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Critical role of an MHC Class I-like/innate-like T cell immune surveillance system in host defense against ranavirus (Frog virus 3) infection.

Edholm, Eva-Stina*[1,2], Francisco De Jesús Andino*[1], Jinyeong Yim[1], Katherine Woo[1], and Jacques Robert[1]

[1] Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, USA.
[2] The Norwegian College of Fishery Science, University of Tromsø, the Arctic university of Norway, Tromsø, Norway.
* These authors contributed equally to this work.
Communicating Author: Dr. Jacques Robert, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY 14642; Phone (585) 275-1722; FAX (585) 473-9573; e-mail: Jacques_Robert@urmc.rochester.edu

Abstract: Besides the central role of classical MHC class Ia-restricted conventional CD8 T cells in antiviral host immune response, the amphibian *Xenopus laevis* critically rely on MHC class I-like (*mhc1b10.1.L* or XNC10)-restricted innate-like (i)T cells (iVα6 T cells) to control infection by the ranavirus Frog virus 3 (FV3). To complement and extend our previous reverse genetic studies showing that iVα6 T cells are required for tadpole survival as well as for timely and effective adult viral clearance, we examined the conditions and kinetics of iVα6 T cell response against FV3. Using a FV3 knock-out (KO) growth-defective mutant, we found that upregulation of the XNC10 restricting class I-like gene and the rapid recruitment of iVα6 T cells depend on detectable viral replication and productive FV3 infection. In addition, by *in vivo* depletion with XNC10 tetramers we demonstrated the direct antiviral effector function of iVα6 T cells. Notably, the transitory iVa6 T cell defect delayed innate interferon and cytokines gene response resulting in long-lasting negative inability to control FV3 infection. These findings suggest that in *Xenopus* and likely other amphibians, an immune surveillance system based on the early activation of iT cells by non-polymorphic MHC class-I like molecules is important for efficient antiviral immune response.

Keywords: Unconventional T cell; nonclassical MHC; antiviral immunity; interferon

1. Introduction

In mammals, potent adaptive immune response against viral pathogens largely depend on conventional CD8 T cell effectors that express a broad T cell receptor (TCR) repertoire capable of recognizing a large array of antigens presented by polymorphic classical MHC class I molecules (class Ia). However, it is increasingly appreciated that other unconventional or innate-like T cell effectors are critically involved in antiviral immunity (Reviewed in (Adams and Luoma, 2013)). These iT cells interact with non-polymorphic MHC class I-like molecules, have a limited or invariant TCR repertoire and are thought to serve as important early responders and immune regulators. One of the best characterized iT cell population are iNKT cells that are restricted by the MHC class I-like molecule CD1d that present lipids (Borg et al., 2007; Rossjohn et al., 2012). The antiviral role of iNKT cells has been shown for murine cytomegalovirus (MCMV; (Wesley et al., 2008), lymphocytic

choriomeningitis virus (LCMV; (Diana et al., 2009)), herpes simplex virus 1 (HSV1;(Raftery et al., 2008)), HIV and influenza (Juno et al., 2012) and even for vaccinia virus (VAC; (Abboud et al., 2016).

In the amphibian *Xenopus laevis*, while adult frogs exhibit an immune system whose conventional class Ia-restricted CD8 T cell compartment is dominant as in mammals, tadpoles are immunocompetent but are naturally class Ia-deficient and primarily rely on MHC class I-like and iT cells to mediate immunity (Edholm et al., 2016). Deep-sequencing repertoire analysis has revealed the over-representation of 6 invariant TCRα rearrangements implying the predominance of 6 larval iT cell subsets (Edholm et al., 2013). One of the 6 putative iT cell subsets, the iV6 T cell subset, expresses the rearranged Vα6-J1.43 TCRα chain and requires the MHC class I-like molecule XNC10 (encoded by *mhc1b10.1.L*) for its thymic development and function (Edholm et al., 2013). Notably, host immune responses against the ranavirus pathogen FV3 is significantly impaired by lack of iVα6 T cells resulting from XNC10 loss-of-function. FV3 is a major pathogen of amphibians, fish and even reptiles (Price et al., 2014). The dramatic worldwide increases in host ranges (i.e., populations and species infected) and amphibian die-offs caused by RVs (see eBook (Gray and Chinchar, 2015)), raise alarming concerns for biodiversity and aquaculture, and pose fundamental issues related to evolution of host/pathogen interactions. As such, the involvement of iT cells in addition to conventional CD8 T cells in antiviral host resistance is of high relevance.

In adult *X. laevis*, XNC10 deficiency considerably delays viral clearance resulting in increased tissue damage (Edholm et al., 2015). As expected from iT cell prevalence in tadpoles, XNC10 deficiency markedly increases the mortality rate, especially at early stage of FV3 infection (Edholm et al., 2013). The generation of XNC10 tetramers by expressing XNC10 fused to *X. laevis* beta 2 (b2m)-microglobulin has permitted a better characterization of the iVα6 T cell response kinetics and show a rapid recruitment (within less than 24 hrs.) at the site of infection. This fast targeted response suggests a sensitive detection mechanism of FV3 infection. However, many facets of this response remain to be investigated including whether iVα6 T cells require stimulation by XNC10 bound to viral or other ligands, or can be activated by XNC10-independent co-stimulation signals (e.g., danger signals, IFN response). To explore some of these issues we have taken advantage of our XNC10 tetramer as well as a FV3 recombinant virus deficient for a putative immune evasion gene to determine the role of productive versus ablated viral infection as well as the effect of transiently impairing iVα6 T cell function at early stage of FV3 infection.

2. Material and methods:

2.1. Animals

All outbred *X. laevis* were from the *X. laevis* research resource for immunology at the University of Rochester (https://www.urmc.rochester.edu/microbiology-immunology/xenopus-laevis.aspx). All animals were handled in accordance with stringent laboratory and University Committee on Animal Research regulations minimizing suffering (Approval number 100577/2003-151). For all experiments, three-weeks old, tadpoles (stage 55, 1.5 cm long; (Nieuwkoop and Faber, 1967)) and 1 year-old young adult frogs were used.

2.2. Frog virus 3 stocks and infection

Baby hamster kidney cells (BHK-21, ATCC No. CCL-10) were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), streptomycin (100µg/mL), and penicillin (100 U/mL) with 5% CO2 at 37°C, then 30ºC for infection. The generation and characterization of the FV3 knock-out (KO) mutant ΔvCARD (or Δ64R) has been detailed elsewhere (Andino Fde et al., 2015; Chen et al., 2011; Jacques et al., 2017). Wild type (WT) and KO FV3 were grown using a single passage through BHK-21 cells and were subsequently purified by ultracentrifugation on a 30% sucrose cushion. Adult frogs were infected by i.p. injection of 1×10^6 PFU in 100 µL of amphibian PBS (APBS) and tadpoles were infected by i.p. injection of 10, 000 PFU in 5 µL APBS. Uninfected control animals were mock-infected with an equivalent volume of APBS. At different days post-infection (dpi), animals were

euthanized using 1 μg/L tricaine methanesulfonate (TMS) buffered with bicarbonate prior to dissection and extraction of nucleic acids from tissues.

