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The Characterization of Zebrafish Natural Killer Cells and Their Role in Immunological Memory

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The characterization of zebrafish natural killer cells and their role in immunological memory

By

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ABSTRACT

Rag1$^{-/-}$ mutant zebrafish lack lymphocytes and were used to study the basis of acquired protective immunity in the absence of lymphocytes to the intracellular bacterium *Edwardsiella ictaluri*. This study morphologically identified and quantified lymphocyte like cells (LLCs) present in the liver, kidney and spleen of these fish. LLCs included Natural Killer (NK) cells and non-specific cytotoxic cells (NCCs) and were discriminated by size, and by the presence of cytoplasmic granules. The antibodies anti-NITR9, anti-NCCRP-1 (5C6) and anti-MPEG-1 were used to evaluate these cell populations by flow cytometry. Gene expression profiles in these tissues were evaluated after the *rag1*$^{-/-}$ mutants were intra coelomically injected with the toll like receptor (TLR)-2 ligand, β glucan, TLR3 ligand, Poly I:C, or TLR 7/8 ligand, R848. The genes interferon γ (*infγ*), expressed by activated NK cells and macrophages, tumor necrosis factor α (*tnfα*), expressed by activated macrophages, myxovirus resistance (*mx*) expressed by cells induced by IFNα, T-cell transcription factor (*t-bet*) expressed by NK cells and novel immune type-receptor 9 (*nitr-9*) expressed by NK cells were evaluated. The TLR

ligands induced different patterns of expression and stimulated both macrophages and NK cells.

Then fish were vaccinated with an attenuated mutant of *E. ictaluri* (RE33®) with or without the TLR ligands then challenged with WT *E. ictaluri* to evaluate protection. RE33® alone and each TLR ligand alone provided protection. Co-administration of β glucan and RE33® or R848 and RE33® resulted in survival higher than RE33® alone showing an adjuvant effect. Tissue specific gene expression of *ifnγ, t-bet, nitr9,* NK cell lysin a (*nkla*)*, nklb, nklc* and *nkld* were correlated to protection.

The final component of this study was the development of tools to discriminate NK cell populations and evaluate the contribution of macrophages. *Rag1*$^{-/-}$ zebrafish were modified to express cherry red in lymphocyte like cells using the Lymphocyte specific tyrosine kinase (*lck*) promotor. Also, *rag1*$^{-/-}$ zebrafish were modified so that the gene encoding the proto-oncogene serine/threonine-protein kinase that is involved in macrophage training (*raf1*) is disrupted. This study indicated that the acquired protection in the absence of lymphocytes likely involves NK cells with possible contribution by macrophages.

DEDICATION

To my late mother (Josephine Pauline Muire) who did not leave a leaf unturned to educate me and encourage me to follow my passion for science. To my father (Melville Vincent Muire) who inspired me to be hardworking and dedicated to my work.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION – LITERATURE REVIEW

1.1 Natural killer (NK) cells

Innate immune cells are extending the frontiers of immunology. Cells that were once thought to be limited in their response to pathogens are now seen as dynamic players in vaccination and protection. Innate immune cells include macrophages, neutrophils, basophils, eosinophils, dendritic cells, non-specific cytotoxic cells, natural killer (NK) cells and innate lymphoid cells (ILCs). NK cells belong to the innate lymphocyte like cell (LLC) group. NK cells distinguish between normal healthy cells and abnormal cells by using a sophisticated repertoire of cell surface receptors. These NK cell receptors control activation, proliferation and functions (Spits, Blom et al. 1998). Germline genes that do not undergo somatic recombination encode these receptors. Thus, NK cells represent an arm of the innate immune system. They lack the ability to generate antigen-specific receptors by somatic cell genetic alterations. NK cells are similar to cytotoxic T cells, with which they share a common bipotential progenitor (Spits, Blom et al. 1998, Colucci, Caligiuri et al. 2003). NK cells can provide protection quicker than T cells, especially in conditions where T cell responses are limited (Bauer, Groh et al. 1999).

NK cells were first identified in 1975 (Kiessling, Klein et al. 1975, Herberman and Holden 1978). They had a lymphocyte like morphology but lacked known surface

