

## Research Article

# TLR8 and TLR7/8 Activations Induce Tissue-specific Natural Killer Cell Gene Expressions in Rag1 Mutant Zebrafish Liver, Kidney, and Spleen

Preeti Judith Muire , Lora Petrie-Hanson\* 

Department of Comparative Biomedical Sciences, College of Veterinary Medicine, Mississippi State University, Starkville, the United States

## Abstract

TLR ligands Resiquimod (TLR7/8) and Motolimod (TLR8) are used to activate NK cells and enhance immune defenses. This study reports the differential gene expressions of innate immune markers and Natural Killer (NK) cell lysins in *rag1* mutant (*rag* MT) zebrafish to TLR8 and TLR7/8 activation in liver, kidney, and spleen tissues. *Rag* MT zebrafish were intracoelomically injected with Motolimod (VTX), Resiquimod (R848), or control saline. Gene expressions of interferon gamma (*ifnγ*), T-box transcription factor 21 (*t-bet*), novel immune type receptor 9 (*nitr9*), and NK lysins a, b, c, and d (*nkla*, *nkla*, *nkla*, *nkld*) were quantified at 6-, 12-, and 24-hours post-injection using quantitative PCR. We observed that the effects of TLR7/8 and TLR8 stimulation vary depending on the tissue type. R848 significantly upregulated *ifnγ*, *t-bet*, *nitr9*, and NK lysins across various tissues. In contrast, VTX had a more limited effect and primarily influenced *nkla* and *nkld* in the kidney and spleen, and *nkld* in the liver, suggesting tissue-specific responsiveness to TLR8. No significant changes in *ifnγ* or *nkla* expression were noted with VTX in any tissues, highlighting the specificity of TLR7 over TLR8 in these responses. Tissue-specific responses revealed dominant activation by TLR7/8 in the liver, particularly influencing *ifnγ*, *t-bet*, *nitr9*, *nkla*, and *nkla*. The kidney had high responsiveness to both TLR7/8 and TLR8. The spleen demonstrated broad gene activation by TLR7/8, but only *nkla* and *nkld* were significantly upregulated by TLR8. These findings demonstrate that TLR8 has selective effects, while TLR7/8 more broadly activates genes across the liver, kidney, and spleen in *rag* MT zebrafish. These NK cell gene expression findings suggest exposure to TLR7/8 and TLR8 ligands elicited differential effects across liver, kidney, and spleen tissues of *rag* MT zebrafish.

## Keywords

Toll Like Receptor Ligands, Natural Killer Cell Activation, *Rag* Mutant Zebrafish, Tissue-specific Responses

## 1. Introduction

Teleosts have well-developed innate and adaptive immune systems with cells morphologically similar to mammals and mediate classically defined immune responses [1]. Due to their phylogenetic position as more ancient vertebrates, fish rely more on innate immune responses than mammals [2],

making them especially valuable for studying conserved pathways of innate immunity [3]. Zebrafish are well-suited for these investigations due to their tractable genetics, transparent embryos and availability of immune-deficient models. Zebrafish innate immune cells include monocytes, macro-

\*Corresponding author: [lp2@msstate.edu](mailto:lp2@msstate.edu) (Lora Petrie-Hanson)

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phages, basophils, eosinophils, mast cells, neutrophils, non-specific cytotoxic cells (NCCs) and Natural Killer (NK) cells [4, 5]. Rag1<sup>-/-</sup> mutant (rag MT) zebrafish lack mature T and B cells and are a good model for investigating fish innate immune cells and mechanisms without the confounding influence of adaptive responses [5, 6]. Natural Killer cells are one of the lymphocyte-like cells (LLCs) described in rag MT zebrafish [7].

Natural Killer (NK) cells recognize infected or stressed cells via pathogen recognition receptors (PRRs), including Toll-like receptors TLR7 and TLR8, which detect viral and bacterial RNAs within endosomes [8]. Synthetic TLR agonists such as Resiquimod (R848) and Motolimod (VTX2337, or VTX) activate these receptors. R848 stimulates both TLR7 and TLR8, and VTX acts selectively on TLR8. R848 demonstrated efficacy in activating NK and dendritic cells and has been explored in human cancer immunotherapy, including skin-related cancers [9]. Motolimod activates NK cells, dendritic cells, and monocytes and is used in general human oncology solid tumor therapy [10]. The utility of TLR7 and TLR8 to dissect NK cell function has been well-documented in human immunological studies [11-14], supporting their application as tools to probe NK cell activation and heterogeneity in other vertebrate models, such as zebrafish.

Teleost NK cells are morphologically similar to mammalian NK cells and contain analogous cytotoxic granules [15]. These granules house NK lysins, which are alpha-helical anti-microbial peptides (AMPs) that equip the host with immune responses against pathogen challenges; they were first identified as anti-bacterial peptides in porcine NK cells and cytotoxic T lymphocytes [16]. When NK cells identify bacterially or virally infected cells, they release lysins into the immunological synapse with the target cell [17]. NK lysins mediate antimicrobial activity by directly attacking pathogen-infected cells and activating dendritic cells, monocytes, and macrophages through chemotaxis and triggering the release of proinflammatory cytokines [18]. NK lysins have been found in a variety of fish, such as Clownfish (*Amphiprion ocellaris*) [19], mud skipper (*Boleophthalmus pectinirostris*) [20], Atlantic salmon (*Salmo salar*) [21], common carp (*Cyprinus carpio*) [22], large yellow croaker (*Larimichthys crocea*) [23], sole (*Cynoglossus semilaevis*) [24], Japanese flounder (*Paralichthys olivaceus*) [25], channel catfish (*Ictalurus punctatus*) [26], and zebrafish (*Danio rerio*) [27].

Significant upregulation of the genes analyzed in the current report has been correlated with NK cell-based immune responses in rag MT zebrafish [28]. Four NK-lysin paralogs (*nkla*, *nkla*, *nkla*, and *nkla*) have been identified in zebrafish [27]. T-bet, also known as Tbox-21, is a transcription factor in T and NK cells that is essential for NK cell maturation [29]. Nitr9 is an activating receptor identified on NK cells in zebrafish [30, 31]. Interferon gamma (*ifnγ*), produced by activated T cells and NK cells, is a cytokine whose levels correlate with cytotoxic activity [32].

