National Institutes of Health
Turning Discovery Into Health

NIHMS PDF Receipt

Journal title:	The Journal of Immunology
Manuscript title:	Distinct host-mycobacterial pathogen interactions between resistant adult and tolerant tadpole life stages of Xenopus laevis
Submitter:	American Association Of Immunologists
NIHMSID:	1540421

Manuscript Files

Type	Label	File Name	Upload Date
Manuscript	1	article_1.docx	2019-09-20 05:34:29
Figure	1	figure_1.tif	2019-09-20 05:34:29
Figure	2	figure_2.tif	2019-09-20 05:34:29
Figure	3	figure_3.tif	2019-09-20 05:34:29
Figure	4	figure_4.tif	2019-09-20 05:34:29
Figure	5	figure_5.tif	2019-09-20 05:34:29
Figure	6	figure_6.tif	2019-09-20 05:34:29
Figure	7	figure_7.tif	2019-09-20 05:34:29
Figure	8	figure_8.tif	2019-09-20 05:34:29
Table	1	table_1.doc	2019-09-20 05:34:29
Table	2	table_2.doc	2019-09-20 05:34:29
Table	3	table_3.doc	2019-09-20 05:34:29

This PDF receipt will be used only as the basis for generating PubMed Central (PMC) documents and will not appear on PMC. The PMC-ready documents will be made available for review after conversion. Any necessary corrections can be made at that time. No materials will be released to PMC without the Reviewer's approval.

Distinct host-mycobacterial pathogen interactions between resistant adult and tolerant tadpole life stages of *Xenopus laevis*

Kun Hyoe Rhoo[1], Eva-Stina Edholm[2], María J. Forzán[3], Adil Khan[1], Anthony W. Waddle[1,4], Martin S. Pavelka Jr and Jacques Robert[1]

[1]Department of Immunology and Microbiology, University of Rochester, Medical Center, Rochester, NY, USA. [2]The Norwegian College of Fishery Science, University of Tromsø, Tromsø, Norway. [3] Cornell Wildlife Health Lab, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University. [4] One Health Research Group, Melbourne Veterinary School, The University of Melbourne, Werribee, VIC 3030, Australia.

Running title: Distinct *Xenopus* tadpole and adult response to mycobacteria

Communicating Author[1]: 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

[1] This research was supported by a predoctoral fellowship to RKH (T32AI118689), as well as R21AI139718 and R24AI059830 from the National Institute of Allergy and Infectious Diseases (NIH/NIAID) and IOS-1456213 from the National Science Foundation.

Abstract

Mycobacterium marinum (Mm) is a promiscuous pathogen infecting many vertebrates including humans, whose persistent infections are problematic for aquaculture and public health. Among unsettled aspects of host-pathogen interactions, the respective roles of conventional and innate-like (i)T cells in host defenses against *Mm* remain unclear. Here, we developed an infection model system in the amphibian *Xenopus laevis* to study host responses to *Mm* at two distinct life stages, tadpole and adult. Adult frogs possess efficient conventional T cell-mediated immunity, whereas tadpoles predominantly rely on innate-like (i)T cells. We hypothesized that tadpoles are more susceptible and elicit weaker immune responses to *Mm* than adults. However, our results show that although anti-*Mm* immune responses between tadpoles and adults are different, tadpoles are as resistant to *Mm* inoculation as adult frogs. *Mm* inoculation triggered a robust pro-inflammatory $CD8^+$ T cell response in adults, whereas tadpoles elicited only a non-inflammatory CD8 negative- and iT cell-mediated response. Furthermore, adult anti-*Mm* responses induced active granuloma formation with abundant T cell infiltration and associated with significantly reduced *Mm* loads. This is reminiscent of local $CD8^+$ T cell response in lung granulomas of human tuberculosis patients. In contrast, tadpoles rarely exhibited granulomas and tolerated persistent *Mm* accumulation. Gene expression profiling confirmed poor tadpole $CD8^+$ T cell response contrasting with the marked increase in transcript levels of the anti-*Mm* iT cell receptor rearrangement (*iVα45-Jα1.14*) and of CD4. These data provide novel insights into the critical roles of iT cells in vertebrate anti-mycobacterial immune response and tolerance to pathogens.

Key points

- *Xenopus tadpoles and adults have adapted distinct immune responses to Mycobacteria*
- *A disease tolerance involving non-inflammatory iT cell response in tadpoles*
- *A disease resistance with granuloma, inflammation and CD8 T cell response in adults*

Introduction

Mycobacteria marinum (Mm) resides in marine and freshwater and is capable of infecting a broad range of aquatic species including fish, reptiles, amphibians (e.g., *Xenopus laevis* [1]), and mammals including humans (reviewed in [2]). *Mm* is difficult to eradicate in aquaculture, especially when introduced to a recirculating water system. This pathogen, which causes fish mycobacteriosis outbreaks, can severely impacts aquaculture as reviewed in [3]. In human, *Mm* causes opportunistic infection in the skin, and poses public health risks [3]. As such, a better understanding of host immune responses to *Mm* remains crucial to improve diagnostics, treatment options, and vaccine strategies.

Importantly, *Mm* often serves as a useful Biosafety Level 2 (BSL2) alternative pathogen for *Mycobacterium tuberculosis* (*Mtb*), the causative agent for important human tuberculosis (TB; [4]). Like *Mtb*, *Mm* has been shown to survive within host macrophages, and is able to induce caseating granulomas in a zebrafish model [5]. Furthermore, studies have shown that *Mtb* and *Mm* share selected virulence determinants such as ESX-1 secretion system for phagosomal arrest in the host macrophages [1]. *Mm*

70 grows optimally at 30 – 33°C, which is lower than the optimal temperature of *Mtb*
71 (~37°C) and replicates every 4 hours, which is markedly shorter than replication time of
72 *Mtb* (~20 hr) and more convenient for *in vitro* studies. Therefore, investigation with *Mm*
73 will further benefit our understanding of host anti-mycobacterial immune responses
74 providing a valuable and practical model that may ultimately lead to the development of
75 new immunotherapeutic-based strategies for TB.

76 *X. laevis* is an attractive comparative immunology animal model due to its fully
77 sequenced genome, and the availability of large genetic and genomic resources, as well
78 as the remarkable similarity of its immune system with that of humans [6, 7]. Unlike
79 mammals, however, *X. laevis* undergoes metamorphosis and has distinct T cell
80 populations prior to and after this developmental transition. *X. laevis* tadpoles lack an
81 optimal protein level of classical MHC class I molecule, yet express multiple *Xenopus*
82 MHC class I-like genes (*XNCs*). In mammals, MHC I-like molecules have been shown to
83 restrict innate-like T (iT) cells that exhibit features of both innate and adaptive immune
84 cell effectors. Similarly, several iT cell subsets have been identified in *X. laevis* [8].
85 These iT cell subsets are predominant in tadpoles representing as much as 80% of
86 $CD8^{low/negative}$ splenic T cells, whereas they represent only a minority compared to
87 conventional T cells in adults *X. laevis*.

88 Generally, tadpoles are considered to have a less efficient immune response and,
89 thus, to be more susceptible to natural pathogens (reviewed in [9]). However, a more
90 detailed investigation *in X. laevis* suggests that distinct specialization of immune response
91 mediated by iT cells allows the immune competence of tadpoles against distinct
92 pathogens [10, 11]. Importantly, one of the iT cell populations exhibiting the invariant

TCRα rearrangement *iVα45-Jα1.14*, has been shown to be critical for tadpole resistance to *Mm* infection [11].

In mammalian models, two iT cell subsets have been studied in the context of anti-mycobacterial immune response: mucosal associated innate T cells (MAIT) and invariant natural killer T cells (iNKT). A few clinical studies, along with *in vitro* studies suggest protective functions of both types of iT cell at least at early stage of mycobacterial infections (reviewed in [12, 13]). However, investigation of the specific regulatory and/or effector functions, activation, and recruitment of iNKT and MAIT cells have been challenged by the lack of suitable animal models (reviewed in [14]). As such *X. laevis,* exhibiting a distinct T cell balance between different life stages, would provide a useful model system to gather new insights into iT cell-mediated immune functions during mycobacterial infection.

Here, we report evidence of a distinct immune response and histopathology between *X. laevis* adults and tadpoles towards *Mm.* In support of our hypothesis that the respective response of iT and conventional T cells is distinct between tadpoles and adult frogs, we found markedly different conventional anti-*Mm* T cell response, histopathology and *Mm* growth between these two life stages.

Materials and Methods

Animals. Two-year-old adult frogs and three-week-old pre-metamorphic outbred tadpoles (stages 52) were obtained from our *X. laevis* research resource for immunology at the University of Rochester, Rochester, New York, USA,

116 http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm. All animals during
117 experiments were carefully handled under the University of Rochester Committee on
118 Animal Resources regulations (approval number 100577/2003-151).
119
120 ***Mm* inoculation.** The *Mm* strain PM2960 was derived from a clinical isolate (stock
121 number PM2690) generously provided by Dr. Hardy, University of Rochester, Rochester,
122 NY. Fluorescent *Mm* strain (PM3495) was generated by transformation of a plasmid
123 (pMV261.Kan.DsRed), kindly provided by W. R. Jacobs, Albert Einstein College of
124 Medicine, Bronx, NY, into the parental PM2960 strain of *Mm*. *Mm* was cultured in
125 Middlebrook 7H9 broth until saturation, and tittered stocks prepared and frozen at -80°C
126 as previously described [11]. A working concentration for intraperitoneal injection into
127 the animals and for *in vitro* infection of leukocytes was prepared by diluting the growth
128 media in the amphibian phosphate buffer solution (APBS) with 0.05% of Tween 80.
129 Tissues were harvested from euthanized animals at indicated days post-inoculation or
130 from postmortem animals for further analyses.
131
132 **Survival analysis of *Mm* inoculated adults and tadpoles** To examine the host
133 resistance of *X. laevis* adults and tadpoles to *Mm* infection, 4-month-old adult frogs and 3
134 week-old pre-metamorphic tadpoles (stage 52) [15] were intraperitoneally inoculated
135 with three different doses of *Mm*: low dose ($5x10^5$ CFU), medium dose ($1x10^6$ CFU), and
136 high dose ($2x10^6$ CFU). The survival of the animals was monitored daily for one month
137 and we investigated the effects of host life-stage (tadpole or adult) and dose on
138 survivorship.

