; Nakajima and Kunita 2005; Nakajima et al. 1999)

5.2 *T Cell Responses and Immunological Memory Against Ranavirus Infections*

X. laevis tadpoles express suboptimal levels of MHC class Ia protein (Du Pasquier et al. 1989) and yet their splenocytes include *bona fide* CD8 T cells that express the pan-*T Xenopus* cell-surface marker CD5 (Jurgens et al. 1995) and exhibit fully rearranged TCR α/β transcripts (Horton et al. 1998). It is possible that suboptimal class Ia protein expression in tadpoles results in a T cell differentiation and selection pathway distinct from that of post-metamorphic animals and relies more heavily on non-polymorphic nonclassical MHC class Ib (class Ib) molecules. Indeed, in the absence of optimal class Ia-mediated T cell selection, larval CD8 T cells may possess a more restricted antigen-binding repertoire, possibly reflected in the relative susceptibility of tadpoles to ranaviruses. However, as discussed later in this section, there are distinct class Ib-mediated T cell selection mechanisms and T cell subsets that may complement conventional class Ia-restricted CD8 T cells in tadpoles.

In contrast to tadpoles, adult *X. laevis* display conventional class Ia-restricted CD8 cytotoxic T cell populations. Despite the absence of available antibodies, CD4 T helper cells are also likely present owing to the presence of all genes involved in differentiation and function of CD4 T cells, the expression of the CD4 gene in CD8⁻/CD5⁺ cells, and the MHC class II-dependent proliferation response obtained by mixed lymphocyte reaction (Du Pasquier et al. 1989). The requirement of T cells for FV3 clearance in *X. laevis* adults has been demonstrated by using sub-lethal γ -irradiation, which depletes thymus-derived T cells. Irradiated T cell-depleted adult frogs do not control FV3 and succumb to infection (Robert et al. 2005). Furthermore, depletion of *X. laevis* CD8 T cells by administration of anti-*X. laevis* CD8 mAb also substantially increases adult frog susceptibility to FV3 infection (Robert et al. 2005). These CD8 T cell-depleted, FV3-infected animals experienced severe edema and hemorrhaging, extensive elevation of viral loads and succumbed to infections, whereas control cohorts effectively cleared the virus (Robert et al. 2005). Thus, cytotoxic CD8 T cell responses are critical for effective FV3 clearance. Intriguingly, administration of the anti-CD8 Ab to tadpoles did not result in either CD8 T cell depletion or in increased susceptibility to FV3 (Robert et al. 2005), again emphasizing the unconventional nature of the tadpole T cell populations.

Frogs re-infected with FV3 exhibit expedited viral clearance concomitant with earlier proliferation of CD5⁺CD8⁺ splenocytes and faster infiltration (3 vs. 6 dpi) of the kidney, the central site of *X. laevis*-FV3 replication (Morales and Robert 2007). This not only underlines the importance of CD8 T cells in ranaviral clearance, but also indicates the presence of a T cell memory responses to ranavirus re-infections in adult

X. laevis. Interestingly, while kidney infiltration was substantially accelerated upon secondary FV3 challenge compared to primary infections, the numbers of recruited CD8⁺ cells were substantially lower during this second immune event (Morales and Robert 2007). This could be attributed to a higher frequency of T cell precursor infiltration upon the primary response and/or the generation of lower number of more effective CD8⁺ memory T cells upon re-infection. It cannot be excluded that this modest secondary response is an inherent property of the evolutionarily primordial amphibian adaptive immune system, considering the relatively meager degree of T cell expansion seen following immunological challenges, the absence of draining lymph nodes and the lack of white pulp-red pulp splenic organization (Du Pasquier et al. 1989). Alternatively, this modest secondary CD8 response could be accounted for by the recruitment and immune involvement of additional effector populations during subsequent anti-ranavirus responses. In support of this notion, during the secondary anti-FV3 response, there is the rapid and robust recruitment, and kidney infiltration of, CD8⁻ MHCII⁺ immune populations, which may be B cells, CD4 T cells, or CD8⁻ nonclassical MHC (class Ib)-restricted invariant T cell populations.

5.3 Roles of Nonclassical MHC-Restricted Cells in Ranavirus Immunity

Nonclassical MHC class Ib (Ib) molecules exhibit structural similarities to class Ia molecules, but typically possess limited tissue distribution and substantially fewer polymorphisms (Flajnik and Kasahara 2001). In mammals, some of these surface glycoproteins are involved in the differentiation and functional regulation of distinct subsets of invariant T (iT) cells, including CD1d-restricted iNKT cells and MR1 restricted mucosal associated iT (MAIT) cells (Bendelac et al. 1995, 1996, 1997; Matsuda and Gapin 2005). Both of these lymphocyte populations undergo unconventional differentiation pathways, exhibit unique semi-invariant T cell receptor rearrangements and are believed to participate in antimicrobial and antiviral immune responses (Behar and Porcelli 2007; Choi et al. 2008; Cohen et al. 2009; Le Bourhis et al. 2010).