Previously, we described cytotoxic cell subpopulations

based on cell morphologies in the liver, kidney, and spleen tissues of rag MT zebrafish and reported tissue-specific gene expressions following TLR ligand exposures [7]. In another study, principal component analysis of kidney and liver gene expressions further suggested that tissue-specific NK cell subpopulations correlated with survival following bacterial exposure after immune training with TLR ligands [28]. The purpose of the current study was to further elucidate kidney, liver, and spleen-specific NK cell subpopulations by determining *ifnγ*, *t-bet*, *nitr9*, *nkla*, *nkla*, *nkla*, and *nkla* expressions following separate exposures to the TLR7/8 ligand R848 and the TLR8 ligand VTX in rag MT zebrafish.

## 2. Materials and Methods

### 2.1. Rag MT Zebrafish Care

The Mississippi State University Institutional Animal Care and Use Committee (MSU IACUC) approved the breeding protocols used to produce the fish in these experiments. Briefly, zebrafish were mated, raised, and maintained at 28 °C in the specific pathogen-free hatchery, as previously described [5].

### 2.2. R848 and VTX Injections

Rag MT zebrafish were IC injected with VTX (0.25 nM or 2.5 nM/0.5 g of fish) and R848 (0.25 nM or 2.5 nM/0.5 g of fish) or endotoxin-free saline. TLR ligands were reconstituted in endotoxin-free saline, and a final volume of 10 µl was injected per fish.

### 2.3. RNA Extraction and cDNA Preparation

Fish were euthanized using a buffered 0.02% Tricaine Methanesulfonate solution (Finquel MS-222; Argent Chemical Laboratories, Redmond, WA). Liver, kidney, and spleen tissues were collected at 6-, 12-, and 24-hours post-injection (hpi) for each TLR ligand (n=5 per time point). Tissues were immediately transferred into 400 µl of Trizol reagent (Zymo Research, USA) and homogenized following standard procedures established in our laboratory [7]. Total RNA was individually extracted from each homogenized liver, kidney, and spleen sample using the RNA extraction kit (Zymo Research, USA), according to the manufacturer's protocol. RNA quantity was assessed using a NanoDrop ND-1000 spectrophotometer, and samples were stored at -80 °C until use. Complementary DNA (cDNA) was synthesized from 100 ng of RNA using the SuperScript III VILO™ cDNA Synthesis Kit (Invitrogen).

### 2.4. Quantifying Gene Expression

*Ifnγ*, *t-bet*, *nitr9*, *nk lysin a*, *b*, *c*, and *d* were measured using real-time quantitative PCR (Takara), as previously described

[28]. The *t-bet*, *nk* *lysin a*, *b*, *c*, and *d* primers and probes (Table 1) were designed by Primer3 plus (GraphPad) software, while primers and probes for *ifny* and *nitr9* (Table 1) were adopted from previous publications. Primers and probes were obtained from Eurofins MWG Operon (Huntsville, Alabama, USA). Amplification of the acidic ribosomal phosphoprotein (*arp*) gene, which is ubiquitously expressed, served as the internal control [7]. PCR reactions were conducted in a final volume of 25  $\mu$ l, comprising 10  $\mu$ l of target cDNA and 15  $\mu$ l of master mix. The master mix included 8.8  $\mu$ l of nucle-

ase-free water (GIBCO, UltraPure™), 1.5  $\mu$ l of 5 mM MgCl<sub>2</sub>, 2.5  $\mu$ l of 10 $\times$  buffer, 0.5  $\mu$ l of dNTPs, 0.2  $\mu$ l of Taq Polymerase HS enzyme (Hot Start PCR Kit, TAKARA, Japan), 0.5  $\mu$ l of forward primer (20  $\mu$ M), 0.5  $\mu$ l of reverse primer (20  $\mu$ M), and 0.5  $\mu$ l of probe (10  $\mu$ M). Thermal cycling was performed under the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 61 °C for 1 minute. Each sample (biological replicate) was analyzed in triplicate.

**Table 1.** Oligonucleotide primers and probes were used for qRT-PCR analysis of gene expression levels of *arp*, *ifny*, *t-bet*, *nitr9*, *nkla*, *nklb*, *nkcl*, and *nkld* in rag mutant zebrafish liver, kidney, and spleen tissues. The housekeeping gene *arp* was used as a reference gene. Primers and probes for *t-bet*, *nkla*, *nklb*, *nkcl*, and *nkld* were designed using Beacon Designer software (Bio-Rad) and Primer3Plus (GraphPad).

Gene	Oligonucleotide Sequences (5'-3')	GenBank Accession No.
<i>arp</i>	Fwd: CTGCAAAGATGCCCGAGGA	NM_131580
	Rev: TTGGAGCCGACATTGTCTGC	
	Probe: [6~FAM] TTCTGAAAATCATCCAAGTCTGGATGACTACC [BHQ1a~Q] [33]	
<i>ifny</i>	Fwd: CTTTCCAGGCAAGAGTGCAGA	NM_212864
	Rev: TCAGCTCAAACAAAGCCTTTTCG	
	Probe: [6~FAM] AACGCTATGGGCGATCAAGGAAAACGAC[BHQ1a~Q] [33]	
<i>t-bet</i>	Fwd: GATCAAGCTCTCTCTGTGATAG	NM_001170599.1
	Rev: GCTAAAGTCACACAGGTCT	
	Probe: [6~FAM] TTCTGAAGGTCACGGTCACA[BHQ1a~Q] *	
<i>nitr9</i>	Fwd: GTCAAAGGGACAAGGCTGATAGTT	AY570237.1
	Rev: GTTCAAACAGTGCATGTAAGACTCA	
	Probe: [6~FAM] CAAGGTTTGAAAAGCAC[BHQ1a~Q] (30)	
<i>nkla</i>	Fwd: TTTCTGGTCGGCTTGCTCAT	NM_001311794
	Rev: TTCTCATTCACAGCCCGGTC	
	Probe: [6~FAM] TCTGCAGCTCACTGGGAGGTTCGTGA[BHQ1a~Q]	
<i>nklb</i>	Fwd: TCCGCAACATCTTTCTGGTCA	NM_001311792
	Rev: AGCCTGCTCATGAATGAAAATGA	
	Probe: [6~FAM] CACGCCTGCAATCTGAACCACCCA[BHQ1a~Q]	
<i>nkcl</i>	Fwd: CTGCTTGTGCTGCTCACTTG	NM_001311793.1
	Rev: AGCACACATGGAGATGAGAACA	
	Probe: [6~FAM] GGGCTTGCAAGTGGGCCATGGGAA[BHQ1a~Q]	
<i>nkld</i>	Fwd: ACCCTGCTCATCTCCTCTGT	NM_212741.1
	Rev: CCCCAGCTAAAGCAAAACCC	
	Probe: [6~FAM] TGCCTGGGATGTGCTGGGCTTGCAA[BHQ1a~Q]	