2.3. Quantitative gene expression analyses

Total RNA was extracted from peritoneal leucocytes and kidneys using Trizol reagent, following the manufacturer's protocol (Invitrogen). cDNA was synthetized with 0.5 μg of RNA in 20 μl using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), and 1 μl of cDNA template was used in all RT-PCRs and 150 ng DNA for PCR. Minus RT controls were included for every primer pair. A water-only control was included in each reaction. The qPCR analysis was performed using the ABI 7300 real-time PCR system with PerfeCT SYBR Green FastMix, ROX (Quanta) and ABI sequence detection system (SDS) software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls were used in conjunction with the ΔΔ CT method to analyze cDNA for gene expression. All primer sequences are listed in Table S1.

2.4. Viral load quantification by qPCR and plaque assay

FV3 viral loads were assessed by absolute qPCR by analysis of isolated DNA in comparison to a serially diluted standard curve. Briefly, an FV3 DNA Pol II PCR fragment was cloned into the pGEM-T Easy vector (Promega). This construct was amplified in bacteria, quantified and serially diluted to yield 10^{10}-10^{1} plasmid copies of the vDNA POL II. These dilutions were employed as a standard curve in subsequent absolute qPCR experiments to derive the viral genome transcript copy numbers, relative to this standard curve. Virus quantification by plaque assay was performed on BHK-21 monolayers in 6-well plates under an overlay of 1% methylcellulose (Morales et al., 2010). Infected cells were cultured 7 days at 30°C in 5% CO_2. Overlay media was aspirated and cells were stained for 10 min with 1% crystal violet in 20% ethanol.

2.5. XNC10 tetramer production

XNC10 tetramers were generated as previously described (Edholm et al., 2013). Briefly, beta 2 microglobulin (b2m) was linked via a 23-aa Glycine rich C-terminal flexible linker to the α1-α3 domains of XNC10 containing a BirA site-specific biotinylation site at the end of the α3 domain and cloned into the pMIBV5-HisA expression vector (Invitrogen). The b2m-linker-XNC10 construct was expressed in Sf9 insect cells and monomeric b2m-linker-XNC10 was purified by Ni-NTA-Agarose Chromatography (Qiagen) and concentrated to 1 μg/μL using Amicon Ultra Centrifugal Filter (Millipore). BirA enzymatic biotinylation was performed for 18 h at 30 °C according to the manufacturer's protocol (Avidity), and the purified biotinylated proteins were extensively dialyzed against APBS, pH 7.5, to remove any unbound biotin. XNC10 tetramers were generated by incubating b2m-linker-XNC10 with fluorochrome-labeled streptavidin at a 5:1 ratio at room temperature for 4 hours before use. Purified XNC10 tetramers (1 μg) were injected intra i.p. in a volume of 5 μl.

2.6. Statistical analysis

The Mann-Whitney U and ANOVA as well as non-parametric Kruskal-Wallis tests were used for statistical analysis of expression and viral load data. Analyses were performed using a Vassar Stat online resource (http://vassarstats.net/utest.html). Statistical analysis of survival data was performed using a Log-Rank Test (GraphPad Prism 6). A probability value of $p<0.05$ was used in all analyses to indicate significance. Error bars on all graphs represent the standard error of the mean (SEM).

3. Results

3.1. Relationships between FV3 infection magnitude and iVa6 T cell response in adult X. laevis

We have previously demonstrated that FV3 infection in adult X. *laevis* elicit a transitory influx of the innate (i)T cell subset iVα6 T into the peritoneal cavity (Edholm et al., 2015). To delineate the

factors governing iVα6 T cell recruitment during a FV3 infection, we took advantage of the FV3 KO mutant ΔvCARD (64R)-FV3 that has previously been shown to have attenuated virulence and growth *in vivo* elicit different host responses (Andino Fde et al., 2015; Jacques et al., 2017). Accordingly, we i.p infected adult *X. laevis* with wild type (WT) FV3 or Δ64-FV3 for 24 hours before collecting peritoneal leukocytes (PLs) and quantified transcript levels of the specific invariant Vα6-Jα1.43 rearrangement. As a control we also challenged frogs with heat killed *E. coli*. This type of bacterial stimulation has been shown to induce a strong nonspecific inflammatory response but does not stimulate the recruitment or accumulation of iVα6 T cells in the peritoneal cavity (Edholm et al., 2015). Because iVα6 T cells interact with the MHC class I like molecule XNC10, we also examined its gene expression profile. To evaluate viral loads, we determined the FV3 genome copy number using absolute qPCR. Consistent with previous findings, Δ64-FV3 infection resulted in significantly (P = 0.005) lower viral load compared to WT-FV3 already at 1 dpi (Jacques et al., 2017). All treatments resulted in a significant increased XNC10 expression compared to PLs collected from APBS injected control frogs at the same time point with no difference among the treatment groups (Fig. 1B). However, only WT-FV3 infection resulted in significantly elevated iVα6-Jα1.43 transcript levels compared to uninfected controls (P = 0.018), whereas in frogs injected with heat killed bacteria iVα6-Jα1.43 expression was lower compared to controls, presumably due to the large influx of immune cells into the peritoneal cavity. Although the high individual variation prevented statistical significance among the two FV3 infected groups, iVα6-Jα1.43 transcript levels observed with Δ64-FV3 mutants was elevated above sham-infect APBS controls in only 20% of frogs (3 out of 15 frogs) compared to 70% (9 out of 13) for WT-FV3 infected frogs, suggesting a correlation between iVα6 recruitment and the level of viral replication.