Histopathology, immunohistochemistry (IHC) and *in situ* hybridization (isH). The liver of post-mortem adult frogs (euthanized at 14 and 50 dpi, n=1 each) and whole tadpoles (euthanized at 30 dpi, n=2) inoculated with *Mm* were fixed in 10% formalin and processed for routine histologic examination. Based on previous observation, the liver serves as the main organ infected by *Mm* [11]. Cross sections of the adult livers and full length longitudinal sections of the tadpoles were stained with Hematoxylin & Eosin [16], as well as special stains for bacteria and acid-fast organisms: Gram [17], Ziehl Neelsen [16] and Fite Faraco [18]. Microgranulomas were counted in three random low power fields (10x) from 4 adults (50 dpi) and 4 tadpoles (30 dpi). The average number of microgranulomas per individual was used to represent the number of microgranulomas per 0.5 mm^2 of liver tissue. Immunohistochemical (IHC) staining with anti-CD3 receptor antibody (Leica Cat # PA0553; [19]) was performed using an automated platform to identify conventional T cells (Bond-Max IHC/ISH platform, Bond polymer refine DAB kit, Bond polymer refine red kit; Leica Biosystems, Newcastle Upon Tyne, United Kingdom). A negative control (staining without the primary anti-CD3 staining) was run along with the test slides. A tissue was considered positive if strong and distinct staining with anti-CD3 antibody was present in the membrane of individual cells and background staining was either absent or clearly distinct from true specific staining. An *in situ* hybridization (isH) probe for *Mycobacterium* sp (ACDBio RNAscope Probe-B-MBovis-23SrRNA, Cat No. 446011, Target region 3-3 – 1286, GenBank NR_076088.1, Brosch 2007) was applied following the manufacturer's specifications to identify the presence of *Mm*.

RNA, and genomic DNA isolation from tissues, RT-PCR, and PCR. Total RNA and genomic DNA were extracted from the animal's tissues by using TRIzol reagent, following the manufacturer's protocol (Invitrogen). Total 2 μg of RNA were used to synthesize complementary DNA (cDNA) by a reverse transcriptase, M-MLV (Invitrogen) with a mixture of oligo(dT) primer (Invitrogen). For reverse transcription (RT)-PCR, 125 ng of cDNA were used to determine the expression levels of genes of interest by ΔΔCT value with ABI 7300 Real-Time PCR System and PerfeCTa SYBR Green FastMix ROX. The expression levels are normalized to that of an endogenous housekeeping gene, *gapdh,* then further normalized against the lowest observed expression. All the primers are validated prior to use. Further, the absolute quantification method was done using the quantitative RT-PCR (qRT-PCR) analysis. Using a *Mm* 16srRNA PCR fragment was cloned into the pGEM-Easy Vector (Promenga), further transformed into DH10β competent bacteria for amplification. The plasmid stock was serially diluted in the range between 10^{10} to 10^{1} plasmid copies of 16srRNA *Mm* in order to generate a standard curve by absolute qRT-PCR. To determine *Mm* loads, the total 250 ng of genomic DNA was used as a template, then the absolute copy number of *Mm* 16srRNA genome was extrapolated from the standard curve. All primer sequences are listed in Table 1.

Statistical Analysis. For studying the kinetics of *Mm* loads, a non-parametric Kruskal-Wallis test followed by Dunn test for multiple comparisons was performed. For RNA expression analyses, a mixed linear regression model analysis was performed to compare the kinetics of adult and tadpoles followed by the Kruskal-Wallis test to compare

individual time points within adults or tadpoles group. For the survival analysis, a Log-Rank test and the Cox Proportional Hazard Model analysis was performed using R (R version 3.5.2, R studio version 1.1.463). GraphPad Prism 6 software (San Diego, CA, USA) was used for all statistical computation except for Cox Proportional Hazard Model analysis.

Results

Comparison of survival rates following *Mm* inoculation between *X. laevis* adults and tadpoles

To examine the respective host resistance of tadpoles and adult frogs to *Mm*, we monitored survival following different doses of *Mm* inoculation. Although tadpole death appeared to start earlier than adults for low and medium *Mm* dose (Fig 1), the mortality rate was not statistically different using a Log-Rank test. To substantiate our analysis, we tested our data using a final statistical model and set different *Mm* doses and two life-stage (adults and tadpoles) as covariates. The analysis indicates survivorship was not significantly impacted by life-stage ($p = 0.1357$), instead survivorship appeared to be *Mm* dose-dependent. The interaction between life-stage and dose was non-significant in our initial model. Further, survivorship decreased with increasing *Mm* dose: medium and high dose both had significantly lower survivorship than low dose ($p = 0.0404$; $p < 0.0001$, respectively) and high dose had significantly lower survivorship than medium dose ($p < 0.0001$). The median survival time for each dose is indicated in Table 2. Collectively, our survival analysis suggests that tadpoles are not significantly more susceptible against *Mm* compare to adult frogs.

208 We also quantified the *Mm* genome copy number of post-mortem animals by
209 qPCR (Figure 1B, C). Relatively modest *Mm* loads were detected in tissues of post-
210 mortem adult frogs (10 to 100 fold higher in respective tissues of post-mortem animals
211 compared to live animals euthanized at 21 dpi). These *Mm* loads were not significantly
212 different across organs or at different time of death (Figure 1B). Similarly, there was no
213 correlation (p=0.9738 by Spearman r analysis) between the total *Mm* loads in tadpoles
214 and time of death. The rapid decay of tadpoles dying from *Mm* inoculation did not allow
215 to determine the bacterial load for particular organ without a risk of contamination.
216
217 **Comparison of T cell-mediated immune responses in *X. laevis* adults and tadpoles**
218 To address our hypothesis that adults and tadpoles have distinct T cell responses
219 during *Mm* infection, we performed flow cytometry using *Xenopus* specific anti-CD8
220 mAb and anti-CD5 mAb to define the response kinetics of two main T cell populations as
221 previously established using adult splenocytes [20]: a $CD8^+$ T cell population defined as
222 cells co-expressing the CD8 and CD5 pan-T cell marker ($CD8^+/CD5^+$) and $CD5^+$ T cell
223 population not significantly expressing CD8 ($CD8^{neg}/CD5^+$) that includes iT cells and
224 presumably CD4 T cells and that we will refer here as $CD8^{neg}$ T/iT cell population
225 (Figure 2A-B). In the absence of lymph nodes, the spleen constitutes both a primary and
226 secondary lymphoid organ in *Xenopus* [21]. Therefore, we monitored the kinetics of the
227 two T cell subsets in spleen (Figure 3). We also examined the liver, which is a major site
228 of mycobacteria infection in *Xenopus* (Figure 4).
229 In adult frogs but not in tadpoles, there was an increase of the total number of
230 splenocytes upon *Mm* inoculation that reached a statistical significance at 21 dpi,

231 suggestive of immune cell expansion (Table 3). Notably, the frequency and the number
232 of CD8 T cells were significantly increased in the adult spleen at 12 dpi (***p=0.001
233 compared to 6 dpi group, and **p=0.0154 compared to the uninfected group,
234 respectively), whereas no significant change was observed in tadpoles (Figure 3A-B). In
235 contrast, both the frequency and the number of the $CD8^{neg}$ T/iT-like cell were
236 significantly reduced in tadpole's spleen at 6 dpi, which suggests an egress of this T cell
237 population (Figure 3C-D). Unlike tadpoles, the $CD8^{neg}$ T/iT cell population remained
238 unchanged following *Mm* inoculation in the adult spleen. (Figure 3C-D).
239 Similarly, the total number of leukocytes recovered from the liver of *Mm*-inoculated
240 adults but not tadpoles significantly increased at 6 dpi, suggestive of immune cell influx
241 and/or expansion (Table 2). The frequency and number of CD8 T cells peaked at 12 dpi
242 only in adult livers, whereas there were no marked changes in tadpoles' liver (Figure 4A-
243 B). However, the frequency of $CD8^{neg}$ T/iT cells significantly increased at 12 dpi in
244 tadpole livers suggesting recruitment of these cell types in the liver (Figure 4C-D). In
245 adult liver, the was a significant drop in the frequency of $CD8^{neg}$ T/iT-like cell at 6 dpi
246 compared to uninfected controls that did not affect their cell number. Taken together, the
247 data suggest that conventional $CD8^{+}$ T cell response is elicited mainly in adults, whereas
248 tadpole anti-*Mm* T cell response appears to rely more on the recruitment of $CD8^{neg}$ T
249 cells that include iT cells.
250
251 **Histopathology of *Mm* inoculated liver of *X. laevis* adults and tadpoles**
252 Differences were noted in the histopathologic appearance of the livers between
253 adults and tadpoles (Figure 5A-H). In the liver of post-mortem adult frogs, distinct

clusters of epithelioid macrophages, indicative of microgranulomas, were scattered randomly throughout the hepatic parenchyma (Figure 5A and D, black arrows). Detailed examination of these microgranulomas in multiple section of different animals revealed that they were composed almost exclusively of histiocytic cells suggestive of epithelioid macrophages as shown in representative sections of Figure 5A and D. Often, the microgranulomas were surrounded by a rim of mononuclear cells with little cytoplasm and darker more condensed nuclear chromatin, consistent with lymphocytes. The largest lesions included basophilic debris, indicative of a poorly demarcated necrotic zone at the core of the microgranuloma (Figures 5A).