## 2.5. Data Analysis and Statistical Evaluation

Relative gene expressions were determined using the Pfaffl method, as previously described [28]. Briefly, data obtained from qRT-PCR were expressed as fold changes relative to

control conditions and were transformed to log<sub>2</sub> values to normalize the distribution. A two-way Analysis of Variance (ANOVA) was performed to assess the independent variables and interactions. Dunnett's multiple comparisons test was performed with TLR ligand type and concentration compared to PBS control. Statistics software used was GraphPad Prism

version 7 for Mac, GraphPad Software, La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com). An alpha level of 0.05 was used to determine the significance of all analyses, and the results are included in Supplemental Tables S1, S2, S3, and S4.

## 3. Results

### 3.1. Liver

Following TLR7/8 stimulation (R848), *ifn $\gamma$*  expression was significantly increased at 6 hpi at the high dose (2.5 mM) ( $p=0.0001$ ), while no significant upregulation occurred with TLR8 (VTX) treatment at any dose or timepoint. *T-bet* expression was significantly elevated at 24 hpi only at the lower R848 dose (0.25 mM) ( $p=0.0030$ ), with no response to TLR8. Expression of *nitr9* increased significantly in the liver following high-dose R848 treatment at 6 hpi ( $p=0.0001$ ) and 24 hpi ( $p=0.0453$ ), and at 24 hpi ( $p=0.0453$ ) following the low dose. No changes were observed with TLR8 at any rate. For *nkla*, expression remained unchanged in all groups. *Nklb* expression increased at 6 hpi with high-dose R848 ( $p=0.0001$ ). *Nklc* was significantly upregulated at 12 hpi with high-dose R848 ( $p=0.0001$ ) and 12 hpi with low-dose R848 ( $p=0.0001$ ) but was not affected by TLR8. *Nkld* expression was not significantly affected by TLR7/8 stimulation but was upregulated by TLR8 (2.5 mM VTX) at 12 hpi ( $p=0.0002$ ). These findings are summarized in Figure 1 and Table 2.

### 3.2. Kidney

In the kidney, *ifn $\gamma$*  expression was significantly increased following TLR7/8 stimulation. High-dose R848 induced expression at 12 hpi ( $p=0.0414$ ), while low-dose R848 induced expression at 12 hpi ( $p=0.0001$ ). No significant increases were observed with TLR8. *T-bet* was significantly upregulated following TLR7/8 treatment at 24 hpi ( $p=0.0001$  for low-dose R848) and with TLR8 at both 6 hpi ( $p=0.0009$ ) and 12 hpi ( $p=0.0049$ ) at low-dose VTX and 24 hpi at high-dose VTX ( $p=0.0270$ ). *Nitr9* expression significantly increased at 24 hpi following high-dose R848 ( $p=0.0001$ ). *Nitr9* was also significantly upregulated by the high dose TLR8 at 6 hpi (0.0046) and 24 hpi ( $p=0.0001$ ), and low dose TLR8 at 6 hpi ( $p=0.0099$ ) and 24 hpi ( $p=0.0064$ ). *Nkla* was significantly upregulated by the low dose of R848 at 24 hpi ( $p=0.0001$ ) and was not upregulated by TLR8. *Nklb* expression was significantly upregulated at 24 hpi following high-dose R848 ( $p=0.0295$ ) and at 24 hpi with low-dose VTX ( $p=0.0295$ ). *Nklc* expression significantly increased at multiple time points following both TLR7/8 and TLR8 stimulation. The high dose R848 increased expression at 6 hpi ( $p=0.0101$ ) and 24 hpi (0.0017), and the low dose at 12 hpi ( $p=0.0031$ ). Low-dose VTX significantly increased *nklc* expression at 6 hpi ( $p=0.0009$ ) and 12 hpi ( $p=0.0031$ ). *Nkld* expression was significantly upregulated following the low-dose TLR7/8 stimulation at 12 hpi ( $p=0.0001$ ) and 24 hpi ( $p=0.0001$ ), and by the

high dose of TLR8 at 6 hpi ( $p=0.0001$ ) and the low dose of VTX at 6 hpi (0.0002) and 12 hpi ( $p=0.0056$ ).

### 3.3. Spleen

In the spleen, *ifn $\gamma$*  expression was significantly upregulated by TLR7/8 exposure. High-dose R848 significantly increased expression at 6 hpi ( $p=0.0261$ ) and 12 hpi ( $p=0.0006$ ), while low-dose R848 significantly upregulated expression at 12 hpi ( $p=0.0001$ ). *T-bet* expression was significantly upregulated at 12 hpi ( $p=0.0169$ ) after low-dose R848 treatment. *Nitr9* was significantly upregulated by TLR7/8. Low-dose R848 increased expression at 12 hpi ( $p=0.0003$ ) and 24 hpi ( $p=0.0001$ ). *Nkla* expression was significantly increased following high-dose R848 at 12 hpi ( $p=0.0103$ ) and low-dose R848 at 24 hpi ( $p=0.0494$ ). *Nklb* was significantly upregulated by high-dose R848 at 12 hpi ( $p=0.004$ ) and 24 hpi ( $p=0.0107$ ), and the low-dose R848 at 12 hpi ( $p=0.004$ ) and 24 hpi ( $p=0.0107$ ). *Nklc* expression was responsive to both TLR7/8 and TLR8. Low-dose R848 significantly increased expression at 12 hpi ( $p=0.0001$ ), and TLR8 (high-dose VTX) significantly increased expression at 24 hpi ( $p=0.0001$ ). *Nkld* was significantly upregulated by both TLR7/8 and TLR8 ligands. Low-dose R848 significantly increased expression at 24 hpi (0.0395) and high dose at 12 hpi ( $p=0.0001$ ) and 24 hpi ( $p=0.0001$ ), while TLR8 significantly increased expression at 24 hpi ( $p=0.0006$ ).