Fig 1

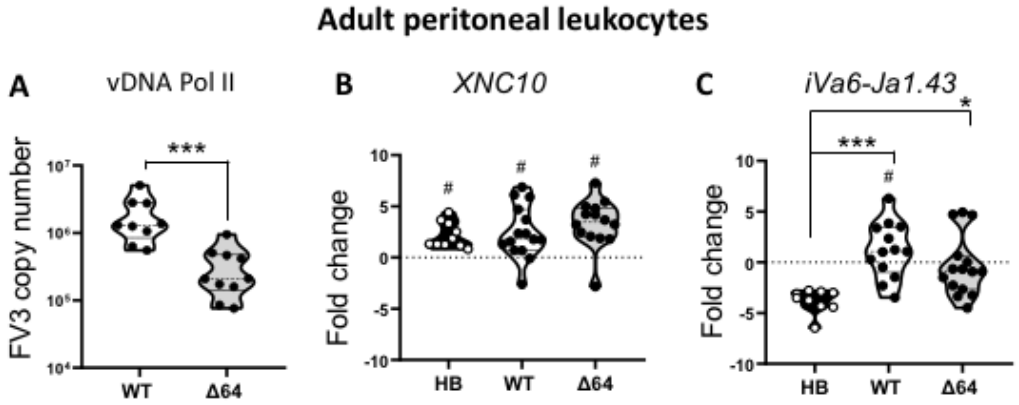

Figure 1. Effects of infection with attenuate KO FV3 recombinant or bacterial stimulation on iVα6 T cell response. PLs were collected at 1 dpi from adult frogs infected with 1 × 10^6 PFUs of WT- FV3 or Δ64-FV3, or 100 µl heat-killed (HB) *E. coli*. (A) Genome copy number using absolute qPCR with primers against FV3 polymerase II. (B) XNC10 relative gene expression and (C) iVα6-Jα1.43 relative gene expression. Gene expression was determined relative to an endogenous control (GAPDH) and fold changes were calculated using the unstimulated sample (injected with equivalent volume of APBS) collected at the same time point. Data are pooled from three independent experiments with n = 4-5 animals in each experiment and each dot represents an individual animal. The line intersecting the *y*-axis at 0 represents the unstimulated control that the fold changes of the treatments are in relation to. # denotes significant differences compared to unchallenged (APBS) injected controls and * p< 0.05 and *** p< 0.001 above the line denotes statistically significant differences between the indicated groups (one way ANOVA and Dunns's multiple comparisons test).

To obtain additional evidence of the correlation between active viral replication and iVα6 T cell recruitment, we i.p infected adult *X. laevis* with WT- and Δ64-FV3, and then monitored the transcript levels of iVα6-Jα1.43 and XNC10 in the peritoneal cavity (the site of infection) and kidney (main site for viral replication) at 1, 3 and 6 dpi (Fig. 2). To evaluate viral replication, we determined the genome copy number by absolute qPCR (Fig. 2A and 2B) and to assess productive infection we performed plaque assays (Fig. 2C and Fig. S1). Again Δ64-FV3 exhibited a severe replication defect preventing the production of infectious particles (Fig. 2C and Fig. S1). Using iVα6-Jα1.43 expression as a proxy for monitoring the kinetics of iVα6 T cell recruitment, we detected an increase in iVα6-Jα1.43 transcript levels in the peritoneal cavity as early as 1 dpi with WT-FV3 compared to uninfected controls, which became significant by 3 dpi, and then returned to low but detectable levels by 6 dpi (Fig. 2D). In contrast, Δ64-FV3 infection did not trigger significant increase in iVα6-Jα1.43 transcript levels. In kidneys, iVα6-Jα1.43 expression was significantly elevated at 3 dpi and then returned to baseline at 6 dpi following infection with WT-FV3 (Fig. 2E). In contrast, no significant changes in iVα6-Jα1.43 expression was detected with Δ64-FV3 infection.

The examination of the expression response of the iVα6 T cell restricting MHC class I-like XNC10 gene revealed some interesting correlations. In PLs, WT-FV3 infection induced a rapid (from 1 dpi) and sustained (until 6 dpi) XNC10 increase (Fig. 2F), which was delayed (from 3 to 6 dpi) in kidneys (Fig. 2G). Consistent with the poorly induced iVα6-Jα1.43 expression, infection with Δ64-FV3 also resulted in impaired induction of XNC10 gene expression. Indeed, Δ64-FV3 infection did not induce any significant change in XNC10 gene expression at 3 and 6 dpi in both PLs and kidneys.

Collectively, these data indicate that XNC10-restricted iVα6 T cell recruitment is relative to viral burden and requires active viral replication.

Fig 2

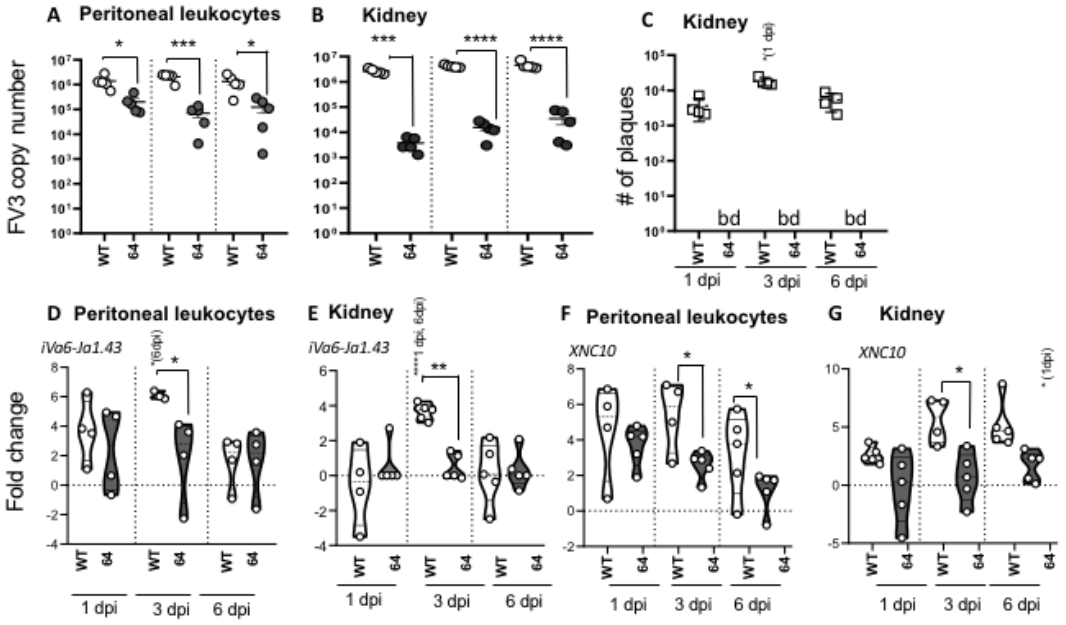

Figure 2. Magnitude of iVa6 T cell response in adult frogs is associated to the level of viral replication and production of infectious particles. PLs and kidneys were collected at 1, 3 and 6 dpi from adult frogs infected with 1 × 10^6 PFUs of WT-or Δ64-FV3. FV3 genome copy number by absolute qPCR in PLs (A) and kidneys (B) were determined and the total number of infectious particles in the kidney was determined by plaque assay (C). Gene expression of iVα6-Jα1.43 and XNC10 in PLs (D, F) and kidney (E, G) were determined relative to an endogenous control (GAPDH) and fold changes were calculated using mock-infected frogs as a control. Each dot represents an individual animal (n = 4-5). The line intersecting the *y*-axis at 0 represents the APBS control that the fold changes of the treatments are in relation to. * p< 0,05; **p<0.01; ***p<0.001 and **** p< 0.0001 above the line denotes statistically significant differences between the different treatment groups, significant differences

between timepoints within each treatment group is indicated in brackets (one way ANOVA and Dunns's multiple comparisons test).