In tadpoles, only a very small number of tiny epithelioid macrophage clusters were present, usually including distinct melanomacrophages or similar cells with nuclei obscured by melanin (representative shown in Figure 5G). Unlike adults, the microgranulomas present in tadpoles had no evidence of a necrotic zone. Based on the number of microgranulomas per unit of liver tissue, adult frogs exhibited a significantly higher number of granulomas compared to tadpoles (non-parametric 2-way ANOVA, $p<0.001$) (Figure 5C).

To visualize T cells in relation to granuloma structures, we used a cross-reactive human anti-CD3 Ab [19]. Following *Mm* inoculation, we detected an accumulation of CD3-positive ($CD3^+$) T cells in both adults' and tadpoles' liver (Figure 5 B, E and H). More specifically, $CD3^+$ T cells were conspicuously present throughout the parenchyma, either individually or forming small clusters. In the liver of *Mm* inoculated adults, the CD3+ T cells were more numerous in the periphery of the microgranulomas (Figure 5B and E, brown staining). In contrast, overall fewer $CD3^+$ T cells were scattered almost

277 uniformly throughout the hepatic parenchyma with little association with
278 microgranulomas in tadpole's liver (Figure 5H). Though very rare, a few $CD3^+$ T cells
279 were present within the core of microgranulomas in tadpoles' liver (Figure 5E).

280 Special stains for bacteria and acid-fast organisms (Ziehl Neelsen and Fite Faraco,
281 respectively) failed to detect the presence of any microorganisms in tissues from both
282 adults and tadpoles [22]. However, an isH probe for *Mycobacterium* sp, produced
283 amorphous staining in the cytoplasm of random cells in the liver of adults, and tadpoles
284 (Figure 5F and I, respectively). Staining was slightly more structured, occasionally
285 forming round vacuoles, in cells within hepatic microgranulomas compared to those
286 scattered in the parenchyma. Subjectively, positive isH staining seemed higher within
287 microgranulomas than in the rest of the tissue. Histological analyses indicated that *Mm*
288 were sequestered in microgranulomas, which were larger and rimmed by abundant CD3+
289 T cells in adult frogs but smaller and less $CD3^+$ rich in tadpoles.

290

291 **Comparison of *Mm* dissemination in *X. laevis* adults and tadpoles**

292 We have previously shown that in tadpoles intraperitoneally inoculated, *Mm*
293 accumulates in the liver concomitant to a decrease of pathogen loads among PLs [23]. In
294 addition, tadpoles that died at different days post *Mm* inoculation had no marked increase
295 of *Mm* loads, suggestive of persistent *Mm* over time (Figure 1C). To determine whether
296 *Mm* has a distinct dissemination pattern in adult frogs, we quantified *Mm* loads in
297 different organs. Following *i.p* injection of $1x10^6$ CFU of *Mm* in adult frogs, we assessed
298 *Mm* loads in PLs that are the first cells to encounter *Mm*; the liver, the main site of *Mm*
299 dissemination; and the spleen that is the major lymphoid organ of *X. laevis*.

300 Similar to tadpoles, we detected persistent *Mm* loads among adult's PLs up until
301 18 dpi, followed by a significant reduction at 21 dpi (Figure 6A). However, unlike our
302 previously published observation of increased of *Mm* loads in tadpoles' liver, we found a
303 significant decrease of *Mm* loads in adult's liver at 21 dpi (Figure 6B). In addition, *Mm*
304 loads significantly dropped at 18 dpi in the adult's spleen (Figure 6C). Taken together,
305 these data indicate that *Mm* disseminates systemically in adult frogs as in tadpoles.
306 Unlike tadpoles, however, *Mm* loads significantly decrease in adult's PLs, liver, and
307 spleen over the course of *Mm* infection.

308 Although the absolute quantification assay using qRT-PCR is a highly sensitive
309 method to detect even low *Mm* genome copy number, the assay does not distinguish live
310 infectious pathogens from dead or inactive *Mm*. As a complementary approach, live *Mm*
311 from liver tissues were recovered on bacterial culture plates. The CFUs of *Mm* were
312 measured from infected livers at 21 dpi, which was the time *Mm* load decrease was
313 detected in adult frogs by qRT-PCR. Due to the size differences of the liver organs
314 between adults and tadpoles, we normalized the total CFUs to mg of total tissue lysates.
315 Notably, we detected significantly higher CFUs of *Mm* in tadpoles' liver than that of
316 adults at 21 dpi per 1 mg of total protein (Figure 6E). These data suggest that adult
317 immune responses actively reduce *Mm* loads in the adults' liver, whereas live *Mm*
318 continues to accumulate in the tadpoles' liver.

319

320 **Changes in expression profiles of relevant immune genes in the liver**

321 Based on the differences in the histology and the kinetics of *Mm* loads between
322 adult frogs and tadpoles, we hypothesized that conventional CD8 T cells and innate

immune cells were recruited and actively induced inflammation to control *Mm* in adult but not tadpoles. To address this, we determined the relative expression kinetics of genes encoding T cell co-receptors (CD3ε, CD4, and CD8β) as a proxy of recruitments in the liver. We used *gapdh* as a mean to normalize the expressions of immune gene transcripts and compared the gene expression profiles between adults and tadpoles. Of note, the Ct value of GAPDH was not markedly different at each time points and between adults and tadpoles (Figure 7E).

No increase in expression levels of CD3ε, CD4, or CD8β gene was observed in tadpoles following *Mm* inoculation (Figure 7A-C). In contrast, CD8β gene expression was drastically increased (more than 3 logs on average) at 6 dpi in adult liver, consistent with infiltration of conventional CD8 T cells in response to *Mm* inoculation (Figure 7C). To assess the response of iT cells following *Mm* inoculation, we determined the transcript levels of the invariant (i) Vα45-Jα1.14 rearrangement that is expressed by critical anti-*Mm* iT cell effectors [11]. In tadpoles, iVα45-Jα1.14 transcripts were undetectable in the uninfected liver, but became rapidly abundant at 6 dpi and remained detectable at 12 dpi (Figure 7D). A similar increase in iVα45-Jα1.14 transcript level occurred in the adult liver during *Mm* infection. These data support our hypothesis of a dominant anti-*Mm* iT cell in tadpoles contrasting with a combined iT and conventional CD8 T cell response in adult frogs.

To assess the involvement of innate immune cell effectors in adult and tadpole liver during *Mm* infection, we monitored the expression of CSF-1 receptor (CSF-1R) as a marker for macrophages; GM-CSF receptor (GM-CSFR) as a marker for neutrophils; and CCR2 as a marker for inflammatory monocytes in the liver (Figure 8A-C). In addition,

346 we determined the expression of pro-inflammatory and anti-inflammatory cytokine genes
347 (Figure 8D-H). Notably, CSF-1R gene expression peaked at 6 dpi in adult frogs and
348 returned the basal level at 12 dpi (Figure 8A). The transcript levels of GM-CSFR and
349 CCR2 did not change at the two time points tested (Figure 8B-C). Interestingly, the
350 increase in CSF-1R expression was correlated with that of TNFα, IL-1β, and iNOS,
351 which are pro-inflammatory cytokines (Figure 8D-F). We did not find a significant
352 increase of anti-inflammatory cytokine expressions such as TGFβ, or IL-10 at 6 and 12
353 dpi (Figure 8G-H). In tadpoles, we observed a reduced expression of CSF-1R at 12 dpi
354 compared to uninfected tadpoles. (Figure 8A). Although the kinetics of TNFα, IL-1β, and
355 iNOS did not drastically change in tadpoles, we found that the expression of TGFβ
356 significantly dropped at 12 dpi in parallel to CSF-1R (Figure 8G). Taken together, these
357 data suggest that the acute influx or activation of macrophages (indicated by CSF-1R)
358 and conventional CD8 T cells (indicated by CD8β cells) may contribute to early
359 inflammation in adult frogs, whereas mainly iT cells (indicated by Vα45-Jα1.14
360 rearrangement) are recruited and persistent in tadpoles without marked inflammation.
361
362
363 **Discussion**
364 The wide range of host species infected by *Mm* poses a concern not only for the
365 aquatic ecosystem and fish industry, but also for its potential risk to public health.
366 Aquatic species notoriously infected by *Mm* includes *X. laevis,* which we utilized in this
367 study as a model to understand host anti-*Mm* immune response. Although the immune
368 system of tadpoles is generally considered more immature and/or less efficient in

369 controlling pathogens than that of adult frogs, our study rather suggests a distinct
370 adaptation and specialization of immune responses for each these two life stages in *X.*
371 *laevis*. Notably, unlike adult frogs that exhibit potent conventional CD8 T cell effectors,
372 tadpoles do not express an optimal level of MHC I protein until the onset of
373 metamorphosis and predominantly rely on immunity driven by MHC class I-like and iT
374 cell subsets expressing [8, 23]. Here, we report evidence that suggests a distinctive host-
375 pathogen interaction between adults and tadpoles during *Mm* infection.