It is intriguing that, as described above, while *Xenopus* larvae are naturally MHC class Ia deficient (Du Pasquier et al. 1989), they express a number of nonclassical class Ib genes (XNCs), with some of these, such as XNC10 displaying preferential thymic expression (Goyos et al. 2009, 2011). Notably, we have recently identified a prominent *X. laevis* iT immune cell subset, which requires XNC10 for both its development and function (Edholm et al. 2013). Using an XNC10 tetramer as well as reverse genetics combining transgenesis and RNA interference, we determined that this iT cell population is CD8⁻/CD4⁻, expresses a semi-invariant T cell receptor consisting of an invariant TCR α (iV α 6-J α 1.43) combined with a limited TCR β repertoire, and fails to develop in the absence of, or with diminished, XNC10 expression (Edholm et al. 2013). Notably, transgenic animals with effectively RNAi-silenced thymic and splenic XNC10 expression failed to develop this iT cell subset.

Moreover, they were also significantly more susceptible to, and more readily succumbed to, FV3 infections (Edholm et al. 2013) suggesting that these cells are important in anti-ranaviral defenses. It is noteworthy that deep sequencing analysis of tadpole TCR α revealed that *Xenopus* larvae possess several additional predominant iT cell populations (Edholm et al. 2013), which are presumably XNC-restricted and most likely participate in immune responses such as those against ranaviruses. Indeed, we have also identified the XNC10-dependent iT cell population in adult frogs; thus it stands to reason that during the primary and secondary anti-FV3 responses, these lymphocyte subsets may be amongst the CD8⁻ kidney infiltrating immune populations (Morales and Robert 2007) discussed above. Table 1 provides a comprehensive summary of host immune strategies.

6 Ranaviral Strategies for Evading Host Antiviral Immunity

As seen with poxviruses and other large DNA viruses, ranaviruses likely encode multiple proteins that function to impede the host antiviral response (Finlay and McFadden 2006; Johnston and McFadden 2003; Seet et al. 2003). However, with the exception of the ranavirus homolog of the largest subunit of eukaryotic initiation factor 2 (vIF-2 α), the functions of these gene products have not been determined. A description of the role of vIF-2 α follows, along with a brief description of other potential immune evasion proteins.

6.1 vIF-2 α Blocks Phosphorylation of eIF-2 α

Protein kinase R (PKR, EIF2 α K2) is a protein kinase that regulates cellular protein synthesis via phosphorylation and inactivation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2 α) in response to a variety of environmental stressors including viral infection (Proud 1995; Toth et al. 2006). PKR is present at low levels within uninfected cells and is induced following cell stimulation by anti-viral IFNs. PKR is an inactive monomer in uninfected cells. However, during viral infection, low concentrations of viral dsRNA bind PKR leading to its dimerization and activation via autophosphorylation (Zhang et al. 2001). Activated PKR subsequently phosphorylates the alpha subunit of eIF-2, an event that results in a global arrest in protein synthesis (Panniers et al. 1988; Rowlands et al. 1988). In addition to the effect of PKR-mediated eIF-2 α inactivation on protein synthesis, activated PKR may play additional roles. Activated PKR phosphorylates an inhibitor, I- κ B, bound to NF- κ B leading to the latter's release and the subsequent activation of pro-inflammatory and interferon genes (Proud 1995). In addition, activated PKR appears to be one of the danger signals that trigger apoptosis in virus infected cells. Because of the adverse effects that translational inhibition, NF- κ B activation, and apoptosis have on virus replication, viruses have evolved numerous approaches for circumventing PKR-mediated antiviral functions (Diener et al. 1993; Katze 1992).