3.2. Relationships between FV3 infection magnitude and iVa6 T cell response in tadpoles

In contrast to adult frogs that exhibit a prominent conventional antiviral CD8 T cell response restricted by classical MHC class Ia, tadpoles have barely detectable class Ia surface protein expression and rely heavily on XNC10-restricted iVα6 T cells (Edholm et al., 2013). Indeed, XNC10-deficient transgenic tadpoles lacking iVα6 T cells succumb to FV3 infection much faster than controls. This suggests that a rapid iVα6 T cell response is required during the early stage of FV3 infection. Similar to adult frogs, we postulated that the efficient recruitment of iVα6 T cells at the site of infection would depend on a productive FV3 infection. Accordingly, we i.p. infected tadpoles with either WT- or Δ64-FV3. Consistent with previous reports, Δ64-FV3 exhibited a severe growth defect in tadpoles as shown by the very low number of plaques detected even at 6 dpi (Fig. 3A and Fig. S2). As expected, WT FV3 infection elicited an iVα6 T cell response that was overall comparable to adults including an early (by 1 dpi) increase in iVα6-Jα1.43 as well as XNC10 transcript levels that were maintained by 3 dpi and then declined close to background levels by 6 dpi. In contrast, there were no significant changes in XNC10 and iVα6-Jα1.43 transcript levels following Δ64-FV3 infection (Fig. 3B).

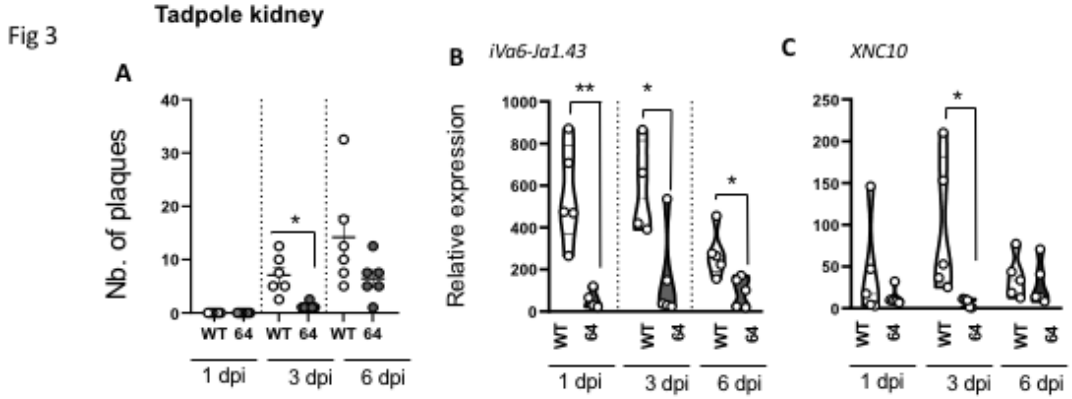

Figure 3. iVa6 T cell response in tadpoles depends on active viral replication and productive FV3 infection. Three week-old (stage 55) tadpoles were infected with 10,000 PFUs of WT-or Δ64-FV3. At 1, 3 and 6 dpi kidneys were collected and the total number of infectious particles were determined by plaque assay respectively **(A)**. Gene expression of iVα6-Jα1.43 (B) and XNC10 (B) was determined relative to an endogenous control (GAPDH) and relative expression was calculated against the lowest observed expression according to the ΔΔCt method (n =5). No iVα6-Jα1.43 transcripts were detected in the kidney uninfected tadpoles. * $p < 0.05$ and **$p<0.01$ above the line denotes statistically significant differences between treatment groups. (one way ANOVA and Dunn`s multiple comparisons test).

3.3. Targeted iVa6T cell depletion in vivo

As mentioned, intraperitoneal (i.p) infection of *X. laevis* tadpoles with FV3 results in a rapid and transitory increase in Vα6-Jα1.43 transcript levels, both at the site of infection and in the kidney (main FV3 target organ), consistent with a direct and active involvement of this specific iT cell population in the early anti-FV3 response (Edholm et al., 2015). In support of this possibility, the developmental disruption of iVα6 T cells in MHC class I-like XNC10- deficient transgenic tadpoles, results in increased susceptibility to FV3 (Edholm et al., 2013). However, silencing of XNC10 from early embryogenesis is likely to result in biological effects other than those inferred via the reciprocal loss of a unique iT cell population. Therefore, to more directly determine the role of iVα6 T cells in anti-viral defense against FV3 in the presence of a functional XNC10 gene, we targeted iVα6 T cells using XNC10 tetramers. XNC10 tetramers have been shown to selectively induce iVα6T cell death *ex vivo*

and to temporarily ablate Vα6-Jα1.43 transcript levels *in vivo* (Banach et al., submitted). Accordingly, we injected *X. laevis* tadpoles with 1 μg of XNC10 tetramers 1 day prior and 1 day following i.p injection with FV3 and quantified transcript levels of the invariant Vα6-Jα1.43 rearrangement in PLs and kidney at different times following infection (Fig. 4). Control groups received injections with an equivalent volume of amphibian PBS at the same time points. To evaluate viral loads and dissemination in the different groups we determined the FV3 genome copy number using absolute qPCR (Fig. 4D-E). In addition, we examined the gene expression of XNC10 to evaluate any impact of the injection regime on the expression of this MHC class I-like gene (Fig. S3).