376 First, in apparent contradiction of the general view of weaker tadpole immunity,
377 we found a comparable survivorship between adults and tadpoles challenged with
378 different doses of *Mm*. Considering the smaller size of the tadpoles compared to the
379 adults, the inoculated tadpoles were remarkably efficient in tolerating high amount of
380 *Mm*. This implies that tadpole and adult immune defenses, albeit distinctive, achieve
381 similar survival against mycobacterial infections. To get further insights into the distinct
382 adaptation of host responses against *Mm* by these two life stages, we first examined the T
383 cell response by flow cytometry. Although specific antibodies to detect iT and CD4 T
384 cells are currently missing in *Xenopus*, we were able to take advantage of CD5, a pan T
385 cell marker in *X. laevis* in combination anti-CD8 mAb to monitor conventional CD8 T
386 cells ($CD8^{+}/CD5^{+}$) versus a population of $CD8^{neg}$ $T/CD5^{+}$ T cells, which have been
387 shown to contain T cell expressing CD4 ([24]). $CD8^{neg}$ $T/CD5^{+}$ T cells also include iT
388 cells (e.g., iVa45 T cells) since they express very low level to no CD8 at all [8, 23].
389 Based on this approach we found that adults frogs exhibit a strong conventional $CD8^{+}$ T
390 cell response against *Mm*, which is not the case in tadpoles where the increase of $CD8^{neg}$
391 T cells and iT cells in the liver concomitant with their drop in the spleen suggests their

392 rapid recruitment upon *Mm* inoculation. It is noteworthy that splenic IgM^+ B cells are not
393 $CD5^+$ in *X. laevis*, except following strong stimulation with PMA [25]. Further
394 investigation by gene expression profiling confirms that *Mm* inoculation induces a
395 significant increase of CD8β transcript levels indicative of CD8 T cell response together
396 with iVα45-Jα1.14 mRNAs in the adult liver. In contrast, only increased of iVα45-Jα1.14
397 but not CD8β transcript levels were detected in tadpoles in response to *Mm* inoculation
398 (Fig 7). It is also interesting to note that CD4 expression significantly decreased, while
399 iVα45-Jα1.14 transcript levels remained persistent in *Mm* inoculated tadpole's liver
400 (Figure 7B, and 7D). This suggests that iVα45 T cells rather than $CD4^+$ T cells are major
401 effector cells during *Mm* infection. This is corroborated by previously published reverse
402 genetic loss-of-function by transgenesis evidence showing that iVα45 cells have a critical
403 host protective function in tadpoles [11]. The mechanisms of activation and functions of
404 these iT cells remain to be elucidated.

405 A striking difference between adult frogs and tadpoles revealed by this study was
406 the granuloma formation resulting from *Mm* inoculation, which consistently occurred in
407 adults but rarely in tadpoles. These granulomas, which consisted of epithelioid
408 macrophages surrounded by adaptive T cells, were common in adult liver and to a lesser
409 extent in other organs such as lung and spleen. Notably, IHC staining revealed an
410 accumulation of CD3+ T cells at the rim of these granulomas, which is suggestive of a
411 vigorous T cell response against an acute *Mm* infection. Histologic examination of the
412 livers of adults and tadpoles occurred at slightly different stages post-inoculation and a
413 definitive conclusion would require further confirmation. However, sampling times
414 included early and late infection for adults and mid-term infection in tadpoles. Thus, we

415 believe our findings support an age-dependent difference in immune response. It is
416 uncertain why the special stains that normally pick out mycobacterial organisms failed to
417 do so in the livers of adults or tadpoles. Confirmation of infection was achieved, however,
418 though PCR and culture results, and with the positive staining of several cells with an isH
419 probe for *Mycobacterium* sp.

420 The formation of granulomas is generally associated with inflammation in
421 mammals, especially in cases of mycobacterial infections [26]. Likewise, in adult *X.*
422 *laevis*, the expression of several inflammatory genes (TNFα, IL-1β, and iNOS) was
423 significantly increased following *Mm* inoculation in the liver. Collectively, the immune
424 histological analyses further strengthen the critical function of T cells in sequestering and
425 controlling *Mm* infection in adult frogs. Importantly, the collective evidence of CD8 T
426 cell-mediated response, inflammation-mediated granuloma formation, and the clearance
427 of *Mm* in adult frogs are highly similar to the critical role of CD8 T cells in granuloma
428 structures from *Mtb* infected human [27]. Therefore, our findings suggest that *Mm*
429 infection in adult *X. laevis* may complement host mycobacterial infection in mammalian
430 models.

431 In sharp contrast, microgranulomas were rarely observed in tadpoles (Figure 3C).
432 In addition, *Mm* inoculation did not induce significant expression of inflammatory genes.
433 Indeed, transcript levels of IL-1β remained low following *Mm* inoculation, whereas
434 TNFα or iNOS expression was not induced by *Mm.* Importantly, the high CFU counts of
435 live *Mm* recovered from the infected liver in this study substantiates the previous report
436 of *Mm* accumulation in tadpoles [23]. Therefore, it is possible that the dominant iT cell
437 response in tadpole allow the larval host to tolerate high and persistent *Mm* load and

consequently to maintain the survivorship at a similar level as that of adult frogs. Alternatively, it also possible that the larval $CD8^{neg}$ T cell population includes regulatory T cells that contribute to maintain tolerance to *Mm* infection. Although, Tregs function has not been characterized in amphibians, gene orthologs encoding cytokines and transcription factors critical for differentiation of Tregs (e.g., FOXP3; CTLA4) are present in the genome of *X. tropicalis* and *X. laevis* (Robert, unpublished observations).

For an evolutionary standpoint is tempting to speculate that the predominant anti-*Mm* iT cell response in tadpole avoid the activation of inflammation, whereas a conventional T cell response in adult is associated with inflammation. As such the adaptive host response against *Mm* in two different life stages occupying distinct environmental niches is fundamentally different: the adult conventional T cell-based system is adapted to eradicate *Mm* pathogens by killing infected cells and generating an inflammatory response, whereas the tadpole iT cell-based system upon detection of the infection is designed to minimize inflammation and tolerate pathogen burden. Consistent with these contrasted immune responses, live *Mm* recovered from tadpole liver did not markedly decrease during infection, whereas it decreased in adults. The high *Mm* loads found in the adults' post-mortem tissues by qPCR, further suggest an insufficient clearance of *Mm* resulting in the death of the adult frogs.

Active tolerance to pathogen has been reported in various cases including mycobacteria infection [28-30] Although not as well defined as immune mechanisms behind host resistance, specific tolerance mechanisms has recently raised attention owing to their relevance for better understanding host-pathogen interactions and potentials for developing more effective treatments for infectious diseases. Our data in tadpoles are

consistent with an adapted tolerance to *Mm* infection that may involve iT cells as specific effectors controlling this tolerance. It will be interesting to examine in more detail how iT cells can establish and control host tolerance to *Mm* in tadpoles by determining the specificity (e.g., ligands presented by MHC-like molecules and recognized by iT cells) and the mechanisms involved (cytokine produced, cell types targeted).

Acknowledgements

We would like to thank Tina Martin for the expert animal husbandry.

References

1. Tobin, D.M. and L. Ramakrishnan, *Comparative pathogenesis of Mycobacterium marinum and Mycobacterium tuberculosis.* Cell Microbiol, 2008. **10**(5): p. 1027-39.
2. Franco-Paredes, C., et al., *Cutaneous Mycobacterial Infections.* Clin Microbiol Rev, 2018. **32**(1).
3. Hashish, E., et al., *Mycobacterium marinum infection in fish and man: epidemiology, pathophysiology and management; a review.* Vet Q, 2018. **38**(1): p. 35-46.
4. Cosma, C.L., D.R. Sherman, and L. Ramakrishnan, *The secret lives of the pathogenic mycobacteria.* Annu Rev Microbiol, 2003. **57**: p. 641-76.
5. Cronan, M.R. and D.M. Tobin, *Fit for consumption: zebrafish as a model for tuberculosis.* Dis Model Mech, 2014. **7**(7): p. 777-84.
6. Session, A.M., et al., *Genome evolution in the allotetraploid frog Xenopus laevis.* Nature, 2016. **538**(7625): p. 336-343.
7. Robert, J. and Y. Ohta, *Comparative and Developmental Study of the Immune System in Xenopus.* Developmental dynamics : an official publication of the American Association of Anatomists, 2009. **238**(6): p. 1249-1270.
8. Edholm, E.S., et al., *Nonclassical MHC class I-dependent invariant T cells are evolutionarily conserved and prominent from early development in amphibians.* Proc Natl Acad Sci U S A, 2013. **110**(35): p. 14342-7.
9. Robert, J. and Y. Ohta, *Comparative and developmental study of the immune system in Xenopus.* Dev Dyn, 2009. **238**(6): p. 1249-70.