## 4. Discussion

In earlier studies, we morphologically characterized lymphocyte-like cell populations in the liver, kidney, and spleen of rag MT zebrafish and identified tissue-specific gene expressions in response to the TLR ligands beta-glucan, Poly I:C, and R848 [7]. Among these, R848 was further investigated for its ability to induce trained immunity and associated NK cell-specific gene responses [28]. The current study builds on these findings to further characterize NK cell-specific gene responses across these tissues. We found tissue-specific differences in the expression of *ifn $\gamma$* , *t-bet*, *nitr9*, *nkla*, *nklb*, *nklc*, and *nkld* in response to TLR7/8 (R848) and TLR8 (VTX) ligands. VTX had no effect on *ifn $\gamma$*  and *nkla* in the liver, kidney (hematopoietic tissue), and spleen, whereas R848 significantly upregulated *ifn $\gamma$*  across all three tissues and *nkla* in the kidney and spleen. The effects of VTX were primarily limited to the kidney, where TLR8 activation significantly upregulated *t-bet*, *nitr9*, and *nklb*. In contrast, VTX did not significantly affect liver expression of *nkla*, *nklb*, or *nklc*. TLR7/8 activation by R848 generally induced broader upregulation across tissues, although *nkla* and *nkld* were not significantly upregulated in the liver. Dose and time-dependent responses were also apparent. Notably, lower concentrations of R848 and VTX often elicited stronger gene expression responses. This may reflect regulatory mechanisms that suppress excessive inflammation, a well-characterized feature of TLR sig-



naling [34].

Although VTX had limited overall effects, it notably induced strong *t-bet* and *nkld* responses in the kidney, indicating a tissue-specific role for TLR8 in modulating kidney immunity. These results suggest that a subpopulation of NK or cytotoxic cells in the kidney responds specifically to TLR8 stimulation. The absence of significant changes in *ifn $\gamma$* , *nkla*, and *nk1b* expression with VTX exposure supports the idea that TLR8 signaling does not broadly activate NK or cytotoxic cell

responses. Interestingly, VTX selectively upregulated *nkld* in the liver and both *nk1c* and *nkld* in the kidney and spleen, demonstrating tissue-specific regulation of NK-lysin genes by TLR8. In human leukocytes, VTX activates TLR8 and the NLRP3 inflammasome, leading to tumoricidal activity in THP-1 cells and monocytes [13]. Since NLRP inflammasomes have also been described in zebrafish [35], it is plausible that VTX similarly activates these pathways in kidney hematopoietic cells.

**Table 2.** Significantly upregulated genes in liver, kidney, or spleen tissues of *rag1* mutant zebrafish at different hours post injection (hpi) with 0.25 mM or 2.5 mM R848 or 0.25 mM or 2.5 mM VTX2337 (VTX) were summarized. Only genes with statistically significant upregulation are included in Table 2. Genes assessed include interferon gamma (*ifn $\gamma$* ), T-box transcription factor 21 (*t-bet*), novel immune-type receptor 9 (*nitr9*), and Natural Killer (NK) cell lysins (*nkla*, *nk1b*, *nk1c*, and *nkld*). Fold changes were considered significant at  $p < 0.05$  (\*) and highly significant  $p < 0.001$  (\*\*), relative to control. The ANOVA data are provided in the supplementary materials.

Treatment	Tissues	Upregulated <10-fold change		Upregulated 10 to 100-fold change		Upregulated >100-fold change	
		0.25mM	2.5mM	0.25mM	2.5mM	0.25mM	2.5mM
R848	Liver			<i>t-bet</i> (24 hpi*)	<i>nitr9</i> (24hpi*), <i>nk1b</i> (6hpi**)	<i>nitr9</i> (24 hpi*)	<i>Ifn<math>\gamma</math></i> (6hpi**), <i>nitr9</i> (6hpi**), <i>nk1c</i> (12hpi**)
	Kidney			<i>t-bet</i> (24 hpi**)		<i>ifn<math>\gamma</math></i> (12 hpi**), <i>t-bet</i> (12 hpi**), <i>nkla</i> (24 hpi**), <i>nk1c</i> (12 hpi**), <i>nkld</i> (12 hpi**), <i>nkld</i> (24 hpi**)	<i>ifn<math>\gamma</math></i> (12 hpi*), <i>nk1b</i> (24 hpi*), <i>nk1c</i> (6 hpi*), <i>nk1c</i> (24 hpi*)
	Spleen	<i>nitr9</i> (12 hpi*), <i>nitr9</i> (24 hpi*)		<i>t-bet</i> (12 hpi*), <i>nkla</i> (24 hpi*), <i>nk1b</i> (12 hpi*), <i>nk1b</i> (24 hpi*), <i>nkld</i> (24 hpi**)	<i>Ifn<math>\gamma</math></i> (6 hpi*), <i>Nk1a</i> (12 hpi*), <i>Nkld</i> (24 hpi*)	<i>ifn<math>\gamma</math></i> (12 hpi**), <i>nk1c</i> (12 hpi**), <i>nkld</i> (12 hpi**)	<i>ifn<math>\gamma</math></i> (12 hpi*), <i>nk1c</i> (24 hpi**),
VTX	Liver		0.25mM <i>Nkld</i> (12 hpi*)	0.25mM	2.5mM	0.25mM	2.5mM
	Kidney	<i>nitr9</i> (6 hpi*), <i>nitr9</i> (24 hpi*),	<i>nitr9</i> (6 hpi*)	<i>Nk1b</i> (24 hpi*)		<i>t-bet</i> (6 hpi*), <i>t-bet</i> (12 hpi*), <i>nk1c</i> (6 hpi*), <i>nk1c</i> (12 hpi*), <i>nkld</i> (6 hpi*), <i>nkld</i> (12 hpi*)	<i>t-bet</i> (24 hpi*), <i>nkld</i> (6 hpi**)
	Spleen				<i>Nkld</i> (24 hpi*)		<i>Nk1c</i> (24 hpi**)