markers/receptors; hence these cells were initially called “null” cells. Later, the null cells were given the name “Natural Killer/NK cells” because in the normal state they can kill tumor and infected cells. Mammalian NK cells are a heterogeneous population of lymphocytes that can be morphologically and phenotypically characterized and isolated based on size and the expression of cell surface activation markers. NK cells can also be characterized by intracellular cytolytic proteins like granzymes and perforins (Voskoboinik, Whisstock et al. 2015). In humans, NK cells are divided into two subsets based on their function. CD56bright/CD16^{-} expressing cells are cytokine producers and CD56dim/CD16^{+} expressing cells are cytotoxic NK cells (Poli, Michel et al. 2009). Murine NK cells do not express CD56. Murine cytokine producers express surface CD27hi and the cytotoxic NK cells express surface CD27lo (Marquardt, Wilk et al. 2010). In contrast to T and B cells, NK cells are larger than T and B cells ranging in size from ~4.8 to 10μ. These cells express cytoplasmic granules containing cytotoxic proteins. NK cells have the ability to kill certain tumor cells and virus infected cells. When in contact with a tumor cell, the NK cell functions by releasing proteins from cytotoxic granules (granzymes and perforins) onto the surface of the target cell. Perforins form pores on the target cells and the cytotoxic granules (granzymes) penetrate the cell surface of the target cell through the pores and cause the target cell to undergo programmed cell death (Lanier 2005). NK cells are the main source of Fas ligand (L). Fas L is a soluble surface marker that is associated with enhanced NK cell dynamics and cytotoxicity via the cytokine mediated regulation Fas L pathway (Medvedev, Johnsen et al. 1997) (Szymanowski, Li et al. 2014).

In vertebrates with a healthy mature immune system, cells of the adaptive immune system, i.e., the T and B cells, mediate a memory response following re-exposure to a homologous antigen (Murphy, Travers et al. 2008). NK cells are very similar to T cells in their cytolytic activity. However, they have activating and inhibiting receptors. These receptors sense the presence or absence of MHC class 1 molecules on the surface of target cells. NK cells are very important in the host as they help control certain viral infections while the adaptive immune system is preparing to mount a response (Murphy and Weaver 2016). Following the recognition of target cells (absence of MHC class 1), activation of NK cells is followed by increased release of proinflammatory molecules like interferons and cytokines, which enhance the cells killing ability (Berrien-Elliott, Wagner et al. 2015).

NK cell activation can occur through one of two pathways, the indirect or the direct pathway. Most activation occurs through the indirect pathway that involves accessory cells. These macrophages, dendritic cells and few epithelial cells, produce cytokines and participate in cell contact dependent signals. The direct pathway is characterized by the direct binding of pathogen associated molecular patterns (PAMPs) to TLRs on the NK cells (Horowitz, Stegmann et al. 2011). During an immune response, TLRs and NK cells provide regulatory roles for each other by direct TLR activation on NK cells or via accessory cells leading to the production of IFNγ and activation of cytotoxicity (Hart, Athie-Morales et al. 2005) (Romee, Schneider et al. 2012).

1.2 NK cell receptor gene families in mammals

Mammalian NK cells have immunoglobulin (Ig) like receptors as well as lectin like receptors. These act as both activating and inhibitory receptors. The killer like

immunoglobulin receptors (KIR) are present in humans and the killer like lectin receptors (KLR) are present in mice. NK cells lack the ability to generate antigen-specific receptors by somatic recombination (Lanier 1998, Parham 2005, Vivier, Tomasello et al. 2008). Instead, they express a repertoire of cell surface receptors that control their activation, proliferation and effector functions (Lanier 2005). These receptors can be classified into two structural types depending on whether their extracellular ligand binding domains are C-type lectin domains or immunoglobulin (Ig) type domains. Gene families that encode the type II transmembrane (TM) receptors with C-type lectin domains are present in the chromosomal region known as the NK complex (NKC). Gene families that encode the type I TM receptors with Ig-like domains cluster in the leucocyte receptor complex (LRC) (Burshtyn and Long 1997, Sawicki, Dimasi et al. 2001, Yokoyama and Plougastel 2003). The NKC and LRC regions, which include both inhibitory and activating receptors, have been well described in rodents and primates. In mice and rats, the Ly49 genes of the NKC encode an entire family of C-type lectin receptors. In humans, the NKC encode the single Ly49 pseudogene. Human KIRs of the LRC functionally replace the rodent Ly49 genes and encode an entire family of Ig-like NK cell receptors. The rat LRC encodes a single KIR, and two murine KIR genes are not linked to the LRC (Nylenna, Naper et al. 2005, Parham 2005). These are the differences. The function, expression and diversity of human KIRs and mouse Ly49 family members are very similar (Kelley, Walter et al. 2005).