10. Edholm, E.S., et al., *Nonclassical MHC-Restricted Invariant Valpha6 T Cells Are Critical for Efficient Early Innate Antiviral Immunity in the Amphibian Xenopus laevis.* J Immunol, 2015. **195**(2): p. 576-86.
11. Edholm, E.S., et al., *Distinct MHC class I-like interacting invariant T cell lineage at the forefront of mycobacterial immunity uncovered in Xenopus.* Proc Natl Acad Sci U S A, 2018. **115**(17): p. E4023-E4031.
12. Le Bourhis, L., et al., *Antimicrobial activity of mucosal-associated invariant T cells.* Nat Immunol, 2010. **11**(8): p. 701-8.
13. Huang, S., *Targeting Innate-Like T Cells in Tuberculosis.* Front Immunol, 2016. **7**: p. 594.
14. Hyoe, R.K. and J. Robert, *A Xenopus tadpole alternative model to study innate-like T cell-mediated anti-mycobacterial immunity.* Dev Comp Immunol, 2019. **92**: p. 253-259.
15. Zahn, N., M. Levin, and D.S. Adams, *The Zahn drawings: new illustrations of Xenopus embryo and tadpole stages for studies of craniofacial development.* Development, 2017. **144**(15): p. 2708-2713.
16. LG, L., *Manual of histologic staining methods of the Armed Forces Institute of Pathology* 1968, McGraw-Hill: New York. p. 40-41, 220.
17. K, B., *A phenol crystal violet stain for differential staining of Gram positive and negative bacteria* Miles Scientific, 1984. **VIII, No. 4**: p. 216.
18. RD, L., *Histologic technic and practical histochemistry* 1954, New York: McGraw-Hill.
19. Gobel, T.W., E.L. Meier, and L. Du Pasquier, *Biochemical analysis of the Xenopus laevis TCR/CD3 complex supports the "stepwise evolution" model.* Eur J Immunol, 2000. **30**(10): p. 2775-81.
20. Morales, H.D. and J. Robert, *Characterization of primary and memory CD8 T-cell responses against ranavirus (FV3) in Xenopus laevis.* J Virol, 2007. **81**(5): p. 2240-8.
21. Neely, H.R. and M.F. Flajnik, *Emergence and Evolution of Secondary Lymphoid Organs.* Annu Rev Cell Dev Biol, 2016. **32**: p. 693-711.
22. do you mean Gram staining and ZN staining? Both ZN and FF staining are for acid-fast bacteria but FF is modified for less acid-fast mycobacteria such as M. leprae
23. Edholm, E.S., et al., *Distinct MHC class I-like interacting invariant T cell lineage at the forefront of mycobacterial immunity uncovered in Xenopus.* Proc Natl Acad Sci U S A, 2018.
24. Chida, A.S., A. Goyos, and J. Robert, *Phylogenetic and developmental study of CD4, CD8 alpha and beta T cell co-receptor homologs in two amphibian species, Xenopus tropicalis and Xenopus laevis.* Dev Comp Immunol, 2011. **35**(3): p. 366-77.
25. Jurgens, J.B., et al., *Identification of a candidate CD5 homologue in the amphibian Xenopus laevis.* J Immunol, 1995. **155**(9): p. 4218-23.
26. Sasindran, S.J. and J.B. Torrelles, *Mycobacterium Tuberculosis Infection and Inflammation: what is Beneficial for the Host and for the Bacterium?* Front Microbiol, 2011. **2**: p. 2.

27. Lin, P.L. and J.L. Flynn, *CD8 T cells and Mycobacterium tuberculosis infection.* Semin Immunopathol, 2015. **37**(3): p. 239-49.
28. McCarville, J.L. and J.S. Ayres, *Disease tolerance: concept and mechanisms.* Curr Opin Immunol, 2018. **50**: p. 88-93.
29. Schneider, D.S. and J.S. Ayres, *Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases.* Nat Rev Immunol, 2008. **8**(11): p. 889-95.
30. Meunier, I., et al., *Unravelling the networks dictating host resistance versus tolerance during pulmonary infections.* Cell Tissue Res, 2017. **367**(3): p. 525-536.

556 Figure legends

557

558 **Figure 1. Survival curves of *Mm* inoculated adults and tadpoles of *X. laevis*.** (A) 4
559 month-old adults (n=5-10/group) and 3 week-old tadpoles (developmental stage of 52,
560 n=10-19/group) were inoculated with different amount of *Mm* intraperitoneally ($5x10^5$
561 CFU for low dose, $1x10^6$ CFU for medium dose, and $2x10^6$ CFU for high dose). The
562 survival rate was dependent on the doses of *Mm* based on the Cox Proportional Hazard
563 model analysis ($p < 0.05$). Comparisons of survival rates between adult frogs and
564 tadpoles for each dose as well as median survival times are listed in table 2. (B) *Mm* loads
565 in different organs from post-mortem adults. (C) *Mm* loads of whole individual post-
566 mortem tadpoles. Viral loads were determined by real-time PCR using *Mm* specific
567 16srRNA gene.

568

569 **Figure 2. Flow cytometric analysis of T cell in spleen and liver of adults and tadpoles.**
570 4 month-old young adults and three-week-old tadpoles were intraperitoneally inoculated
571 with $1x10^6$ CFU, or $3x10^5$ CFU of *Mm,* respectively (n=5-6 per each time points). Then,
572 the total lymphocytes at different dpi were stained with *Xenopus* specific CD5 mAb, and
573 CD8 mAb to analyze two subsets of T cell populations (A-B). After gating on live cells,
574 $CD8^+CD5^+$ cells and $CD8^{neg}CD5^+$ cells were defined (black boxes).

575

576 **Figure 3. Comparison of the frequency and the number of CD8 and $CD8^{neg}$ T/iT**
577 **cells in the spleen of adults and tadpoles following *Mm* inoculation.** Using the flow
578 cytometric strategy shown in the Figure 2, the kinetics of CD8 T cell (A) frequency, and

(B) number were determined in adults (white) and tadpoles (black) at different days post inoculation (n=6-7/group from 2 independent experiments). Further, the kinetics of $CD8^{neg}$ T/iT cell (C) frequency, and (D) number were determined. C: uninfected control. Asterisks indicated statistical significance by the Kruskal-Wallis test, non-parametric.

Figure 4. Comparison of the frequency and the number of CD8 and $CD8^{neg}$ T/iT cells in the liver of adults and tadpoles during *Mm* infection. Using the flow cytometric strategy shown in the Figure 2, the kinetics of CD8 T cell (A) frequency, and (B) number were determined in adults (white) and tadpoles (black) at different days post inoculation (n=6-7/group from 2 independent experiments). Further, the kinetics of $CD8^{neg}$ T/iT cell (C) frequency, and (D) number were determined. C: uninfected control. Asterisks indicated statistical significance by the Kruskal-Wallis test, non-parametric.

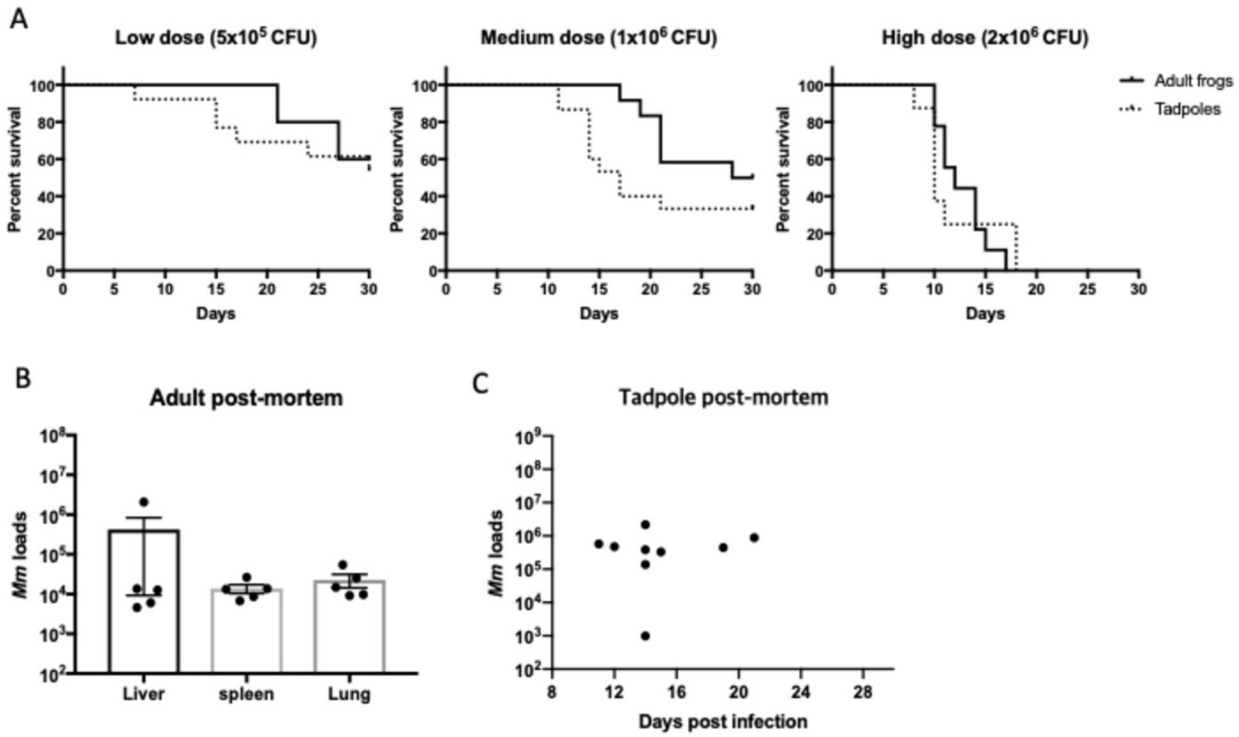
Figure 5. Histopathology of the liver of *Mm* inoculated adults and tadpoles and comparison between microgranuloma numbers. 4 month-old young adults and three-week-old tadpoles were intraperitoneally inoculated with $1x10^6$ CFU, or $3x10^5$ CFU of *Mm,* respectively (n=5-6 per each time points). Representative of liver sections that were stained with hematoxylin and eosin (A, D and G), an anti-CD3 mAb (B, E and H, brown staining), or an *in situ* hybridization (isH, red staining) probe for *Mycobacterium* sp (F and I). Adults inoculated with 10^6 CFU of *Mm* and euthanized 14 and 50 days post-infection (dpi): Larger granulomas (A and D, black arrows) surrounded by CD3+ cells in moderate (B) to high numbers (E) and positive isH for *Mycobacterium* sp in the