Overall, R848 induced a broader gene response than VTX, likely due to its strong and early induction of *ifn $\gamma$*  in liver and spleen tissues, as *ifn $\gamma$*  is known to enhance NK-lysin expression [36]. In another study of *rag* MT zebrafish, R848 simi-

larly upregulated *ifn $\gamma$* , *t-bet*, and *nitr9* in the liver, kidney, and spleen [7], although splenic *t-bet* and *nitr9* were not significantly induced. Our data highlight clear differences in gene expression across tissues, as well as variable responses to

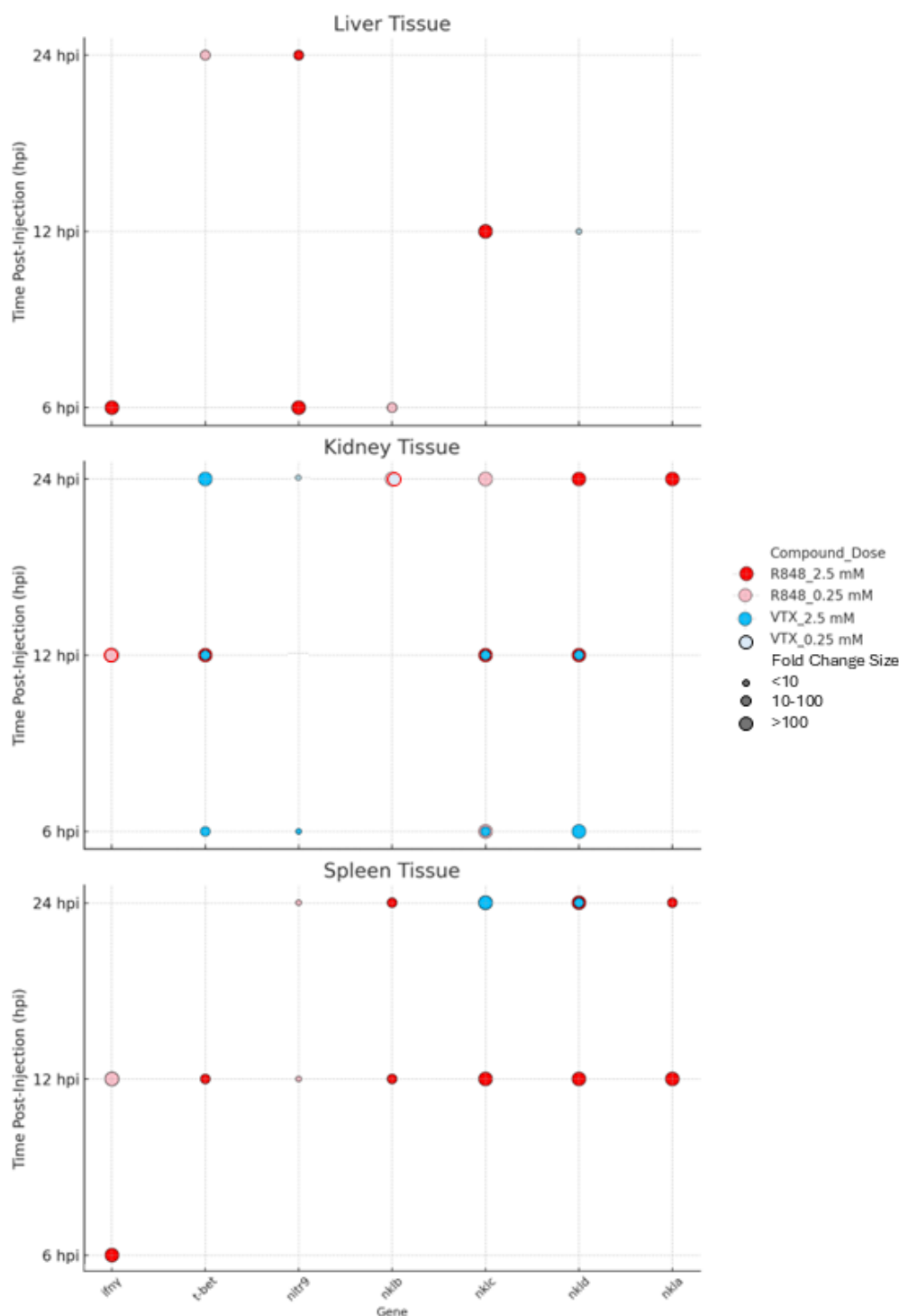
ligand concentration and type. These results suggest that liver, kidney, and spleen tissues harbor different proportions or subtypes of NK cells with distinct sensitivities to TLR7/8 (R848) and TLR8 (VTX). In human leukocytes, TLR7 and TLR8 activation trigger divergent pathways depending on cell type [12], and TLR8 selectively enhances the activity of the CD56 bright NK subset [11]. Although zebrafish NK cell subset markers have not yet been defined, our results imply the existence of functionally distinct subpopulations.