The KIR and Ly49 genes belong to small gene families of 10-15 closely linked genes that demonstrate genetic polymorphism. These genes encode inhibitory receptors that inhibit NK cell function. Inhibitory receptors have immune receptor tyrosine based

inhibition motif (ITIMs) in their cytoplasmic domains. Phosphorylation of the specific tyrosine motif in the ITIM involves activation and recruitment of phosphatases like *src* homology -1 (SHP-1), SHP-2 and SHIP for the delivery of inhibitory signal to the cell interior to silence NK cell mediated cytotoxicity. The KIR and Ly49 genes also encode activating receptors that possess a positively charged amino acid in their transmembrane region. This amino acid associates with immuno-receptor tyrosine based activation motif (ITAM) that contains adaptor proteins. This activates protein tyrosine kinases such as Syk/ZAP70 or PI3K that leads to target cell lysis. KIRs and mouse Ly49 are expressed primarily on NK cells, and interact with MHC-I molecules (Lanier, Corliss et al. 1998, Yokoyama and Plougastel 2003, Kelley and Trowsdale 2005, Lanier 2005, Barrow and Trowsdale 2006, Lanier 2008).

Other receptors that are common to both humans and mice include the C-type lectin, NKG2 and NKRP1 families and the activating NKG2D receptor. Single copy activating receptors such as NKp46 and NKp30 that recognize viral hemagglutinins are also present in both humans and mice (McQueen and Parham 2002, Litman, Yoder et al. 2003, Lanier 2008). The fact that various species use different genetic solutions to conserve NK cell receptor function through evolution indicates the importance of these receptor gene families and their functions. It has been hypothesized that NK cell receptor function is also conserved in zebrafish, where the NITR gene family, described later, may be involved in mediating NK cell receptor function.

MHC-I is a ubiquitous cell surface marker expressed on normal host cells. Inhibitory receptors on the NK cells recognize MHC-1 molecules as ‘self’ ligand and inhibit destruction of normal host cells. In contrast, transformed, infected or stressed cells

can lose expression of self-MHC class 1 molecules. If MHC class I is not present, the inhibitory KIR receptor is not activated and NK cell activation and target cell lysis is initiated. This phenomenon is known as missing-self recognition. Most tumor or virus infected cells down regulate MHC I as a means of evading cytolysis by CD8 T cells. But this escape mechanism makes these cells targets for NK cells (Lanier 2005). In some cases, untransformed or uninfected cells that do not express a full complement of MHC I molecules are susceptible to NK cell lysis. M157 is a protein encoded by MCMV and has a putative major histocompatibility complex class I (MHC I) structure (Tripathy, Smith et al. 2006) that is recognized by the murine Ly49H NK cell activation receptor.

1.3 NK cell receptors in zebrafish

Teleost possess receptors that are structurally homologous to the mammalian Ig-type and lectin-type receptors (Panagos, Dobrinski et al. 2006, Yang 2009). Immune related, lectin like receptors (*illrs*) and novel immune type receptors (NITRs) have been identified in zebrafish. These receptors are homologous to the immunoglobulin like receptor and the lectin like receptor and have an activating role. The NK cell receptors that recognize target cells and either induce killing or prevent killing have a C-type lectin like domain (CTLD). This domain has highly conserved cysteine residues that bind to carbohydrates or protein ligands, either Ca^{2+} dependently or independently (McQueen and Parham 2002). Studies on zebrafish illrs show a multi gene family of type-II immune related *illrs*. Their members contain activating and/or inhibiting signaling motifs similar to the mammalian type V NK activating or inhibitory receptors (Panagos, Dobrinski et al. 2006). *Illrs* include a type II transmembrane receptor with an extracellular CTLD and a coiled region. They are found in myeloid and lymphoid cells in the hematopoietic tissue

(Panagos, Dobrinski et al. 2006). *Illrs* play a role in triggered immune responses and specifically in NK cell immune functions during those responses in zebrafish. The zebrafish *illr* gene has two additional cysteines forming a characteristic extra disulphide bond specific to zebrafish NK cells receptors (Weis, Taylor et al. 1998). RT-PCR revealed isoforms of *illrs*. *Illr1* (inhibitory receptor), illr3 (activating receptor) and *illrL* (inhibitory receptor) were expressed in zebrafish kidney and spleen, but not in liver (Panagos, Dobrinski et al. 2006). Additionally, while *illr2* did not show any expression, *illrL* was expressed in intestine and myeloid cells. *Illr1* was expressed in lymphoid cells and *illr3* was expressed in both myeloid and lymphoid lineages (Panagos, Dobrinski et al. 2006). *Illr3* is a major NK cell receptor that plays a role in targeting cell for killing, similar to human NK cell cytotoxic function (Yang 2009). The zebrafish genes for group II CTLD receptors are *illrs* on NK like cells, and are similar to mammalian NK cell receptors (Panagos, Dobrinski et al. 2006).