602 cytoplasm of cells within the granuloma at 50 dpi (F). Tadpole inoculated with $3x10^5$
603 CFU of *Mm* and euthanized 30 dpi: Small microgranuloma including cells with
604 intracytoplasmic melanin granules (G, black and white arrows, respectively), with rare
605 CD3+ cells (H) and positive isH for *Mycobacterium* sp in the cytoplasm of cells within
606 the microgranuloma (I, arrow) Bars = 50 μm. The average numbers of microgranulomas
607 in adults at 50 dpi (n=4) were significantly higher than those in tadpoles at 30 dpi (n=4)
608 (C, non-parametric 2-way ANOVA, $P<0.001$).
609
610
611 **Figure 6. Determination of *Mm* loads and dissemination using an absolute**
612 **quantification method and a recovery of live *Mm* in culture from adult and tadpoles**
613 **of *X. laevis*.** Adult frogs were intraperitoneally inoculated with $1x10^6$ CFU of *Mm* then,
614 *Mm* loads were determined by using a real-time PCR with *Mm* specific 16srRNA gene
615 (A-D). (A) peritoneal leukocytes (PLs), (B) liver, and (C) spleen tissues were taken at the
616 indicated days post infection. The dashed line indicates the level of detection by real-time
617 PCR. In order to measure only the live and replicating *Mm*, we cultured kanamycin
618 resistant *Mm* from the inoculated adults and tadpoles at 21 dpi using a Middlebrook 7H10
619 media supplemented with 50 μg/mL kanamycin. (D) Quantitative measurement of CFUs
620 was then normalized to total mg of homogenates.
621
622 **Figure 7. Relative expression of T cell-related immune genes in the liver of *Mm***
623 **inoculated adults and tadpoles.** Four month-old young adults (white bar) and three-
624 week-old tadpoles (grey bar) were intraperitoneally inoculated with $1x10^6$ CFU, or $3x10^5$

625 CFU of *Mm,* respectively (n=5-6 per each time points). Relative gene expression in liver
626 for (A) CD3ε, (B) CD8β, (C) CD4, and (D) iVα45-Jα1.14 was normalized to the
627 housekeeping gene *gapdh*. (E) Ct values for *gapdh* of each time point of between adults
628 and tadpoles. C: uninfected control, u.d: undetected value, asterisks indicate a significant
629 difference by the Kruskal-Wallis test.
630
631 **Figure 8. Relative expression of immune receptor genes and pro- and anti-**
632 **inflammatory cytokine genes in the liver of *Mm* inoculated adults and tadpoles.** Four
633 month-old young adults (white bar) and three-week-old tadpoles (grey bar) were
634 intraperitoneally inoculated with $1x10^6$ CFU, or $3x10^5$ CFU of *Mm,* respectively (n=5-6
635 per each time points). The relative gene expression in the liver was determined for the
636 immune gene receptors: (A) CSF-1R (macrophage recruitment marker), (B) GMCSF-R
637 (neutrophil recruitment marker), (C) CCR2 (inflammatory monocyte marker); for the
638 pro-inflammatory cytokine genes: (D) TNFα, (E) IL-1β, (F) iNOS; and for the anti-
639 inflammatory cytokine genes: (G) TGFβ and (H) IL-10. All the data were normalized to
640 the housekeeping gene *gapdh*. C: uninfected control, u.d: undetected value, asterisks
641 indicate significant difference by the Kruskal-Wallis test.
642
643

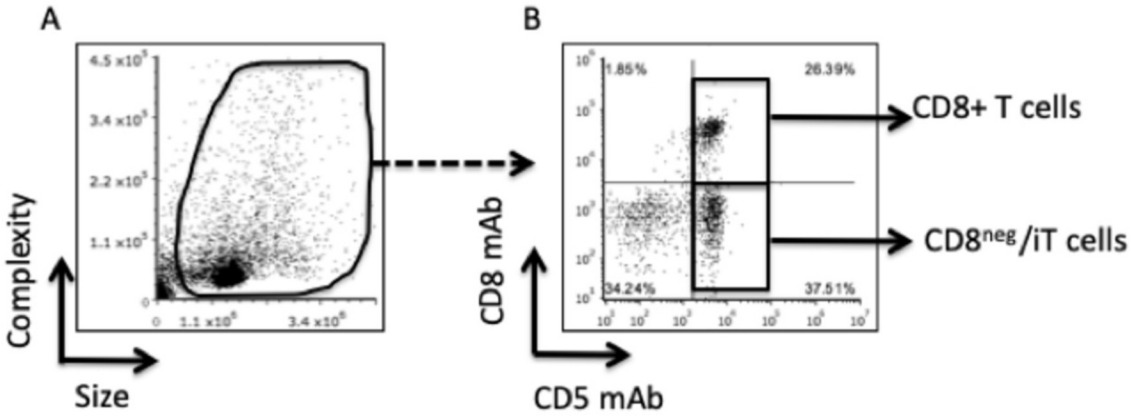
File type: Figure
Label: 1
File name: figure_1.tif

Figure 1


A
Low dose (5x10^5 CFU)
Medium dose (1x10^6 CFU)
High dose (2x10^6 CFU)
Percent survival
Days
Adult frogs
Tadpoles
B
Adult post-mortem
Mm loads
Liver
spleen
Lung
C
Tadpole post-mortem
Mm loads
Days post infection

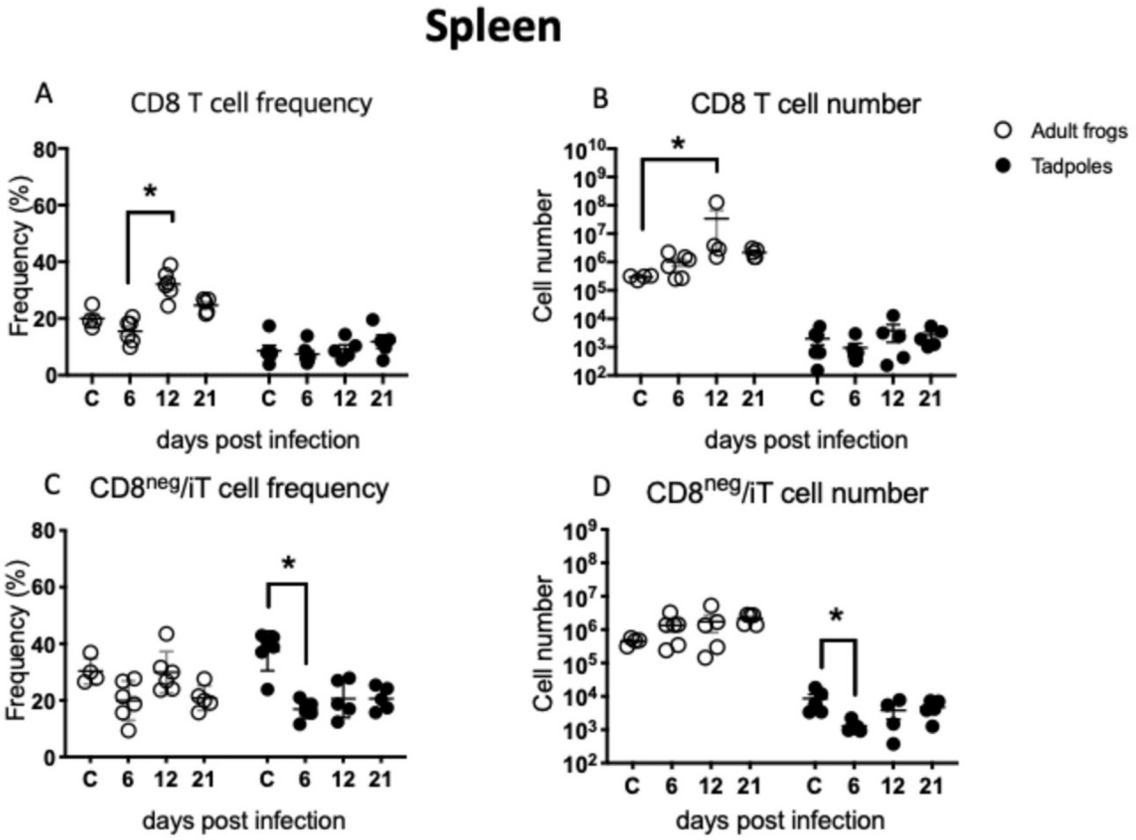
File type: Figure
Label: 2
File name: figure_2.tif

Figure 2


A
B
Complexity
Size
CD8 mAb
CD5 mAb
1.85%
26.39%
34.24%
37.51%
CD8+ T cells
CD8neg/iT cells