In the liver, upregulation of *ifn $\gamma$* , *t-bet*, *nitr9*, *nklb*, and *nkcl* was driven by TLR7/8 activation. TLR8 activation did not induce upregulations in these genes, suggesting TLR7-specific signaling plays a dominant role in this tissue. *Ifn $\gamma$*  is associated with initial antiviral and antibacterial responses, but also plays a role in modulating inflammation. The principal producers of *ifn $\gamma$*  are NK cells [37]. Our findings suggest that in the liver, there is an *ifn $\gamma$* -producing NK cell subpopulation that is highly responsive to TLR7. *T-bet*, *nitr9*, *nklb*, and *nkcl* are essential for NK cell and cytotoxic cell function and inflammatory responses in the liver. TLR7/8 activation did not affect liver *nkla* and *nkld*. This suggests that the cell types or immune functions initiated by these genes or signaling pathways are not responsive to TLR7/8 in the liver. *Nklb* and *nkcl* were highly upregulated by TLR7/8 activation. TLR8 activation did not affect liver *ifn $\gamma$* , *t-bet*, *nitr9*, *nkla*, *nklb*, and *nkcl*, but did significantly upregulate *nkld*, suggesting TLR8 is less expressed or signaling is mediated differently in the liver. In wild-type (WT) zebrafish liver, the relative basal expression of *nkla*, *nklb*, *nkcl*, and *nkld* was 54%, 41%, 5%, and <1%, respectively [27]. TLR7/8 activation induced significantly upregulated liver *ifn $\gamma$* , *t-bet*, and *nitr9* in a similar study [7].

*Ifn $\gamma$*  was highly upregulated in the kidney by TLR7/8, but not by TLR8 activation. *T-bet* was moderately upregulated by TLR7/8 activation and highly upregulated by TLR8 activation in kidney innate immune cells, in the absence of T cells. This is interesting because *t-bet* is traditionally recognized as a T-cell transcription factor [37]. This upregulation suggests that in rag MT zebrafish, *t-bet* plays a role in NK cells, suggesting NK/T cells [27, 38] and lymphocyte-like innate cells activation and population expansion. Our findings also suggest that in zebrafish, *t-bet* may be involved in the transcription of multiple innate immune cell types in the kidney envi-

ronment. *Nkla* and *nkld* were highly upregulated by TLR7/8 activation in the kidney, suggesting that signaling components activated by TLR7/8 are particularly effective at inducing these genes in the kidney environment. Pereiro [27] indicated that in the rag MT zebrafish kidney, CTLs were specialized in producing *nkla* and *nkld*. In our study, TLR8 activation resulted in highly differentially expressed *nkld* but not *nkla* in the kidney, suggesting that an NK or cytotoxic cell subset expressing TLR8 produces *nkld* in the kidney. Single-cell transcriptional analysis of *rag2*<sup>E450fs</sup> mutant zebrafish kidney marrow determined that *nk1.4+* (*nkld+*) cells were cytotoxic T/NK cells, and this population significantly expanded after viral infection [38]; this study did not examine *nkla*, *nklb*, or *nkcl*. In our study, *nklb* and *nkcl* were highly upregulated by TLR7/8 activation, suggesting that these pathways are effective at activating these genes to enhance cytotoxic functions in the kidney interstitial tissue. TLR8 activation induced *nkcl* upregulation but not *nklb*. TLR7/8 activation induced significantly upregulated kidney *ifn $\gamma$* , *t-bet*, and *nitr9* in a similar study [7].

In the spleen, *ifn $\gamma$*  was upregulated by TLR7/8, but not by TLR8, suggesting TLR7 plays a critical role in *ifn $\gamma$*  induction in the spleen. Spleen cells can differentially express TLR7 and TLR8, or the cells may have different sensitivities to TLR7 and TLR8. This finding also suggests that TLR7 is more involved in pathways leading to *ifn $\gamma$*  production than TLR8, or that TLR7 and TLR8 pathways might have negative regulatory effects on each other in the spleen tissue environment. This mechanism could control inflammation. *Nkla* and *nklb* were moderately upregulated by TLR7/8 but not TLR8. This suggests that TLR7 plays a key role in initiating the expression of *nkla* and *nklb* and that TLR8 does not effectively activate the *nkla* and *nklb* signaling pathways or is possibly suppressed by TLR7 activation. TLR7/8 and TLR8 are highly upregulated *nkcl* and moderately upregulated *nkld*, suggesting that the activation of TLR7 and TLR8 either alone or synergistically induces pathways for *nkcl* and *nkld* production. A broader activation potential may indicate that *nkcl* and *nkld* may have more critical roles in splenic NK cell functions. Overall, TLR7-mediated pathways appear to play the dominant role in activating NK-lysin genes and modulating NK cell functions in the spleen. TLR7/8 activation induced significantly upregulated splenic *ifn $\gamma$*  in a similar study [7].



**Figure 1.** Significantly upregulated NK cell-related genes in the liver, kidney, and spleen of *rag1* mutant zebrafish following injection with 0.25 mM or 2.5 mM R848 or VTX2337 (VTX) were summarized and presented in a dot matrix plot. Each dot represents a gene that was significantly upregulated at the indicated time point post-injection (hpi). The size of the dot indicates the fold change category: <10-fold, 10 to 100-fold, or >100 fold. Genes assessed include interferon gamma (*ifnγ*), T-box transcription factor 21 (*t-bet*), novel immune-type receptor 9 (*nitr9*), and Natural Killer (NK) cell lysins (*nkla*, *nkly*, *nkcl*, and *nkld*).

To better understand the divergent gene expression profiles observed among tissues, it is important to consider the immune microenvironments of the zebrafish liver, kidney, and spleen tissues. In zebrafish, the kidney is the primary hematopoietic tissue and contains a diverse population of pluripo-

tent, developing, and mature immune cells, including innate lymphoid cells, monocytes, and granulocytes. This diversity may allow the kidney to respond more selectively to TLR stimulation, particularly TLR8, which broadly affected gene expression in this tissue. In contrast, the liver is both an im-

mune and metabolic organ, with resident macrophages and cytotoxic cells that may be adapted to tolerate frequent exposure to antigens. This may influence the sensitivity of NK cell activation by TLR7 and 8. The spleen is a secondary lymphoid organ, with lymphocyte-like cells and antigen-presenting cells, resulting in selective gene expression responses. Together, these factors may contribute to the gene expression patterns observed following TLR7/8 or TLR8 stimulation.