Table 1 Current understanding of anti-ranaviral immune defenses

| Immune parameter | Species | Immune outcome | Reference |
|---|-----------------------|---|--|
| <i>Cellular immunity</i> | | | |
| Macrophage-lineage cells | <i>X. laevis</i> | M ϕ recruitment to FV3 inoculum | Morales et al. (2010) |
| | <i>X. laevis</i> | FV3 reservoirs | Robert et al. (2007) |
| | <i>E. lanceolatus</i> | TGIV reservoirs | Chao et al. (2004) |
| | <i>S. glanis</i> | TGIV inhibits kidney phagocyte ROI | Siwicki et al. (1999) |
| Innate immunity | <i>A. mexicanum</i> | Innate immune responses to ATV | Cotter et al. (2008) |
| NK cell response | <i>X. laevis</i> | NK cell recruitment to FV3 | Morales et al. (2010) |
| CD8 responses | <i>X. laevis</i> | Recruitment to and clearance of FV3 | Morales et al. (2010) |
| Absence of lymphocyte responses | <i>A. mexicanum</i> | Lack of adaptive immunity linked to ATV susceptibility | Cotter et al. (2008) |
| Nonclassical MHC Ib restricted iT cells | <i>X. laevis</i> | Poorly understood protection against FV3 in tadpoles and adult frogs | Edholm et al. (2013) |
| <i>Humoral immunity</i> | | | |
| Antimicrobial peptides | <i>R. pipiens</i> | Disruption of FV3 viral envelopes | Chinchar et al. (2001) |
| | <i>R. dybowskii</i> | Inhibition of RGV infectivity | Yang et al. (2012) |
| IgY | <i>X. laevis</i> | FV3 clearance; memory response to re-infection | Du Pasquier et al. (1989, 2000); Hsu (1998) |
| <i>Inflammatory cytokines</i> | | | |
| TNF α | <i>X. laevis</i> | Expression correlates with anti-RV protection; more modest and delayed in tadpoles | Morales et al. (2010) |
| | EPC cell line | Induced by FV3 | Holopainen et al. (2012) |
| IL-1 β | <i>X. laevis</i> | Expression correlates with anti-FV3 protection; more modest and delayed in tadpoles | Morales et al. (2010) |
| IFN γ | <i>X. laevis</i> | Expression correlates with anti-FV3 protection; more modest and delayed in tadpoles | De Jesús Andino et al. (2012) |
| <i>Antiviral immunity</i> | | | |
| Mx | <i>S. aurata</i> | Inhibits ESV replication | Alvarez-Torres et al. (2013); Fernandez-Trujillo et al. (2013) |
| IFN inducible genes | <i>A. mexicanum</i> | ATV-elicited expression | Cotter et al. (2008) |
| IFITM1 | <i>P. olivaceus</i> | Cellular antiviral response to RGV | Zhu et al. (2013) |
| Type I IFN | <i>X. laevis</i> | Expression correlates with anti-FV3 protection; more modest and delayed in tadpoles | Grayfer et al. (2014) |

To block phosphorylation of eIF-2 α , ranaviruses encode a pseudosubstrate of eIF-2 α designated vIF-2 α . In most ranaviruses, vIF-2 α is present as protein, of about 250 amino acid residues in length, that contains a sequence motif (V[L/I]RVDxxKGY[V/I]D) common to multiple ranaviruses, host cell eIF-2 α , and the K3L protein of vaccinia virus (Majji et al. 2006). K3L has been shown to block the phosphorylation and subsequent inactivation of eIF-2 α by acting as a pseudosubstrate for PKR (Beattie et al. 1995), and vIF-2 α has been shown to function in similar fashion (see below). By using an ATV KO mutant lacking vIF-2 α , Jancovich and Jacobs (Jancovich and Jacobs 2011) showed that this mutant virus was more sensitive to IFN-mediated inhibition and failed to block the phosphorylation of eIF-2 α . Moreover, whereas wt ATV degraded fish PKZ, an IFN-inducible kinase similar to PKR, the KO mutant did not. Lastly, the KO mutant displayed reduced virulence *in vivo* suggesting that vIF-2 α is a virulence gene. A similar attenuation of virulence was observed following infection of *Xenopus laevis* with a FV3 KO mutant lacking a truncated version of vIF-2 α (Chen et al. 2011). Truncated versions of vIF-2 α are found in FV3, soft-shell turtle iridovirus, and *Rana grylio* virus and are missing the N-terminal half of the native molecule. Since this region contains the VxRVDxxKGYxD motif described above, the reduction in virulence cannot be due to an effect of vIF-2 α on PKR, but to some element present within the C-terminal half of the protein.

Rothenburg et al. (2011), using a yeast model, demonstrated that transfection of a vector expressing either human or zebrafish PKR into yeast cells resulted in marked cell death. Furthermore, in confirmation of the role of vIF-2 α as an antagonist of PKR, co-transfection of a vector expressing the full-length vIF-2 α gene from *Rana catesbeiana* virus (RCV) along with PKR blocked the toxic effects of both human and zebrafish PKR. Indicative of species specificity, vaccinia virus K3L was only able to block the activity of human PKR. Although the above study indicates a role for vIF-2 α in maintaining protein synthesis in virus-infected cells, the observation that FV3 and other closely related ranaviruses contain truncated vIF-2 α genes lacking critical N-terminal motifs indicates that vIF-2 α may not be the only ranavirus protein that plays a role in maintaining protein synthesis in virus infected cells.