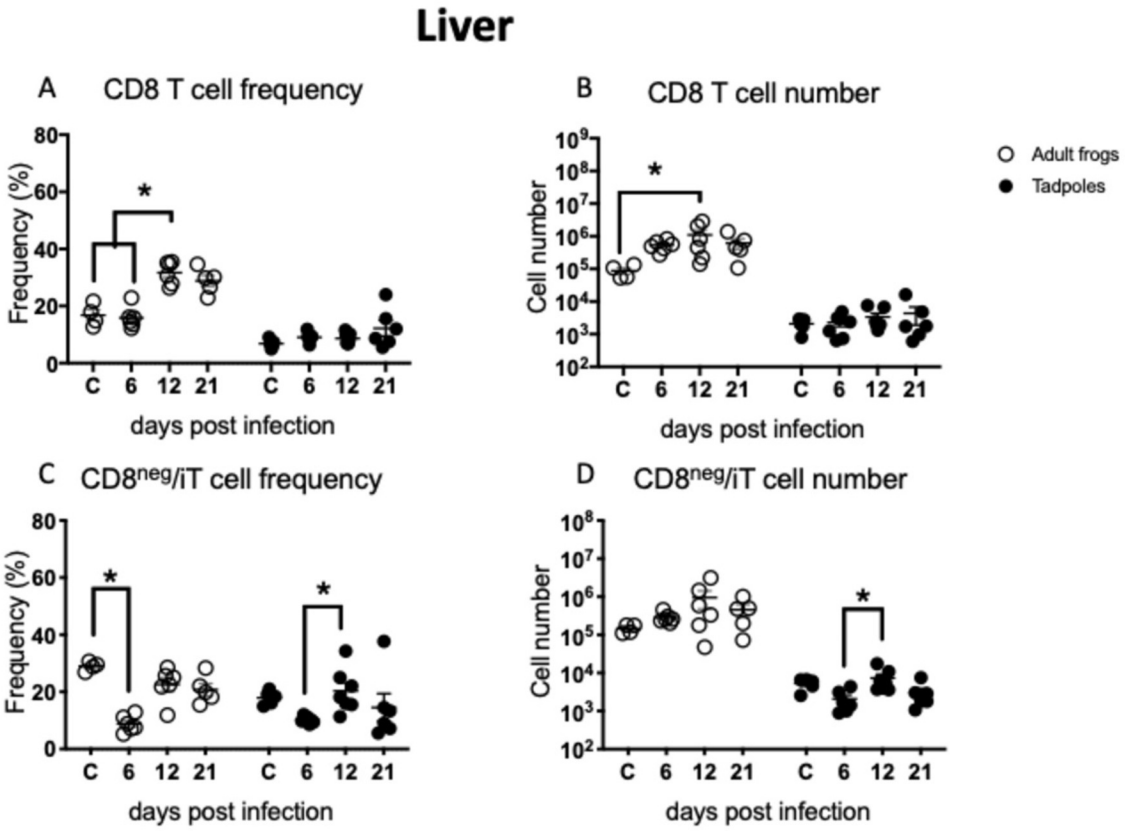
File type: Figure
Label: 3
File name: figure_3.tif

Figure 3

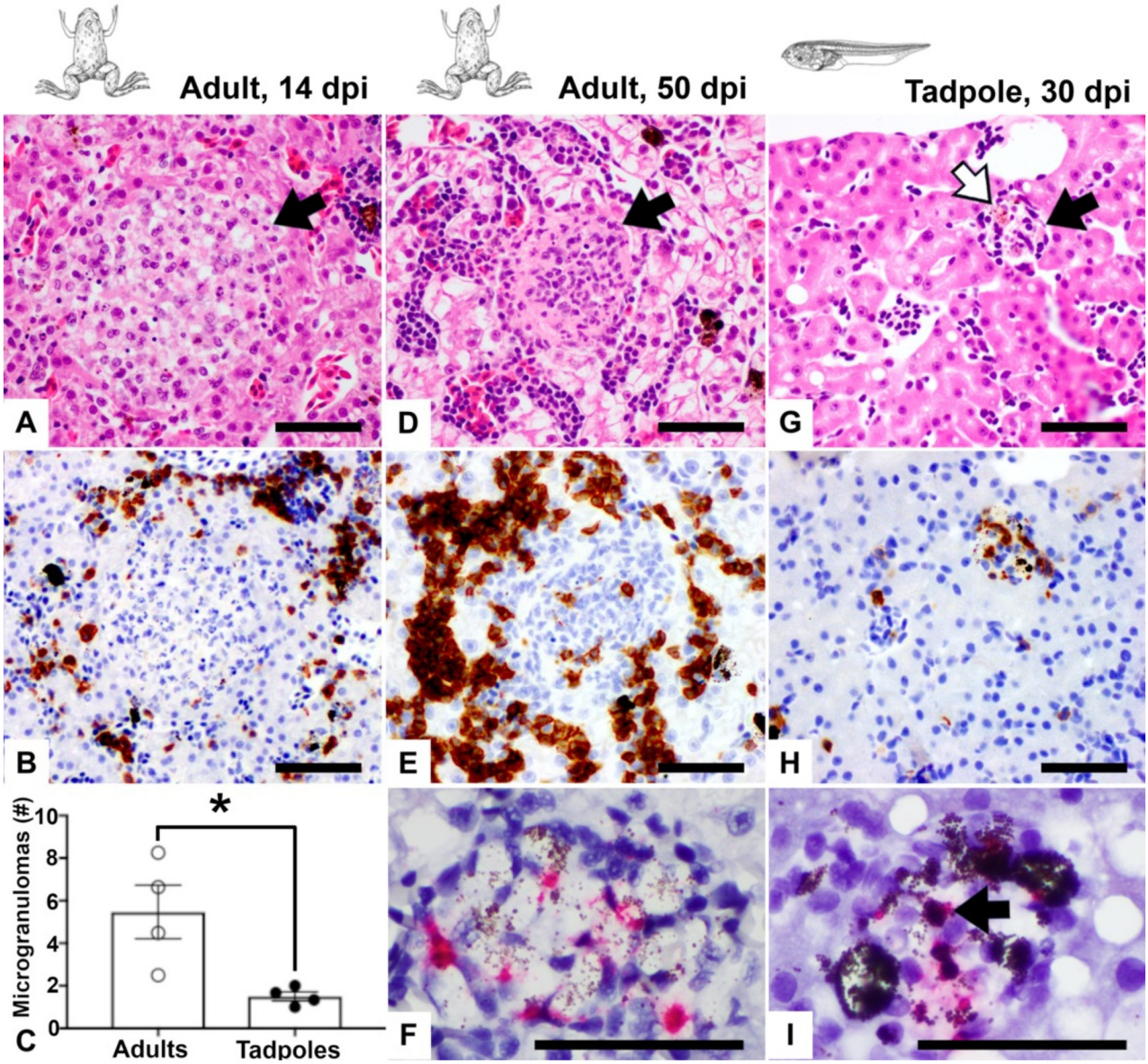

Spleen
A
CD8 T cell frequency
Frequency (%)
days post infection
B
CD8 T cell number
Cell number
days post infection
Adult frogs
Tadpoles
C
CD8neg/iT cell frequency
Frequency (%)
days post infection
D
CD8neg/iT cell number
Cell number
days post infection
C 6 12 21 C 6 12 21

File type: Figure
Label: 4
File name: figure_4.tif

Figure 4

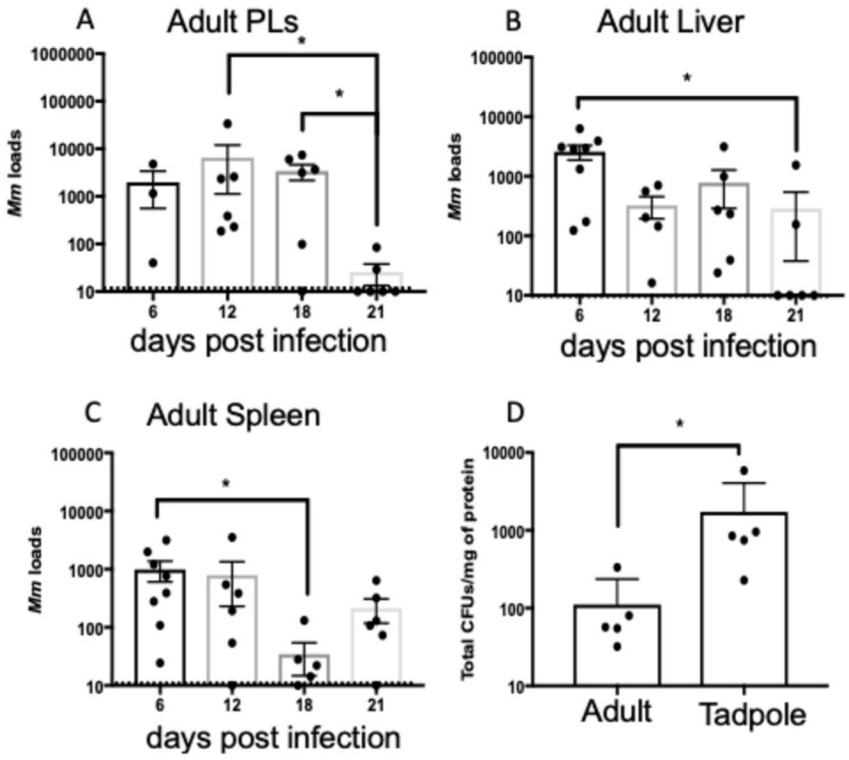

Liver
A
CD8 T cell frequency
Frequency (%)
days post infection
B
CD8 T cell number
Cell number
days post infection
Adult frogs
Tadpoles
C
CD8neg/iT cell frequency
Frequency (%)
days post infection
D
CD8neg/iT cell number
Cell number
days post infection
C 6 12 21 C 6 12 21

File type: Figure
Label: 5
File name: figure_5.tif


Adult, 14 dpi
Adult, 50 dpi
Tadpole, 30 dpi
A
D
G
B
E
H
C
Micrograulomas (#)
10
8
6
4
2
0
*
Adults
Tadpoles
F
I

