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Susceptibility of *Xenopus laevis* tadpoles to infection by the ranavirus Frog-Virus 3 correlates with a reduced and delayed innate immune response in comparison with adult frogs

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ABSTRACT

Xenopus laevis adults mount effective immune responses to ranavirus Frog Virus 3 (FV3) infections and clear the pathogen within 2–3 weeks. In contrast, most tadpoles cannot clear FV3 and succumb to infections within a month. While larval susceptibility has been attributed to ineffective adaptive immunity, the contribution of innate immune components has not been addressed. Accordingly, we performed a comprehensive gene expression analysis on FV3-infected tadpoles and adults. In comparison to adults, leukocytes and tissues of infected tadpoles exhibited modest (10–100 time lower than adult) and delayed (3 day later than adult) increase in expression of inflammation-associated (TNF-α, IL-1β and IFN-γ) and antiviral (Mx1) genes. In contrast, these genes were readily and robustly upregulated in tadpoles upon bacterial stimulation. Furthermore, greater proportions of larval than adult PLs were infected by FV3. Our study suggests that tadpole susceptibility to FV3 infection is partially due to poor virus-elicited innate immune responses.

Introduction

The tadpole and adult forms of the amphibian *Xenopus laevis* each display distinct immune systems. This peculiarity affords a unique opportunity to compare and contrast immune responses in the same organism. Although both tadpoles and adults are immunocompetent, both B and T cell responses are weaker in larvae (Reviewed in (Du Pasquier et al., 1989; Robert and Ohta, 2009). In particular, there is no consistent expression of MHC class I protein until metamorphosis, although thymic derived CD8 T cells are present (Flajnik and Du Pasquier, 1988; Flajnik et al., 1986). Further weakness of larval adaptive immunity includes a poor switch from IgM to IgY, an affinity of antibody lower than adult, and incomplete skin graft rejection capacity (Chardonnens and Du Pasquier, 1973; DiMarzo and Cohen, 1982b; Hsu and Du Pasquier, 1984). Besides the observed absence of NK cells until metamorphosis (Horton et al., 2003), little is known about tadpole innate immune responses.

We have developed *X. laevis* as a reliable model system to explore the evolution of viral immunity as well as to better evaluate host factors involved in susceptibility to emerging infectious diseases caused by ranavirus (RV) pathogens (Chinchar et al., 2009). RVs have become a major concern for captive and wild amphibians, fish, and other ectothermic species worldwide. In fact, ranavirus infections were the leading causes of amphibian mortality in the US between 1996–2001 (Green et al., 2002; Schloegel et al., 2010). We have focused our study on Frog Virus 3 (FV3), which is the main member and the type species of the RV genus. FV3 is a large (200 nm) poxvirus-like, double stranded DNA virus that is infectious in both enveloped and non-enveloped form (reviewed in Chinchar et al. (2009)). FV3 or FV3-like viruses are now found worldwide, infecting many different amphibian species, making it a serious global threat (Duffus et al., 2008; Gray et al., 2007; Mazzoni et al., 2009; Pearman et al., 2004).

The *Xenopus* adaptive immune response elicited during FV3 infection has been well characterized (Gantress et al., 2003; Robert et al., 2005). Adult frogs develop an effective CD8 T cell responses and clear FV3 within 2–3 weeks (Morales and Robert, 2007). Potent specific antibodies are also generated against FV3 in adults (Maniero et al., 2006). Recently, we began to characterize innate immune responses at an early stage of FV3 infection in adults that includes a rapid up-regulation of genes encoding the pro-inflammatory cytokines TNF-α and IL-1β (Morales et al., 2010). In contrast to adults, most *Xenopus* tadpoles (~90%) are unable to clear the virus and die within a few weeks after infection (Gantress et al., 2003). The high susceptibility of larval stages to FV3 infection is also documented for other anuran species in natural (Gray et al., 2009; Gray et al., 2007) and captive population (Mazzoni et al., 2009). The weaker or