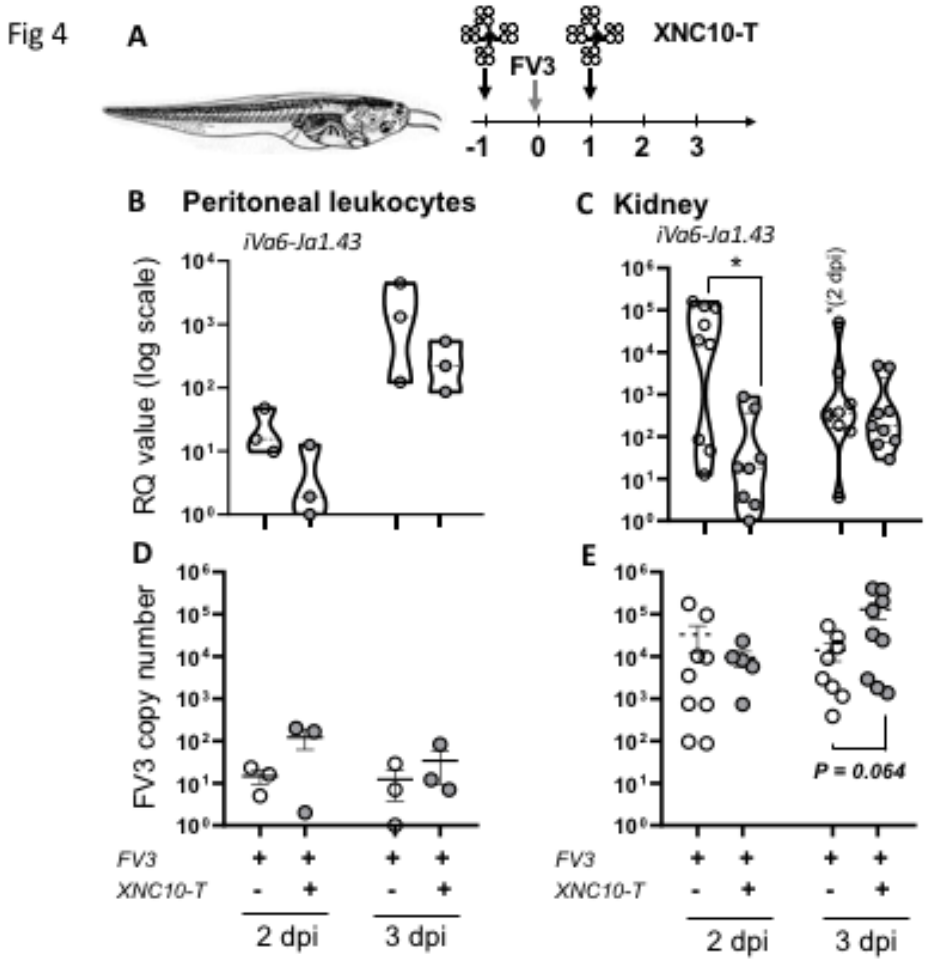

Figure 4. XNC10 tetramer-mediated iVa6T cell depletion in tadpoles affects iVα6-Jα1.43 transcript levels and viral replication. PLs and kidneys were collected from three weeks-old (stage 55) tadpoles that had been injected with 1 μg XNC10 tetramers or vehicle control 1 day pre and 1 day post i.p injected with 10,000 PFUs of FV3, at the indicated time points (n = 9). A schematic of the injection regime is shown in A. Gene expression of iVα6-Jα1.43 in PLs (B) and kidney (C) is shown. Results are normalized to an endogenous control and presented as relative expression compared with the lowest observed value according to the ΔΔCt method. FV3 loads in PLs (D) and kidney (E) were measured using absolute qPCR with primers against FV3 polymerase II. For peritoneal leucocytes each dot represents a pool of 3 tadpole while for kidney each dot represents a single tadpole. * $p<0.05$ denotes statistically significant differences between the indicated groups (One way ANOVA followed by Tukey's multiple comparisons test).

Consistent with XNC10 tetramers inducing iVα6 T cell death, iVα6-Jα1.43 transcripts were markedly reduced, albeit not significantly, in the peritoneal cavity (site of infection) at 2 dpi, and remained lower than controls at 3 dpi. In the kidney, iVα6-Jα1.43 transcripts were significantly lower in the XNC10 tetramer treated group at 2 dpi, reaching levels equivalent to controls by 3 dpi. It is

noteworthy that consistent with previous reports, iVα6-Jα1.43 transcripts were either undetected or near threshold levels in uninfected tadpoles. Thus, XNC10 tetramer treatment provides an efficient transient depletion of iVα6 T cells *in vivo* providing us with a system to directly investigate the functional roles of iVα6 T cells. No significant difference was observed in XNC10 expression between the two groups (Fig. S3).

In regards to viral loads, while there was no significant difference in viral genome copy numbers in the peritoneal cavity, XNC10 tetramer treated tadpoles displayed a marginally significant (*P =0.064*) higher viral load in the kidney by 3 dpi. By 6 dpi, using the same injection scheme as described in Fig. 4A, there were significantly higher viral loads in the kidney of the XNC10-tetramer treated group compared to vehicle control (Fig. 5). Notably, viral loads were not increased in tadpoles injected with XNC10 monomers, which do not bind to iVα6 T cells (Edholm et al., 2013). In addition, XNC10 tetramer treatment had only a transitory effect on iVα6 T antiviral activity as shown by comparable viral loads detected when FV3 infection occurred 3 days rather than 1 day after XNC10 tetramer injection (Fig 5A). To assess whether this increase in viral loads had an impact on host resistance, we determined the cumulative mortality and mean survival of XNC10 tetramer injected and FV3 challenged tadpoles (Fig 6). Notably, transitory depletion of antiviral iVα6 T cells within 24 hours of FV3 infection resulted in increased mortality rate compared with FV3 infected controls (*P = 0.0568*). These data support the conclusion that iVα6 T cells are intricately involved in *X. laevis* anti-FV3 immune responses and highlights the importance of an efficient onset of appropriate anti-viral immune response in order to control and efficiently combat FV3 infection.