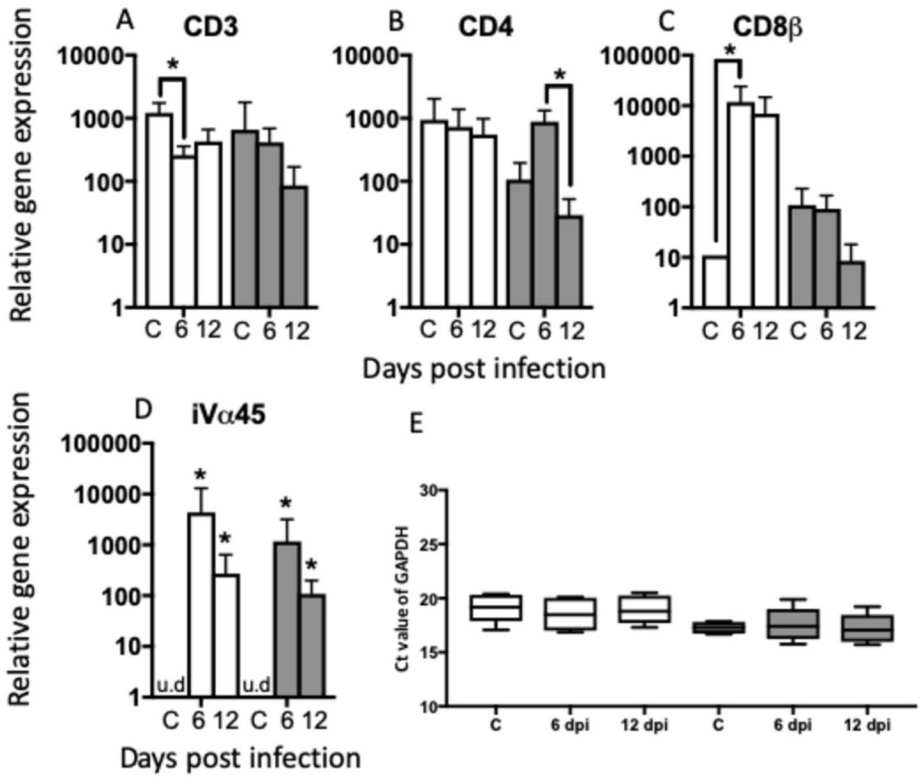
File type: Figure
Label: 6
File name: figure_6.tif

Figure 6


A
Adult PLs
*
*
1000000
100000
10000
1000
100
10
Mm loads
6
12
18
21
days post infection
B
Adult Liver
*
100000
10000
1000
100
10
Mm loads
6
12
18
21
days post infection
C
Adult Spleen
*
100000
10000
1000
100
10
Mm loads
6
12
18
21
days post infection
D
*
10000
1000
100
10
Total CFUs/mg of protein
Adult
Tadpole

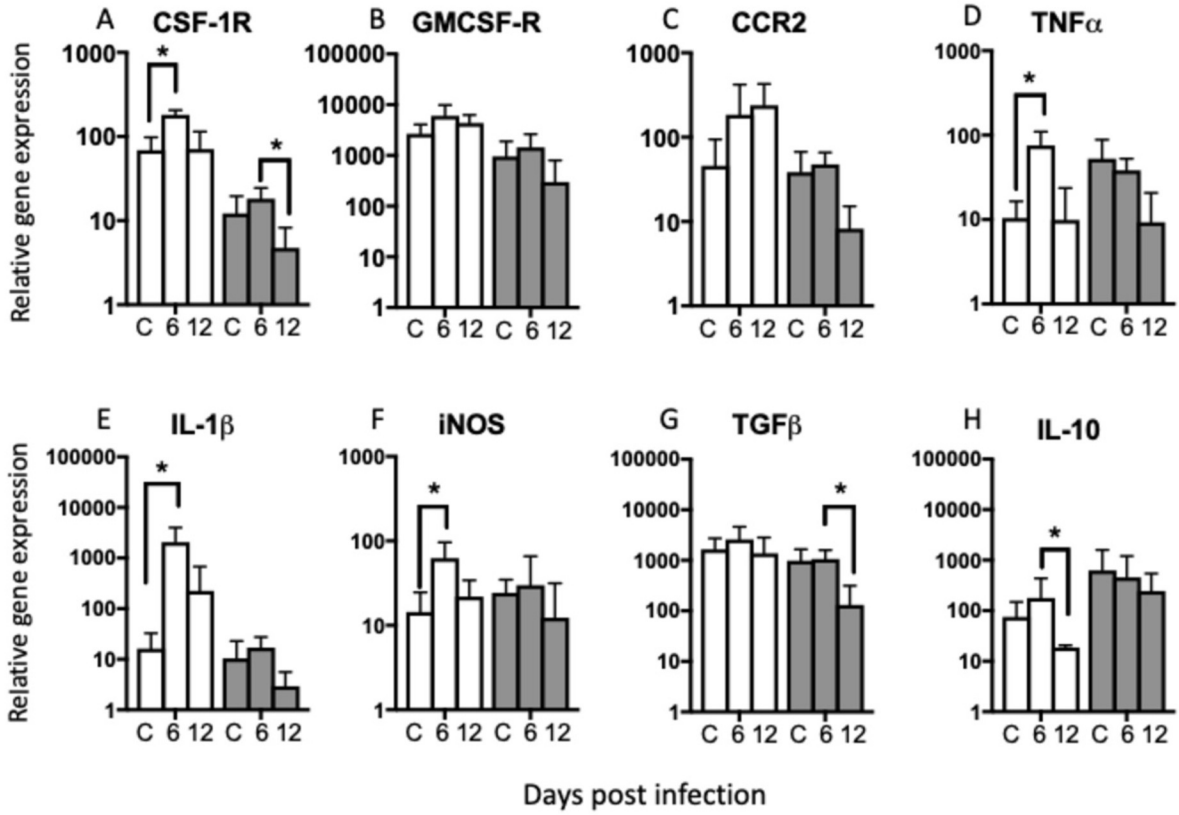
File type: Figure
Label: 7
File name: figure_7.tif

Figure 7


A
CD3
B
CD4
C
CD8β
Relative gene expression
10000
1000
100
10
1
100000
C 6 12 C 6 12
Days post infection
D
iVα45
u.d
E
Ct value of GAPDH
30
25
20
15
10
C
6 dpi
12 dpi

File type: Figure
Label: 8
File name: figure_8.tif

Figure 8


A
CSF-1R
B
GMCSF-R
C
CCR2
D
TNFα
E
IL-1β
F
iNOS
G
TGFβ
H
IL-10
Relative gene expression
Relative gene expression
1000
100
10
1
100000
10000
1000
100
10
1
C 6 12 C 6 12
*
Days post infection

File type: Table
Label: 1
File name: table_1.doc

Table 1: List of qPCR primer sequences

PRIMER	SEQUENCE (5'- 3')
CCR2	F:ATTGGGCAGAACTGTGGTAG
	R:GGGCGAGTAATCTGAGTCATAA
CD3	F: TTGGGCTCAGTGTGGAATG
	R: GGTCCCGGTATCCATCTCTAT
CD4	F: CATAGTGGTTTCCCTCTGGTTTAG
	R: CGCAGAGCGTCCATTCATTA
CD8β	F: GGAACACGTTTACCCTGAAGA
	R: GGGAGGTTCCATTCCCAAAT
CSF-R1	F: TGTATTCTTTGGACTTGCCGTATCTGG
	R: TTGTTTAGCTTCAAATTCTGGGTAATA
GAPDH	F: GACATCAAGGCCGCCATTAAGACT
	R: AGATGGAGGAGTGAGTGTCACCAT
GMCSF-R	F: ACGTGCCAGCTAAACCTCACAGAT
	R: TGACACAGCCTGGGCGAGAAATAA
IL-1β	F: CATTCCCATGGAGGGCTACA
	R: TGACTGCCACTGAGCAGCAT
IL-10	F: TGCTGGATCTTAAGCACACCCTGA
	R: TGTACAGGCCTTGTTCACGCATCT
iNOS	F: AACCGTAAGCCAAAGAAGGA
	R: TGGTTCTGGCAGCCACAGT
Mm 16S rRNA	F: AGAGTTTGATCCTGGCTCAG
	R: CACTCGAGTATCTCCGAAGA
TGFβ	F: CCCACAGGCCAAAGATATAGAC
	R: CATCAGGTAGGGTTTCGTGTT
TNF-α	F: TGTCAGGCAGGAAAGAAGCA
	R: CAGCAGAGCAAAGAGGATGGT
	R: CCGACACACTGAGCGGAAA
Vα45-Jα1.14	F: TCCGTTAACGAGAAGGATTCCCAG
	R: CTCCCAGCCACTACCAGAATAAG

F: Forward; R: Reverse

File type: Table
Label: 2
File name: table_2.doc

Table 2. Median survival days for adult frogs and tadpoles infected with different doses of *Mm* intraperitoneally (dpi).

Life Stage	Doses for intraperitoneal infection			
	$3x10^5$ CFU	$5x10^5$ CFU	$1x10^6$ CFU	$2x10^6$ CFU
Adult frogs	N/A	33	29	12
Tadpoles	30	30	17	10
P value for Log-rank test	N/A	0.7164	0.1176	0.9449

N/A means no data

File type: Table
Label: 3
File name: table_3.doc

Table 3. Total number of lymphocytes ($x10^3$) ± standard error.

Organ	Stages	Control	6 dpi	12 dpi	21 dpi
Spleen	Adult	1500 ±158	6000 ±1450	4860 ±2104	9580 ±1632 *p=0.0226
	Tadpole	24 ±11	12 ±4	34±16	22 ±4
Liver	Adult	505±63	3363 ±309 *p=0.0316	3650 ±1656	2100 ±710
	Tadpole	30 ±4	25 ±6	44 ±15	32 ±15

***** Compared to control using non-parametric Kruskal-Wallis test followed by Dunn test.