The novel immune type receptors (NITRs) belong to the immunoglobulin super family (IgSF) and have structural similarities with mammalian NK cell receptors (KIRs) (van den Berg, Yoder et al. 2004). NITR cDNA was originally cloned from puffer fish (Rast, Haire et al. 1995). NITRs have been identified in 13 different species: zebrafish, rainbow trout, channel catfish, Japanese flounder, medaka, fugu and *Tetraodon nigroviridis* (Strong, Mueller et al. 1999, Hawke, Yoder et al. 2001, Yoder, Mueller et al. 2001, Yoder, Mueller et al. 2002, Yoder, Litman et al. 2004, Piyaviriyakul, Kondo et al. 2007, Yoder, Cannon et al. 2008, Yoder 2009). NITRs encode a type I transmembrane glycoprotein with an extracellular variable (V) and intermediate (I) domain, a transmembrane region and a cytoplasmic tail. Both ecto-domains may contain joining (J)

like sequences, even though there is no evidence of somatic recombination (Yoder, Litman et al. 2004, Yoder 2009). The NITR variable domains possess highly conserved residues that are also conserved in T cell receptors (TCR) and immunoglobulin (Ig) V domains. The V domains include three hypervariable regions that correspond to the complementarity determining regions in TCRs and Igs (Litman, Hawke et al. 2001, Yoder, Litman et al. 2004, Wei, Zhou et al. 2007). The NITR V domains are structurally similar to the V domain of antigen receptors (Yoder, Litman et al. 2004, Yoder 2009). The NITR I domain contains six conserved cysteine residues and many of the same conserved residues observed in the V domains of NITRs, TCRs and Igs (Giudicelli, Duroux et al. 2006). It has been hypothesized that NK cell receptor function is also conserved in zebrafish, where the NITR gene family may be involved in mediating NK cell receptor function. It is likely that NITRs represent either modern forms of progenitor genes or derived products of TCR and Ig loci of innate function.

The *NITRs* were identified in and are best characterized in zebrafish (Yoder, Mueller et al. 2001, Trede, Langenau et al. 2004). An extracellular immunoglobulin like V domain, a transmembrane region and a cytoplasmic tail containing an immune receptor tyrosine base motif structurally characterize them. Twelve different NITR gene families (*nitr1 – nitr12*) encode approximately 36 different zebrafish NITR genes. These genes are located within the NITR gene cluster on chromosome 7. Chromosome 7 may be derived from the ancient chromosome that gave rise to the human chromosome that encodes the LRC region 19q (Yoder, Litman et al. 2004). Additionally, nitr13 and nitr14, located on chromosome 14 have also been described. Observations and results from previous studies on NITR gene families have led to a model suggesting that NITRs are

functionally linked to human KIRs. NITRs and KIRs are encoded by rapidly evolving multigene families that include both inhibitory and activating forms. Like mammalian NKRs, NITRs can be classified into two primary functional groups: 1) inhibitory and activating. Inhibitory receptors have a cytoplasmic ITIL or ITIM like sequences. Activating receptors are based on the presence of a positively charged residue (ITAM like) within the transmembrane domain (Yoder, Mueller et al. 2001). Similar to mammalian NKRs, fish NITRs have more inhibitory functions than activating functions. There are 12 distinct NITR families. *Nitr5* and *nitr9* are the only receptors that play activating roles (Wei, Zhou et al. 2007, Yoder 2009). The remaining *nitrs* are inhibitory. 1 out of 39 NITRs in zebrafish have activating potential. The remaining are either inhibiting or a combination of both. A quantitative estimation of NITRs on a single cell is unclear. It is hypothesized that the higher number of inhibitory receptors may contribute to a functional redundancy ensuring that activating pathways are normally suppressed. It is not known if NITRs are ligand specific. However, due to the high level of diversity within each NITR family, NITRs could recognize a wide range of ligands. It has been postulated that similar to mammalian NK cell receptors, inhibiting NITRs may recognize viral and stress related proteins and/or MHC-1 molecules. Channel catfish express IpNITR11 (activating receptor) on a clonal cytotoxic NK-like cell line. IpNITR11 binds to a ligand on the surface of a non-self allogeneic target B cell line. Like the polygenic and polymorphic nature of the KIR loci, the NITR genes show high interindividual heterozygosity and are mostly expressed in the lymphoid cell population in zebrafish. *Nitr14* is expressed in lymphoid and myeloid cells (Yoder, Turner et al. 2010).

Of the several inhibitory NITRs, the *nitr3* gene family is interesting since it is expressed during embryogenesis and in mature lymphocytes (Yoder, Turner et al. 2010, Moore, Garcia et al. 2016). In addition, *in vitro* studies have demonstrated that cross-linking of *nitr3* in transfected human NK92 cells (a NK cell line), leads to down-regulation of the MAPK pathway, when incubated with Raji tumor cells (Yoder, Mueller et al. 2001). This inhibition is regulated at the level of the ITIM, indicating that inhibitory NITRs may utilize similar intracellular signaling pathways as mammalian inhibitory NKRs in zebrafish (Yoder, Mueller et al. 2001). Both activating NITRs and KIRs utilize Dap12 as an adaptor protein for signaling. The only inconsistency to this model is that the extracellular domains of NITRs contain (V) and (I) domains whereas KIRs contain C2 type domains. Nevertheless, NITRs and KIRs possess similar signaling domains so that the receptors can couple with appropriate signaling adaptors (DAP10 or DAP12) (Kelley, Walter et al. 2005).