Fig 5

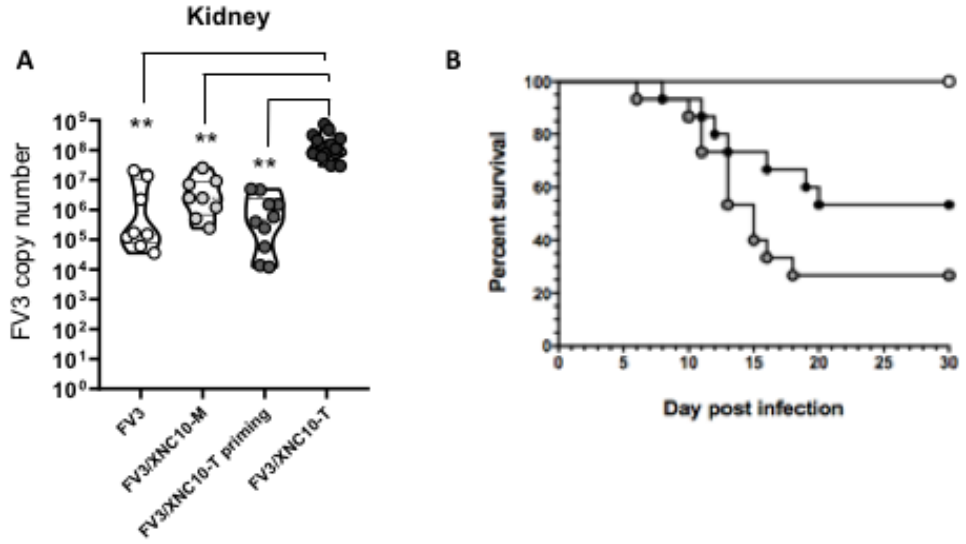

Figure 5. Specificity and long term impact of transitory iVa6T cell depletion. (A) Three week-old (stage 55) tadpoles were injected with either 1 μg XNC10- tetramers, 1 μg XNC10-monomers or vehicle control 1 day pre and 1 day post i.p injected with 10,000 PFUs of FV3 (n=8) and kidneys were collected at 6 dpi. Viral loads were assessed by absolute qPCR with primers against FV3 polymerase II. The results are combined from two separate experiments and each dot represents an individual tadpole. ** $p< 0.01$ and *** *p>0.0005* above the line denotes statistically significant differences between the indicated groups (One way ANOVA followed by Tukey`s multiple comparisons test). (B) Three week-old (stage 55) tadpoles were injected with either 2 μg XNC10 tetramers or vehicle control 1 day pre and 1 day post i.p injected with 10,000 PFUs of FV3 (n=X) and survival was monitored daily over a 30-day period. Survival was determined using Kaplan-Meier, * $p< 0.05$, ** *p>0.005 and* *** *p>0.*0005. Uninfected controls (white circle), FV3 infected tadpoles (black circle) and XNC10 tetramer treated FV3 infected tadpoles (grey circle).

3.4. Effects of iVa6T cell depletion on PLs and kidney antiviral responses in tadpoles.

We have previously shown that the compromised anti-FV3 response observed in XNC10-deficient transgenic frogs correlates with the induction of macrophages exhibiting a less potent antiviral state (Edholm et al., 2013). In particular, XNC10 deficiency hampered the expression of the macrophage growth factor IL-34. Notably IL-34 has been shown to elicit the differentiation of mononuclear phagocytes into robust type I interferon producing macrophages exhibiting strong FV3 antiviral activity (Grayfer and Robert, 2014). These findings prompted us to hypothesize that iVα6 T cells promote timely and efficient type I interferon (IFN)-mediated anti-FV3 responses by influencing the polarization of macrophages into a more potent antiviral state. Thus, to further delineate the putative functional roles of iVα6 T cells during FV3 infection we next determined if direct and transient iVα6 T cell depletion has an impact on macrophage effector functions. To address this possibility, we monitored the expression profiles of the two macrophage growth factors CSF-1 (Grayfer and Robert, 2013) and IL-34 (Grayfer and Robert, 2014) in peritoneal leucocytes and kidney (Fig. 6) from FV3 infected tadpoles with or without XNC10 tetramer treatment. Both IL-34 and CSF-1 transcription levels were significantly lower in the kidney of XNC10 tetramer treated tadpoles at 2 dpi, correlating with the reduced iVα6-Jα1.43 expression and reached levels comparable to controls, by 3 dpi.

Fig 6

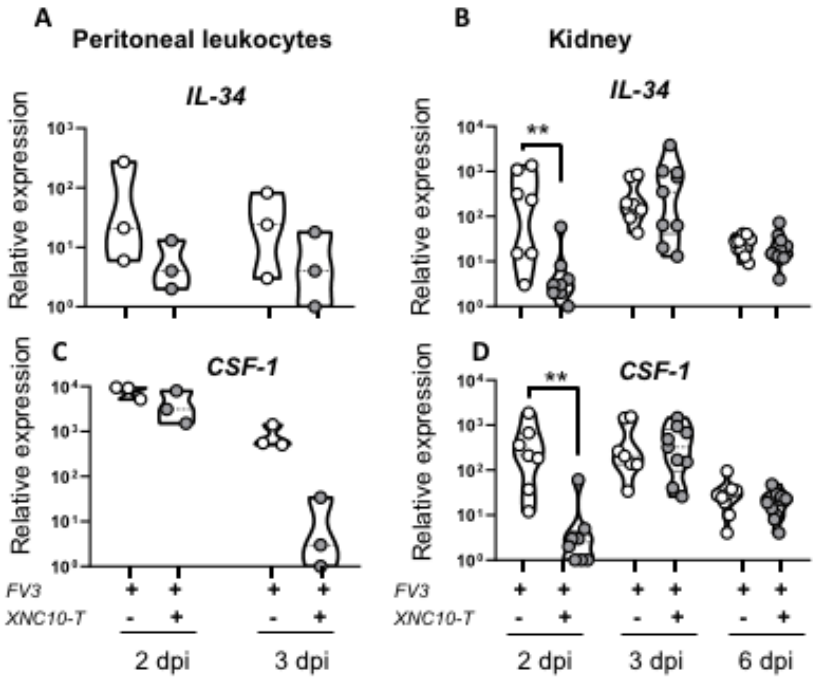

Figure 6. Effect of XNC10 tetramer treatment on the expression of the macrophage stimulating factor genes CSF1 and IL-34 in peritoneal cavity and kidney of FV3 infected tadpoles. PLs and kidneys were collected from three weeks-old (stage 55) tadpoles that had been injected with 1 μg XNC10 tetramers or vehicle control 1 day pre- and 1 day post-ip injected with 10,000 PFUs of FV3, at the indicated time points (n= 8-9). Quantitative gene expression analysis of IL-34 (A, B) and CSF-1(C, D) were determined relative to a endogenous control (GAPDH) and relative expression was calculated against the lowest observed expression according to the ΔΔCt method (n =9), * p< 0,05, ** p<0.005 and ***p>0.001 above the line denotes statistically significant differences between the different treatment groups, significant differences between timepoints within each treatment group is indicated in brackets (One way ANOVA followed by Tukey's multiple comparisons test).

Given that the type I IFN response is critical in controlling viral infections and is compromised in XNC10-deficient animals (Edholm et al., 2015), we postulated that a direct impairment of iVα6 T cells should affect type I IFN gene expression to a similar extent. Indeed, unlike control animals, both type I IFN and type III IFN gene expression were magnitudes lower at 3 dpi in PLs of XNC10 tetramer treated animals compared to controls, whereas the levels of type II IFN was highly variable between animals with no apparent difference in expression between the two groups (Fig 7A-C). Similarly, in kidneys the expression of both type I and type III IFN genes was significantly impaired in XNC10 tetramer treated animals at 2 dpi, whereas by 3 dpi both treated and control groups exhibited high transcript levels of type I and type III IFN (Fig. 7D-E).