## 5. Conclusions

Our findings demonstrate differential gene expression in response to TLR7 and TLR8 activation. Together with previous research [7, 28], these findings can infer NK cell and NK/cytotoxic cell subpopulations in liver, kidney, and spleen tissues of rag MT zebrafish, and these cells respond differently based on different tissue environments. TLR7/8 activation resulted in significantly upregulated *nklb*, *nklc*, and *nkld* in the liver, *nklb*, *nklc*, and *nkld* in the kidney, and *nkla*, *nklb*, *nklc*, and *nkld* in the spleen. TLR8 activation resulted in significantly upregulated *nklc* and *nkld* in the kidney, spleen, and *nkld* in the liver. The only cells that significantly upregulated *nkla* or *nklb* were activated by TLR7/8; these cells were not activated by TLR8 alone. NK cell and cytotoxic responses vary by tissue due to the functional differences of cell populations in different tissues in response to a wide spectrum of pathogens and insults.

## Abbreviations

NK	Natural Killer
Rag MT	Rag1 Mutant (rag MT) Zebrafish
ifn $\gamma$	Interferon Gamma
t-bet	T-box Transcription Factor 21
Nitr9	Novel Immune Type Receptor-9
Nkla	NK Lysin a
Nklb	NK Lysin b
Nklc	NK Lysin c
Nkld	NK Lysin d
NCC	Non-specific Cytotoxic Cell
LLC	Lymphocyte-like Cell
PRR	Pathogen Recognition Receptor
PAMP	Pathogen Associated Molecular Pattern
R848	Resiquimod
VTX	Motolimod VTX2337
AMP	Anti-microbial peptides
MSU	Mississippi State University Institutional
IACUC	Animal Care and Use Committee
cDNA	complementary DNA
PCR	polymerase chain reaction
Arp	acidic ribosomal phosphoprotein
ANOVA	Analysis of Variance

## Supplementary Materials

The supplementary material can be accessed at <https://doi.org/10.11648/j.iji.20251303.11>

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## Author Contributions

**Preeti Judith Muire:** Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing

**Lora Petrie-Hanson:** Conceptualization Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing - review & editing

## Institutional Review Board Statement

This study was approved by the Institutional Animal Care and Use Committee of Mississippi State University protocol #17-028.

## Data Availability Statement

Data available on request from the authors. The raw data supporting the conclusion of this article will be made available by the authors on request.

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## Conflicts of Interest

The authors declare no conflict of interest.

## References

- [1] Sunyer JO. Fishing for mammalian paradigms in the teleost immune system. Vol. 14, Nature Immunology. Nature Publishing Group; 2013. p. 320-6.
- [2] DeWitte-Orr S, Edholm ES, Grayfer L. Editorial: Innate Immunity in Aquatic Vertebrates. Vol. 10, Frontiers in Immunology. Frontiers Media S. A.; 2019.
- [3] Bailone RL, Fukushima HCS, Ventura Fernandes BH, De Aguiar LK, Corrêa T, Janke H, et al. Zebrafish as an alternative animal model in human and animal vaccination research. Vol. 36, Laboratory Animal Research. BioMed Central Ltd; 2020.