Nitr9 is the only zebrafish gene that encodes an activating receptor. It contains a positively charged amino acid, arginine, in its transmembrane region. The nitr9 gene encodes three splice variants, which encode three distinctly different receptor isoforms: Nitr9-long (Nitr9-L), Nitr9-short (Nitr9-S) and Nitr9-supershort (Nitr9-SS). All three splice variants possess identical trans membrane and cytoplasmic domains as well as an extracellular Ig intermediate (I) domain. Nitr9-L transcripts possess an additional exon encoding an extracellular N-terminal Ig variable (V) domain whereas nitr9-S transcripts include only part of this exon. The n*itr9-SS* gene encodes only the (I) ecto-domain as the entire V domain is eliminated due to internal splicing (Yoder, Nielsen et al. 2002, Kärre 2008). Similar to human activating KIRs, zebrafish Nitr9 shares a similar down-stream

signaling pathway. RT-PCR analysis indicated that both zebrafish Nitr9 and the adaptor protein, DAP12 are expressed in lymphocytes.

The identification, purification and characterization of different blood cell lineages in humans and mice have become possible with the abundance of commercial monoclonal antibodies available. However, the availability of monoclonal antibodies against blood cells of zebrafish is limited making it difficult to isolate and generate cell lines. Generation of zebrafish monoclonal antibodies is a challenging process possibly due to the different glycosylation patterns that are recognized as foreign by the rodent immune system (Traver, Paw et al. 2003), which has led to a paucity of antibodies to study zebrafish blood cells (Yoder 2008). In order to identify NK cells and elucidate their role in fish immune responses, Shah et al 2012 identified a cell surface marker (Nitr9) expressed on teleost NK cells and then generated antibodies against it (Shah 2009, Shah, Rodriguez-Nunez et al. 2012). They developed and characterized mouse anti-nitr9 monoclonal antibody (IgG isotype) that is raised against the zebrafish NK cell putative receptor Nitr9 (Shah, Rodriguez-Nunez et al. 2012). 293T cells were transfected with plasmids encoding FLAG-tagged Nitr9 isoforms and EGFP. The results generated from their study suggest the labeling of FLAG tagged Nitr9 proteins with anti-FLAG mAb, the 90.10.5 anti-Nitr9 mAb and the 19.1.1 anti-Nitr9 mAb. After FACS analysis in zebrafish, no antibody labeling was observed. For this reason, the above study could not justify the application of the anti-Nitr9 antibody to purify and characterize zebrafish NK cells, suggesting that the anti-Nitr9 antibody was not working in zebrafish or it needed further characterization and optimization. Based on its predicted protein structure, Shah et al

hypothesized that Nitr9 is an activating transmembrane receptor and is expressed on NK cells or cytotoxic T cells.

Similar to activating receptors previously described in human NK cells, antibody cross linking *Nitr9* functions via the Dap 12 intracellular adaptor molecule (Wei, Zhou et al. 2007) and results in increased phosphorylation of AKT and ERK. AKT is downstream of PI3K, thereby indicating the activation of the PI3K-AKT-ERK pathway. Mutation of the ITAM present in the cytoplasmic tail of Dap12 fails to activate this pathway, indicating that this pathway is dependent on the presence of the ITAM (Barclay 1999, Hawke, Yoder et al. 2001, Yoder, Mueller et al. 2001, van den Berg, Yoder et al. 2004, Yoder, Litman et al. 2004, Wei, Zhou et al. 2007). These observations led to the hypothesis that Nitr9 is expressed on NK cells or cytotoxic T cells and can activate similar downstream signaling pathways as mammalian activating NKRs. Thus, it would follow that Nitr9 provides cytotoxic function, killing virally infected and tumor cells as is the case with activating mammalian NKRs.