Abbreviations: ANOVA, one-way analysis of variance; IE, immediate-early; FV3, Frog Virus 3; MOI, multiplicity of infection; PFU, plaque forming units; i.p., intraperitoneal injection; Qpcr, quantitative real-time PCR; p.i., post-infection; dpi, days post-infection; RV, Ranavirus

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immature adaptive immune effector functions in tadpoles may explain this higher susceptibility. Indeed, our attempts to generate protective immunity by immunization and to detect an anti-FV3 antibody response have so far been unsuccessful. However, the variability of survival times observed among individuals suggests that the tadpole immune system is not completely inactive or ignorant of FV3 infection. Therefore, we postulate that in *Xenopus* tadpoles, some innate immune responses are elicited upon FV3 infection.

To assess this possibility and begin to characterize innate immunity in tadpoles, we determined the expression profiles of several relevant inflammation-associated genes (TNF-α, IL-1β, IFN-γ) and the type I IFN-inducible Myxovirus-resistance 1 (Mx1) gene during the early phase of FV3 infection. Surprisingly, the expression changes of these genes upon FV3 infections is delayed and of lower in magnitude in tadpoles compared to adults, which may be one of the reasons for the high susceptibility of tadpoles to FV3.

Results

Changes in inflammation-associated gene expression during FV3 infection

In adult *X. laevis*, increased expression of the pro-inflammatory genes IL-1β and TNF-α by peritoneal leukocyte (PL) can be detected as early as 1 day post-infection (dpi; (Morales et al., 2010). We investigated whether a similar gene expression kinetics are elicited in tadpoles upon FV3 infections. For this purpose, we used outbred pre-metamorphic tadpoles at developmental stage 56 (3–4 week post-fertilization; Supplementary Fig. 1), when the spleen is well developed and immune responses can be detected (review in Robert and Ohta (2009)). Tadpoles were infected for 1 to 9 days by a single i.p. injection of 1×10^4 PFU of FV3, and PLs and tissues were collected from 3 individuals at each time point for qPCR analysis. To obtain sufficient amounts of RNA from PLs and spleens, we pooled respective samples from three tadpoles. In three independent experiments using this approach (Fig. 1), we detected a consistently delayed (6 dpi) increases of TNF-α expression in PLs. Similarly, IL-1β gene expression by PLs exhibited delayed increases (6 dpi), albeit with greater variation in the magnitude of response (Fig. 1). For the spleen, which represents both a primary and the only secondary lymphoid organ in *Xenopus*, similar delayed increases of TNF-α gene expression were observed, whereas the mRNA levels of IL-1β were elevated by 1 dpi and subsided subsequently (Fig. 1).

Since activated leukocytes expressing pro-inflammatory genes may have accumulated at the sites of infection, we examined the immune gene expression profiles in several tadpole organs. Given that the adult kidney is the main target of FV3 infection (Robert et al., 2005), we focused on this tissue to compare gene expression between adults and tadpoles during FV3 infections. As expected, adults exhibited rapid and marked increases of TNF-α (1000× on average) and IL-1β (100× on average) mRNA levels as early as 1 dpi, with further increases at 3 and 6 dpi (Fig. 2). Interestingly, the basal mRNA levels of TNF-α and IL-1β in uninfected tadpole kidneys were significantly higher than those seen in uninfected adult kidneys (100× and 10×, respectively; Fig. 2). In addition, the expression levels of these two genes remained at basal at 1 and 3 dpi, and only modestly increased at 6 dpi (10×; Fig. 2). Similarly, relatively delayed and modest increases of TNF-α, IL-1β and IFN-γ expression were found in tadpole liver tissues (Supplementary Fig. 2).