Fig 7

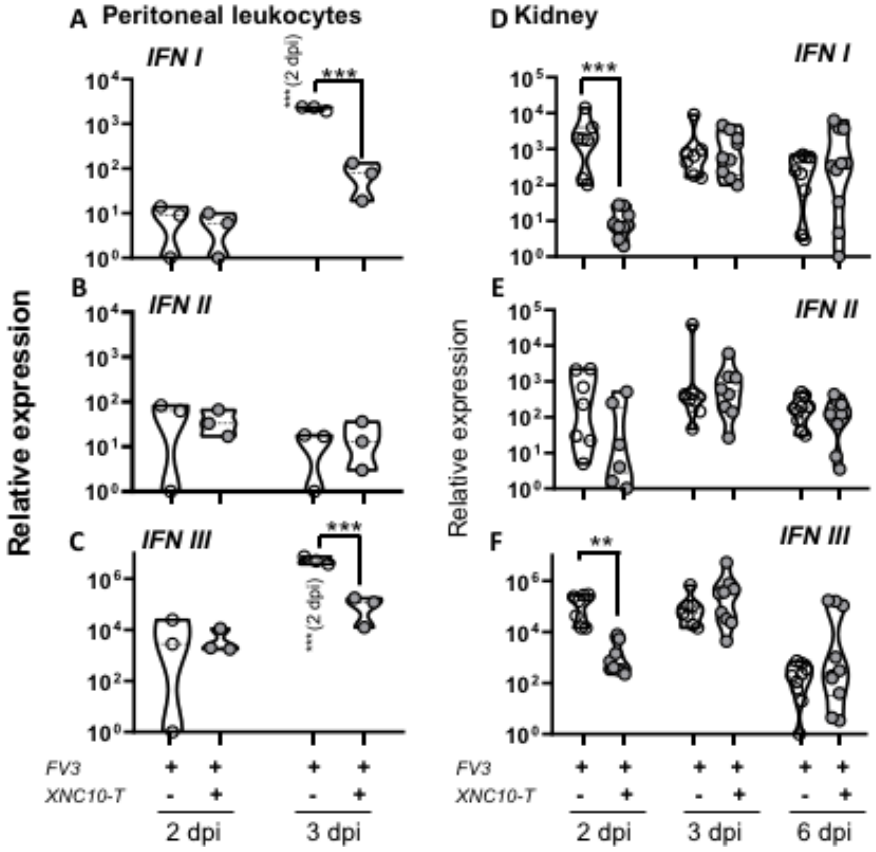

Figure 7. XNC10 tetramer treatment results in a delayed antiviral response in peritoneal cavity and kidney of FV3 infected tadpoles. PLs and kidneys were collected from three weeks-old (stage 55) tadpoles that had been injected with 1 μg XNC10 tetramers or vehicle control 1 day pre- and 1 day post-ip injected with 10,000 PFUs of FV3, at the indicated time points (n= 8-9). Quantitative gene expression analysis of type I (A, D), type II (B, E) and type III IFN (C, F) were determined relative to a endogenous control (GAPDH) and relative expression was calculated against the lowest observed expression according to the ΔΔCt method (n =9), * p< 0,05, ** p<0,005 and ***p>0,001 above the line denotes statistically significant differences between the different treatment groups, significant differences between timepoints within each treatment group is indicated in brackets (One way ANOVA followed by Tukey's multiple comparisons test).

In response to a viral infection, protective host response relies on the onset of appropriate cell mediated immunity to mediate viral clearance. Thus, we next examined the expression of interleukin18 (IL-18) and interleukin 12 (IL-12) upon FV3 infection in PLs and kidney of control and iVα6 T cell deficient tadpoles. Both IL-18 and IL-12 are produced by macrophages and act in synergy to promote the development of innate and specific Th1 type immune responses (Barbarin et al., 2017; Huber et al., 2015; Zajonc and Girardi, 2015). Given that iVα6 T cell deficiency hampers the induction of macrophage differentiating factors resulting in a delayed interferon response, we postulated that IL-18 and IL-12 cytokine production would be similarly impaired. Indeed, unlike control animals, IL-

18 expression was significantly lower at 2 dpi in kidney of XNC10 tetramer treated animals, whereas at 3 dpi XNC10 tetramer treated tadpoles exhibited elevated IL-18 transcript levels on par with that of controls that were significantly reduced by 6 dpi in both groups (Fig. 8). While there was a slight decrease in IL-12 gene expression at 2 dpi in XNC10 tetramer treated animals, it was not statistically significant.

Fig 8

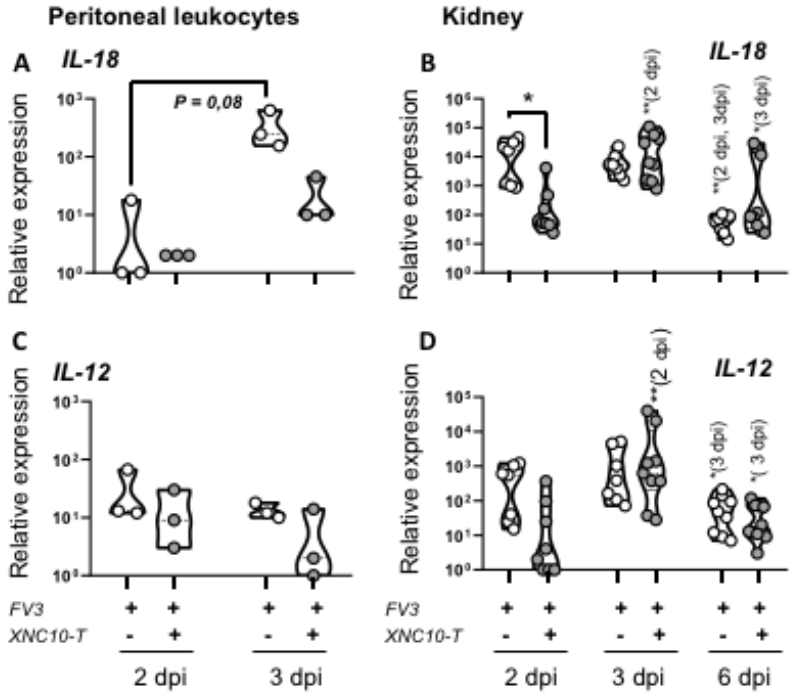

Figure 8. Effects of iVa6T cell depletion on IL-18 and IL-12 responses. PLs and kidneys were collected from three weeks-old (stage 55) tadpoles that had been injected with 1 μg XNC10 tetramers or vehicle control 1 day pre and 1 day post i.p injected with 10,000 PFUs of FV3, at the indicated time points (n= 8-9). Quantitative gene expression of IL-18 (A-B) and type IL-12 (C-D) was determined relative to an endogenous control (GAPDH) and relative expression was calculated against the lowest observed expression according to the ΔΔCt method (n =9), * $p< 0.05$; ** $p<0.005$ and ***$p>0.001$ above the line denotes statistically significant differences between the different treatment groups, significant differences between timepoints within each treatment group is indicated in brackets (One way ANOVA followed by Tukey's multiple comparisons test).

Overall, these findings are in accordance with what was previously been reported for XNC10-deficient transgenic animals (that lack both XNC10 and iVα6 T cells, (Edholm et al., 2015)) suggesting that iVα6 T cells are directly and critically involved in mounting an anti-FV3 response, and that acute loss or impairment of these cells result in both a delayed type I and type III IFN antiviral response as well as a delayed IL-18 response.