1.4 Toll Like Receptors (TLRs) in mammals

Pathogen Associated Molecular Patterns (PAMPs) are specific conserved structural motifs on microbial pathogens. They play vital functional roles and are essential for the survival and proliferation of the pathogen. Key players in the immune responses are special extracellular or intracellular receptors known as Pattern Recognition Receptors (PRRs) that bind to PAMPs (Goldstein 2004). There are several classes of PRRs: RIG-1 like receptors (RLRs), NOD-like receptors (NLRs), DNA receptors (cytosolic sensors for DNA) and Toll Like Receptors (TLRs). The receptors that recognize the most diverse array of PAMPs are the TLRs. TLRs are present on innate

immune cells like antigen presenting cells (APCs) and NK cells (Lauzon, Mian et al. 2007). These type I transmembrane receptors activate a TLR signal transduction event via a MyD88 dependent or independent pathway (Akira and Takeda 2004). The toll gene was first identified in *Drosophila melanogaster* (fruit fly) and was responsible for embryonic development for dorso-ventral patterning embryo (Belvin and Anderson 1996). Up-regulation of Toll genes induce antimicrobial peptide secretion and defended against gram-positive bacteria and fungal pathogens (Netea, Van der Meer et al. 2007). In *Drosophila* larvae, Toll signaling is required for melanization (Belvin and Anderson 1996). Receptors analogous to the toll receptor have been found in both vertebrates and invertebrates and represent an ancient and evolutionarily conserved host defense mechanism. Nine Toll related receptors have been identified in the *Drosophila* genome. *Toll*, or *Toll1* was the first *Toll* identified and is responsible for antimicrobial protein (AMP) induction via *Toll* pathway (Belvin and Anderson 1996). The *Drosophila* Toll 9 has only one cysteine residue between the leucine rich repeat and the transmembrane domain, a structure very similar to the human TLRs.

Similarities to the *Toll* receptor have been shown to a lesser degree in proteins encoded by plant resistance (R) genes as the N gene in *Nicotiana glutinosa.* Vertebrate TLRs arose from an ancient gene duplication that has given rise to two large gene families of TLRs. This observation was based on the calculation of phylogenetic reconstructions of the TLRs from both full-length gene and amino acid sequence. Subsequent evolution of the two large families has resulted from a complex interaction between gene duplication, gene conversion and co-evolution. PAMP recognition specificities in vertebrates are determined by the N terminal extracellular leucine rich

repeats (LRR) domain. Signal transduction takes place through a Toll/interleukin 1 receptor (TIR) domain (Baratin, Roetynck et al. 2005).

Toll like receptors are structurally characterized by an extracellular domain containing leucine rich repeats (LRR) and a cytoplasmic tail having conserved regions called toll/IL-1 receptor (Baratin, Roetynck et al. 2005) domains (Jault, Pichon et al. 2004). It is a type I transmembrane receptor. Each TLR can recognize and bind to a specific PAMP. So far, 10 human TLRs (Takeda, Kaisho et al. 2003), 12 murine TLRs and 22 zebrafish TLRs have been characterized (Meijer, Gabby Krens et al. 2004). TLRs are found on antigen presenting cells (macrophages, dendritic cells and B-cells), NK cells and certain epithelial cells. When TLRs bind to a specific ligand, it drives an intracellular signal transduction cascade of events that ultimately leads to activation of transcription factors including (but not limited to) AP-1 (Hu, Paik et al. 2006), NF-κB (Frantz, Kelly et al. 2001, Kawai and Akira 2006) and IRF3 (Doyle, Vaidya et al. 2002, Fitzgerald, Rowe et al. 2003, Moynagh 2005). The activated transcription factors present in the cytoplasm translocate into the nucleus to induce transcription of pro-inflammatory cytokines, interferons and a variety of cellular responses and effector cytokines that eventually direct an enhanced immune response. In mammals all TLRs except TLR3 can mediate an MyD88 dependent or MyD88 independent adaptor signaling pathway (Kawai and Akira 2007). Mammalian TLR3 is the only TLR that cannot induce a MyD88 pathway.

Drosophila Toll 9 is the only member of the toll family that groups with the vertebrate *Toll*. Following phylogenetic analysis of Toll/IL-1 (TIR) domains of the TLRs together with their adaptor molecules, it suggests that the adaptor molecule usages and signaling pathways are ancestral characteristics of TLRs. Further, phylogenetic analyses

indicated an independent evolutionary origin for the MyD88-dependent and MyD88 independent pathways. Roach et al 2013, reported a common ancestor for vertebrate and invertebrate MyD88, suggesting a very ancient origin for the MyD88 dependent pathway (Roach, Racioppi et al. 2013). This work reported the importance of the adaptor molecules in TLRs to mediate an immune response and how these adaptor molecules are evolutionary conserved.

1.5 Specific binding of TLRs to their ligands

TLR2/TLR1 or TLR2/TLR6 recognize lipoteichoic acid, a component of gram-positive bacterial membranes and di and tri acyl lipoproteins. The 2 chains of the PAMP bind onto the convex of LRR of TLR2 and the third chain binds to the convex of LRR of TLR1 dimerizing the two TLRs and bring their TIR cytoplasmic domains in close proximity to each other in the cytoplasm. A similar interaction occurs for TLR2/TLR6 that binds to long chain fatty acids and a cell wall component of yeast (B glucan). Binding to TLR2/TLR6 requires the help of a scavenger receptor CD36 (88KD glycoprotein) for diacylglyceride recognition or a co-receptor Dectin-1 for binding B glucan (Hoebe, Georgel et al. 2005, Stewart, Stuart et al. 2010). Both cooperate with TLR2 in binding the ligand. CD36 is required by B cells and is expressed by myeloid cells, platelets, endothelial cells, erythroid precursors and adipocytes (Hoebe, Georgel et al. 2005). TLR2 and TLR4 require a second adaptor molecule called TIRAP in the MyD88 pathway.