- [4] Moss LD, Monette MM, Jaso-Friedmann L, Leary JH, Dougan ST, Krunkosky T, et al. Identification of phagocytic cells, NK-like cytotoxic cell activity and the production of cellular exudates in the coelomic cavity of adult zebrafish. *Dev Comp Immunol*. 2009; 33(10): 1077-87.
- [5] Petrie-Hanson L, Hohn C, Hanson L. Characterization of rag1 mutant zebrafish leukocytes. *BMC Immunol*. 2009 Feb 3; 10.
- [6] Tokunaga Y, Shirouzu M, Sugahara R, Yoshiura Y, Kiryu I, Ototake M, et al. Comprehensive validation of T- and B-cell deficiency in rag1-null zebrafish: Implication for the robust innate defense mechanisms of teleosts. *Sci Rep*. 2017 Dec 1; 7(1).
- [7] Muire PJ, Hanson LA, Wills R, Petrie-Hanson L. Differential gene expression following TLR stimulation in rag1-/- mutant zebrafish tissues and morphological descriptions of lymphocyte-like cell populations. *PLoS One* [Internet]. 2017; 12(9): 1-22. Available from: <http://dx.doi.org/10.1371/journal.pone.0184077>
- [8] Souza-Fonseca-Guimaraes F, Parlato M, Fitting C, Cavaillon JM, Adib-Conquy M. NK Cell Tolerance to TLR Agonists Mediated by Regulatory T Cells after Polymicrobial Sepsis. *The Journal of Immunology*. 2012 Jun 15; 188(12): 5850-8.
- [9] Rook AH, Gelfand JM, Wysocka M, Troxel AB, Benoit B, Surber C, et al. Topical resiquimod can induce disease regression and enhance T-cell effector functions in cutaneous T-cell lymphoma. *Blood*. 2015 Sep 17; 126(12): 1452-61.
- [10] Chow LQM, Morishima C, Eaton KD, Baik CS, Goulart BH, Anderson LN, et al. Phase Ib trial of the toll-like receptor 8 agonist, motolimod (VTX-2337), combined with cetuximab in patients with recurrent or metastatic SCCHN. *Clinical Cancer Research*. 2017 May 15; 23(10): 2442-50.
- [11] Veneziani I, Alicata C, Pelosi A, Landolina N, Ricci B, D'Oria V, et al. Toll-like receptor 8 agonists improve NK-cell function primarily targeting CD56 bright CD16 - Subset. *J Immunother Cancer*. 2022 Jan 28; 10(1).
- [12] Bender AT, Tzvetkov E, Pereira A, Wu Y, Kasar S, Przetak MM, et al. TLR7 and TLR8 Differentially Activate the IRF and NF- $\kappa$ B Pathways in Specific Cell Types to Promote Inflammation. *Immunohorizons*. 2020 Feb 1; 4(2): 93-107.
- [13] Dietsch GN, Lu H, Yang Y, Morishima C, Chow LQ, Disis ML, et al. Coordinated activation of toll-like receptor8 (TLR8) and NLRP3 by the TLR8 agonist, VTX-2337, ignites tumoricidal natural killer cell activity. *PLoS One* [Internet]. 2016; 11(2): 1-18. Available from: <http://dx.doi.org/10.1371/journal.pone.0148764>
- [14] Dietsch GN, Randall TD, Gottardo R, Northfelt DW, Ramnathan RK, Cohen PA, et al. Late-stage cancer patients remain highly responsive to immune activation by the selective TLR8 agonist motolimod (VTX-2337). *Clinical Cancer Research*. 2015 Dec 15; 21(24): 5445-52.
- [15] Fischer U, Koppang EO, Nakanishi T. Teleost T and NK cell immunity. Vol. 35, *Fish and Shellfish Immunology*. Academic Press; 2013. p. 197-206.
- [16] Andersson M, Gunne H, Agerberth B, Boman A, Bergman T, Olsson B, et al. NK-lysin, structure and function of a novel effector molecule of porcine T and NK cells. Vol. 54, *Veterinary Immunology and Immunopathology*. 1996.
- [17] Stinchcombe J, Griffiths G. Secretory Mechanisms in Cell-Mediated Cytotoxicity. *Annual Review of Cell and Developmental Biology*. 2007; 23: 495-517.
- [18] Zhang M, Li MF, Sun L. NKLP27: A teleost NK-lysin peptide that modulates immune response, induces degradation of bacterial DNA, and inhibits bacterial and viral infection. *PloS One*. (2014) 9(9): e106543-52. <https://doi.org/10.1371/journal.pone.0106543>
- [19] Yu D, Zhao H, Wen Y, Li T, Xia H, Wang Z, et al. Characterization and Functional Evaluation of NK-lysin from Clownfish (*Amphiprion ocellaris*). *Fishes*. 2023 Nov 1; 8(11).
- [20] Ding FF, Li CH, Chen J. Molecular characterization of the NK-lysin in a teleost fish, *Boleophthalmus pectinirostris*: Antimicrobial activity and immunomodulatory activity on monocytes/macrophages. *Fish Shellfish Immunol*. 2019 Sep 1; 92: 256-64.
- [21] Acosta J, Roa F, González-Chavarría I, Astuya A, Maura R, Montesino R, et al. In vitro immunomodulatory activities of peptides derived from *Salmo salar* NK-lysin and cathelicidin in fish cells. *Fish Shellfish Immunol*. 2019 May 1; 88: 587-94.
- [22] Wang GL, Wang MC, Liu YL, Zhang Q, Li CF, Liu PT, et al. Identification, expression analysis, and antibacterial activity of NK-lysin from common carp *Cyprinus carpio*. *Fish Shellfish Immunol*. 2018 Feb 1; 73: 11-21.
- [23] Zhou QJ, Wang J, Liu M, Qiao Y, Hong WS, Su YQ, et al. Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish *Larimichthys crocea*. *Fish Shellfish Immunol*. 2016 Aug 1; 55: 195-202.
- [24] Zhang M, Long H, Sun L. A NK-lysin from *Cynoglossus semilaevis* enhances antimicrobial defense against bacterial and viral pathogens. *Dev Comp Immunol*. 2013; 40(3-4): 258-65.
- [25] Hirono I, Kondo H, Koyama T, Arma NR, Hwang JY, Nozaki R, et al. Characterization of Japanese flounder (*Paralichthys olivaceus*) NK-lysin, an antimicrobial peptide. *Fish Shellfish Immunol*. 2007; 22(5): 567-75.
- [26] Wang Q, Wang Y, Xu P, Liu Z. NK-lysin of channel catfish: Gene triplication, sequence variation, and expression analysis. *Mol Immunol*. 2006 Apr; 43(10): 1676-86.
- [27] Pereiro P, Varela M, Diaz-Rosales P, Romero A, Dios S, Figueras A, et al. Zebrafish NK-lysins: First insights about their cellular and functional diversification. *Dev Comp Immunol*. 2015 Jul 1; 51(1): 148-59.
- [28] Muire PJ, Hanson LA, Petrie-Hanson L. Rapid Natural Killer Cell Gene Responses, Generated by TLR Ligand-Induced Trained Immunity, Provide Protection to Bacterial Infection in rag1-/- Mutant Zebrafish (*Danio rerio*). *Int J Mol Sci*. 2025 Feb 1; 26(3).

- [29] Klose CSN, Blatz K, d'Hargues Y, Hernandez PP, Kofoed-Nielsen M, Ripka JF, et al. The transcription factor T-bet is induced by IL-15 and thymic agonist selection and controls CD8 $\alpha\alpha$ <sup>+</sup> intraepithelial lymphocyte development. *Immunity*. 2014 Aug 21; 41(2): 230-43.
- [30] Shah RN, Rodriguez-Nunez I, Eason DD, Haire RN, Bertrand JY, Wittamer V, et al. Development and characterization of anti-Nitr9 antibodies. *Adv Hematol*. 2012; 2012.
- [31] Wei S, JM Z, Chen X, RN S, Liu J, TM O, et al. The zebrafish activating immune receptor Nitr9 signals via Dap12. *Immunogenetics*. 2007; 59(10): 813-21.
- [32] Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Vol. 9, *Nature Immunology*. 2008. p. 503-10.
- [33] Vojtech LN, Sanders GE, Conway C, Ostland V, Hansen JD. Host immune response and acute disease in a zebrafish model of Francisella pathogenesis. *Infect Immun*. 2009 Feb; 77(2): 914-25.
- [34] Fitzgerald KA, Kagan JC. Toll-like Receptors and the Control of Immunity. Vol. 180, *Cell*. Cell Press; 2020. p. 1044-66.
- [35] Chang MX. Emerging mechanisms and functions of inflammasome complexes in teleost fish. Vol. 14, *Frontiers in Immunology*. Frontiers Media S. A.; 2023.
- [36] Walker FC, Sridhar PR, Baldrige MT. Differential roles of interferons in innate responses to mucosal viral infections. Vol. 42, *Trends in Immunology*. Elsevier Ltd; 2021. p. 1009-23.
- [37] Murphy K, Weaver C. Janeway's Immunobiology, 9th edition. 2016. Garland science New York, New York. ISBN: 978-0815345053.
- [38] Moore FE, Garcia EG, Lobbardi R, Jain E, Tang Q, Moore JC, et al. Single-cell transcriptional analysis of normal, aberrant, and malignant hematopoiesis in zebrafish. *J Exp Med [Internet]*. 2016; 213(6): 979-92. Available from: <http://www.jem.org/lookup/doi/10.1084/jem.20152013>