6.2 RNase III-Like Proteins

Similar to poxviruses, ranaviruses may also contain at least two genes whose function is to prevent the activation of PKR. Vaccinia virus encodes both the aforementioned K3L gene and a second gene, E3L, which binds dsRNA and prevents the dimerization and activation of PKR (Langland and Jacobs 2002; Langland et al. 2006). Although no ranavirus protein with homology to E3L has been detected, an RNase III-like protein has been identified. RNase-III targets dsRNA and it is possible that ranavirus homologs bind virus-induced dsRNA and degrade it, or block its ability to interact with and activate PKR. Experiments to directly test this hypothesis have not been reported. Moreover, knock down of RNase III-like protein

expression using antisense morpholino oligonucleotides resulted in a 40% reduction in virus yield (K. Cheng and V.G. Chinchar, University of Mississippi, unpublished data) suggesting that the RNaseIII-like protein plays a role in virus replication.

6.3 *β -Hydroxysteroid Dehydrogenase and vCARD*

Ranaviruses, like poxviruses, contain proteins with homology to β -hydroxysteroid dehydrogenase (β HSD). β HSD plays a role in steroid synthesis and expression of a β HSD homolog by vaccinia virus results in suppression of immunity and an increase in viral replication (Sroller et al. 1998). vCARD is a 10 kDa, virus-encoded protein that contains a Caspase Activation and Recruitment Domain (CARD) motif that modulates interaction between proteins bearing similar domains (Kawai and Akira 2009, 2010). Because proteins involved in apoptosis or in the induction of IFN and pro-inflammatory molecules such as RIG-I, MDA5, and MAVS contain CARD motifs, it is postulated that vCARD interacts with one or more of these signaling molecules and short-circuits cellular antiviral immunity (Besch et al. 2009; Meylan et al. 2005).

6.4 *vTNFR, dUTPase, DMTase*

In addition to the four viral gene products mentioned above, ranaviruses also contain homologs of Tumor Necrosis Factor (TNF) receptor (vTNFR) and dUTPase (Chinchar et al. 2009; Eaton et al. 2007) and a unique virus-encoded DNA cytosine methyltransferase (DMTase). Similar to their poxvirus counterparts, ranavirus vTNFR could function as a decoy molecule and block protection mediated by TNF α . Although dUTPase is generally considered to be a protein that plays a role in viral DNA synthesis (e.g., by increasing dTTP pools or blocking the incorporation of dUTP into DNA), a herpesvirus dUTPase was shown to also block antiviral immunity (Glaser et al. 2006; Oliveros et al. 1999). Lastly, the ranavirus DMTase may play a role in immune evasion by methylating cytosine residues within CpG motifs and blocking recognition by TLR-9 or cytoplasmic DNA sensors and preventing the subsequent induction of IFN and pro-inflammatory cytokines (Krieg 2002; Krug et al. 2004).

The above list of putative immune evasion proteins is based on ranavirus proteins with detectable homology to proteins in other systems with known antiviral effects. While this serves as a useful starting point in the identification and characterization of ranaviral immune evasion genes, there are approximately a dozen additional ORFs of unknown function, which are unique to ranaviruses. Whether these ORFs encode proteins that control virus replication in specific hosts or whether they encode proteins that modulate host-specific immune responses remains to be determined.

Knock-down experiments using antisense morpholino oligonucleotides or siRNA and infections using knock-out mutants will be needed to resolve the function of these unique ranavirus-specific proteins.

7 Concluding Remarks and Future Directions

It is evident from the studies described here that anti-ranaviral immunity is multifaceted, complex, and likely species- and developmental stage-specific. Also evident are the many gaps in our understanding of the immune response to these pathogens as well as possible defects in the host's ability to mount effective responses that contain and eliminate these infections. It is particularly worrisome that *Ranavirus* and other genera within the family *Iridoviridae* have devised numerous, highly efficient strategies for evading, and even utilizing host immune components, to achieve persistence, facilitate dissemination and expand host range. Clearly, ranaviruses encode a large number of putative gene products, which represent both potential virulence factors as well as promising targets for future therapeutic interventions.

While it is easy to dismiss lower vertebrate immune systems as functionally analogous to those of mammals, there is a growing literature suggesting otherwise. It is through the fundamental understanding of the physiological and ecological pressures governing these unique immune systems that we may begin to comprehend ranavirus infection strategies and the immune systems that may, or may not have adequately co-evolved to stop them.

The investigation of ranavirus infection and immune subversion strategies should be approached not only by taking into account well-defined mammalian pathogens, but also by considering the possibility that ranaviruses may represent unique viral agents. In contrast to the majority of homeothermic vertebrate pathogens, ranaviruses are extraordinary in their ability to overcome cell and host tropism barriers, while their mechanisms of pathogenicity appear to be (at least partially) much less dependent on viral loads. Indeed, the immune systems of ectothermic hosts have evolved as the result of, and are subject to, different physiological and pathogenic pressures than those that have shaped the mammalian immune system. It stands to reason that ranavirus pathogens have co-evolved with these unique immune systems, thus we must garner greater insights into both to fully understand either.

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