4. Discussion

The present study provides new insights into the requirement for an effective amphibian host response to the ranavirus FV3 trough an early detection by the MHC class I-like XNC10 and the rapid recruitment of the activated XNC10-restricted innate T cell subset iVα6. A remarkable feature of this novel antiviral immune surveillance system is that it is prominent in naturally classical MHC class Ia-deficient tadpoles, whereas it is a component in class Ia and conventional T cell competent adults. Nevertheless, while this antiviral system is central for tadpole survival at early stage of FV3 infection, it is also critical for a timely control of viral burden in adult frogs. To better functionally define this novel antiviral immune surveillance system, we examined the initial conditions of FV3 infection that trigger the iVα6 T cell response by using a deficient FV3 recombinants and we defined the antiviral immune response initiated by iVα6 T cells.

385 The critical role of iNKT cells in antiviral immunity is well documented in mammals, where
386 their pre-activated or memory phenotypes allow them to serve as early responder (within hours
387 following infection) without requiring expansion as conventional T cells (Juno et al., 2012; Kohlgruber
388 et al., 2016). Similarly, in *X. laevis* we have shown a rapid increase of iVα6-Jα1.43 transcript levels
389 (within 1 dpi) at the site of FV3 infection in the peritoneum and in the main site of viral replication in
390 the kidney, concomitant with a decrease in the spleen. Notably, the recruitment of these iVα6 cells
391 occurs days before significant proliferation of conventional T cells, which become significant only
392 from 3 dpi onward in the spleen (Morales and Robert, 2007). This strongly suggests that iVa6 T cell
393 are rapidly activated and recruited from the spleen by FV3 infection (Edholm et al., 2015).
394 Here, we provide further evidence that iVα6 T cell recruitment occurs in direct response to FV3
395 infection and is not indirectly triggered by inflammation induced by heat-killed bacteria or by a
396 replication-defective Δ64-FV3 recombinant. Furthermore, while both attenuated Δ64-FV3 and heat-
397 killed bacterial stimulation induced an early transcriptional induction of the MHC class I-like XNC10
398 that restricts iVα6 T cells, these elevated expression levels were only maintained in WT-FV3 infected
399 frogs. This suggests a coordinated role of XNC10 and iVα6 T cells in detecting and eliciting a rapid
400 response against ranavirus pathogens. This coordinate response of XNC10 and iVα6 T cells only
401 induced by a productive viral infection is also consistent with the detection of virally-derived
402 products or ligands. Whether XNC10 bind ligand(s) and if so the identity of such putative XNC10
403 ligand(s) is currently unknown, but nucleotide sequence comparisons and 3D-modelling of the
404 putative ligand binding regions indicate that XNC10 could accommodate lipid ligands in a way
405 reminiscent to CD1d (Edholm et al., 2014; Goyos et al., 2011; Rossjohn et al., 2012). Thus, it is tempting
406 to speculate that XNC10 can be upregulated and bind products/ligands from early FV3 replication
407 and/or release of assembled viruses to activate iVα6 T cell response. In this scenario, infection by Δ64-
408 FV3 would not lead to sufficient production and release of FV3-derived products/ligands to initiate
409 iVα6 T cell recruitment and activation.
410 Interestingly, the correlation between defective viral replication, blunted XNC10 gene
411 expression response and impaired iVα6 T cell recruitment was more robust in tadpoles. This may be
412 due to the heavier reliance on iT cells in tadpoles. In absence of an efficient classical MHC-I restricted
413 conventional CD8 T cell response, iVα6 T cells are likely to be more directly and critically involved
414 in tadpole host response against FV3. This is evidenced by marked vulnerability of XNC10-deficient
415 transgenic tadpoles lacking iVα6 T cell to FV3 infection, especially at early stages on infection
416 (Edholm et al., 2013). Our findings using XNC10 tetramer treatment substantiate this initial
417 requirement of iVα6 T cell response for controlling viral infection and host resistance to FV3. Indeed,
418 a partial and transient depletion of iVα6 T cells prompted by injection of XNC10 tetramers shortly
419 before and after infection impairs tadpole immune defenses, which not only leads to more severe
420 viral burden but also decrease survival rate.
421 While the effector functions of iVα6 T cells are likely to be multifaceted, our previous findings
422 support a role of these cells as immune modulators influence the polarization of peritoneal
423 macrophages into a more robust anti-viral state (Edholm et al., 2015). It is noteworthy that the
424 expression of type I and III IFN as well as cytokine such as CSF-1 and IL-18 are transiently altered in
425 parallel to iVα6 T cells depletion. Little is known about the iVα6 T cell mechanism of action, but
426 previous findings have hinted at a modulation of macrophages (Edholm et al., 2013; Edholm et al.,
427 2015). While type I and III IFN is likely produced and released by many cell types, the rapid increase
428 of their transcript levels in the peritoneum and kidneys upon FV3 infection coincide with the
429 recruitment of monocytic phagocytes both in tadpoles and adult frogs (De Jesús Andino et al., 2012;
430 Grayfer et al., 2015; Morales et al., 2010). Infiltration of MHC class II+ leukocytes in kidneys at early
431 stage of FV3 infection is also consistent with infiltration of macrophages (Morales and Robert, 2007).
432 CSF1 and IL-34 are two key macrophage growth factors acting through the same CSF-1 receptor
433 across jawed vertebrates (Droin and Solary, 2010). In *X. laevis*, CSF1 is required for the commitment
434 and maturation of mature macrophage lineage cells (Grayfer and Robert, 2013), whereas IL-34
435 stimulates stronger antiviral effector function by macrophages both in tadpoles and adult frogs
436 (Grayfer and Robert, 2014, 2015). The acute decrease in type I and III IFN as well as CSF1 and IL-34

at 2 dpi concomitantly with the XNC10 tetramer-mediated iVα6 T cell defect reinforce the postulated functional link between iVα6 T cell and macrophages. This, of course does not preclude possible interaction of iVα6 T cells with other immune cell effectors as well as a reciprocal effect of macrophages on iVα6 T cells. Indeed, macrophages in mammals are known to be important producer of IL-12 and IL-18, two cytokines that stimulate iNKT cells and promote Th1 adaptive immune response (Barbarin et al., 2017; Huber et al., 2015; Yasuda et al., 2019). The transitory blunted expression of IL-18 and to a lesser extent IL-12 that accompanied iVα6 T cell transient defect, besides being consistent with decreased macrophage function may have amplified XNC10 tetramer-mediated iVa6 T cell impairment. In any case, it is remarkable that the brief interference of iVα6 T cell function has a long-lasting negative impact on the tadpole ability to control FV3 infection and ultimately to survive infection.

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