In mammals, IFN-γ is a critical effector cytokine initially produced by activated NK cells during innate immune response, and later on during adaptive immune responses by CD8 T and CD4 T helper 1

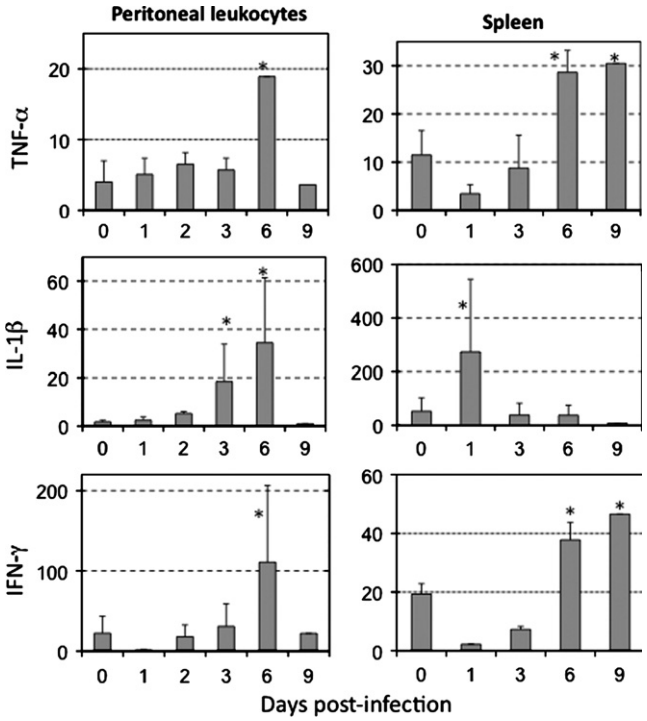

Fig. 1. Low and delayed increases of inflammation-associated gene expression by tadpole peritoneal and splenic leukocytes during FV3 infection. Quantitative gene expression analysis (qPCR assay) was performed on PLs and spleen harvested from the same pre-metamorphic tadpoles (st 56) infected by i.p. injection of FV3 (1×10^4 PFU) for 1, 2, 3, 6, and 9 day. The primers used were specific for *Xenopus* TNF-α, IL-1β, and IFN-γ. Control cells for this experiment were the peritoneal and splenic leukocytes removed on the day of infection (day 0). Each data point represents 3 different experiments where cells from 3–5 tadpoles were pooled. The expression level was determined using the delta delta CT method and the results are expressed as the means ± SD of relative quantification, with GAPDH used as endogenous control. *Significantly greater expression of cytokines in cells of infected tadpoles in comparison with uninfected controls by Student's *t*-test, $p \leq 0.005$.

(Th1) cells (Schoenborn and Wilson, 2007). An IFN-γ homologue has been identified and partially characterized in *Xenopus tropicalis,* using its fully sequenced genome (Qi and Nie, 2008). Using this sequence we cloned, sequenced and characterized by phylogenetic analysis the *X. laevis* IFN-γ homologue (data not shown, GenBank accession number: JN634068). We designed and validated primers specific for this gene and here report the first expression analysis of the *X. laevis* IFN-γ in FV3-infected adults (Fig. 2). Significant increases of IFN-γ gene expression were already detectable at 1 dpi (20× on average above non-infected controls), whereas greater increases (>1000×) occurred at the peak of the response, 6 dpi.

We then examined the IFN-γ gene expression in various tissues of FV3-infected pre-metamorphic tadpoles. In several experiments, we detected no significant increases in IFN-γ mRNA levels above uninfected controls at 1 and 3 dpi in kidneys, whereas at 6 and 9 dpi this cytokine was consistently increased 30–40 fold over respective controls (Fig. 2). Significant increases of IFN-γ expression were also observed in larval PLs and spleen (Fig. 1, bottom panel), and to a lesser degree in liver tissues (Supplementary Fig. 2).

Kinetics of virus load in tadpoles

Since the less robust and more delayed inflammation-associated gene expression changes observed in infected tadpoles could be attributed to slower infection kinetics and/or lower virus loads, it was important to evaluate the degree of FV3 infections in tadpoles. For this purpose, we monitored the virus load over time for different tadpole tissues by qPCR using primers specific for the FV3 DNA polymerase II (*vPol,* 60R). Significant amplification of