TLR 3 binds to dsRNA in virus (rotavirus). The mammalian RNA is not dsRNA and so the TLRs can distinguish the dsRNA and bind to it. The TLRs that recognize nucleic acid are located in the endosomal membrane and are transported via the

endoplasmic reticulum. The dsRNA is internalized either by endocytosis of virus or by phagocytosis of dying cells in which virus are replicating. The ectodomain of TLR 3 binds the dsRNA in two contact sites: one the amino terminal and the other the carboxy end. Because of the 2-dimensional nature of the ds-RNA, it can bind to two TLR3 molecules and form a homodimer. TLR4 recognizes lipopolysaccharide (LPS) a glycolipid in the cell wall of gram-negative bacteria.

TLR7, 8 and 9 are intracellular TLRs in the endosomal membranes. TLR 7 and 8 (in mice) recognize and bind to ssRNA (flavivirus and rabies). This is the RNA in the endosomal compartment and not the host ssRNA. The ssRNA of healthy host cells is normally confined to the nucleus and cytoplasm and is not present in the endosomes. When extracellular viruses are endocytosed by macrophages and plasmacytoid DC, they are uncoated in the acidic environment of the endosomes and lysosomes and the ssRNA is presented to TLR7/8. Normally, extracellular RNase releases and degrades the ssRNA that is released from injured tissues. In the human genome, TLR 7 and TLR 8 are on the X chromosome. TLR 3, 7, 8 and 9 are transported to the endosome from the endoplasmic reticulum by a specific protein UNC93B1.

1.6 TLRs in zebrafish

TLR ligands function as immunostimulants and are promising tools to enhance animal production in intensive aquaculture. They boost the natural defenses already present, increase survival rates and can also enhance protection offered by vaccination. TLR ligands have been shown to activate both specific and non-specific immune responses (Sakai 1999). The observed induction of zTLR1, zTLR2 and zTLR18 (zTLR1 and zTLR18 are homologous to human TLR1) in response to *Mycobacterium marinum*

suggest that the recognition mechanisms of microbial patterns were already established in the common vertebrate ancestor and that the functions of receptors in the TLR1 and TLR2 groups may be conserved between mammals and teleost (zebrafish). Microbial infection also induced the expression of zTLR20a and zTLR22 genes, suggesting that receptors of the fish specific TLR cluster may also function in microbial infections. Meijer et al 2004, have shown that the zebrafish genome contains at least 17 expressed TLR genes as well as expressed homologues of IL-1R, IL-18R and four different TIR domain adaptors genes. Previously it was suggested that early vertebrates contained few TLRs and that the TLR4 gene, which is the LPS sensor in mammals, might have emerged as a distinct entity only 180 million years ago (Du, Poltorak et al. 1999). Further, a recent analysis of the TLR family in the genome of pufferfish, *F. rubripes*, suggested that TLR4 is absent from the fish lineage. These hypotheses are contradicted by data showing that zebrafish contains 2 sets of TLR4 genes in tandem duplication (Meijer, Gabby Krens et al. 2004). The full-length gene corresponding to one of the zTLR4 copies was cloned, and this demonstrated that zTLR4 and hTLR4 display conserved intro-exon structure. These findings supported evolutionary calculations indicating that TLR4 diverged more than 400 million years ago. The zebrafish genome also contains a conserved homologue of TLR2, which like its human counterpart is encoded by a single exon. The phylogeny reconstruction indicates the presence of a fish-specific TLR cluster, consisting of zTLR19, zTLR20a/b, zTLR21 and zTLR22 and their homologues in *Fugu*. This cluster is closely related to the *Drosophila Toll-9* gene, which is the only member of the *Drosophila* Toll family that groups with vertebrate TLRs. More recently, TLRs have been identified in NK cells. TLRs can activate NK cells either directly or indirectly

(Adib-Conquy, Scott-Algara et al. 2014). Direct activation is by NK cell specific receptors. Indirect activation is by TLR-ligand association on NK cells or by accessory cells like dendritic cells, macrophages or by accessory cytokines like IL-2, IL-12, IL-15 and IL-18.

1.7 Background

In *rag2*$^{-/-}$ mutant murine models, adoptive transfer of previously exposed NK cells resulted in an adaptive immune like response coupled with a memory response to chemical haptens 2.4 dinitroflurobenzene (DNFB) and oxazolone (OXA) (O'Leary, Goodarzi et al. 2006). Adoptive transfer of NK cells in the same model resulted in protection to re-infection with murine cytomegalovirus (MCMV) encoded m157 protein (Sun, Beilke et al. 2009). M157 is a protein encoded by MCMV and has a putative major histocompatibility complex class I (MHC I) structure (Tripathy, Smith et al. 2006) that is recognized by the murine Ly49H NK cell activation receptor. *Rag2*$^{-/-}$ mutant mice showed that previously exposed NK cells follow 4 phases of adaptive immune cells i.e., expansion, proliferation, contraction and memory cell formation, followed by the expansion phase on re-exposure to MCMV (Sun, Beilke et al. 2009). Protective secondary responses against bacteria were observed in *rag1*$^{-/-}$ mutant zebrafish (Hohn and Petrie-Hanson 2012). Adoptive transfer of kidney leukocytes isolated from vaccinated *rag1*$^{-/-}$ mutant zebrafish provided protection for naïve mutants when they were exposed to bacteria. The mechanism mediating the memory response has not been determined. The murine NK cell mediated adaptive/memory immune response has been reported against virus (Sun, Beilke et al. 2009), but how zebrafish NK cells mediate a similar response to intracellular bacteria, such as *Edwardsiella ictaluri* is not clear.

The innate immune system in zebrafish is like the mammalian innate immune system and this makes it a powerful model to study the function of immune cells and immune related genes. In the case of a wild type (healthy subject) with fully functional T and B cells, the NK cell memory response would act synergistically with adaptive immunity. It is difficult to dissect the role of NK cells in a protective immune response in the wild type model. In comparison to wild types, the *rag1*$^{-/-}$ mutant zebrafish have a similar phagocytic (monocytes/macrophage) population, higher numbers of granulocytes (neutrophils), a higher number of myelomonocyte cells and lymphocyte like cells (Petrie-Hanson, Hohn et al. 2009). The lymphocyte like cell population is comprised of NK cells and Nonspecific cytotoxic cells (NCCs) (Petrie-Hanson, Hohn et al. 2009). Fish kidney (mammalian bone marrow equivalent) is where antigen phagocytosis and processing occurs. Like the spleen, the fish kidney houses plasmacytes, lymphocytes (T cells, B cells and NK cells), monocytes, granulocytes and natural cytotoxic cells (Fischer, Koppang et al. 2013).

A better understanding of the basis of protective immunity in T and B cell deficient zebrafish could be possible by carrying out adoptive transfers of sensitized NK cells. But this process is not feasible because in-spite of the vast resources available to study various aspects of the immune system in a variety of model organisms, zebrafish research is still lacking the tools to do basic immunology research. Availability of a pure NK cell population would help us define the phases of the memory response following reinfection with intracellular bacteria. Anti-Nitr9 antibody is available to detect zebrafish NK cells. However, the application of this monoclonal antibody has not been extensively explored in zebrafish due to its inconsistent positive cell labeling. It is critical to

characterize specific zebrafish NK cell markers to study the detailed functions of NK cells and to understand how these cells mediate a secondary memory response.

1.8 Objectives

1. To determine differential expressions of immune cell genes *ifnγ, mx, tnfa, t-bet* and *nitr9* following targeted TLR stimulation in *rag1*$^{-/-}$ mutant zebrafish tissues.
2. To determine differential expression of NK cell genes *ifnγ, t-bet, nitr9, NK-lysin (nkl)a, nklb, nklc* and *nkld* in previously exposed and then challenged (4 weeks later) *rag1*$^{-/-}$ mutant zebrafish tissues.
3. To administer R848 and VTX at two doses to further define differential expression of NK cell specific marker genes *ifnγ, t-bet, nitr9, nkla, nklb, nklc* and *nkld* in *rag1*$^{-/-}$ mutant zebrafish, and nitr9 expression
4. To characterize the morphology of zebrafish NK cells.
5. To develop transgenic zebrafish models that will enable visualization of NK cell development and functions.

The main objectives of this project were designed to identify and describe zebrafish NK cells. Immunological reagents or tools were not commercially available, so indirect methods had to be used. The first objective exposed zebrafish to TLR ligands and evaluate gene expression to determine the ligand to which NK cells and myelomacrophages are stimulated. These gene expressions were quantified in liver, kidney and spleen tissues. These findings gave us an initial understanding of the NK cell populations in different tissues. The TLR ligands we used were β-glucan, Poly I:C and R848. β glucan binds to TLR2, Poly I:C binds to TLR3 and R848 binds to TLR 7/8. We

used *ifnγ* expression as an indicator of NK cell activation, *tnfα* expression as an indicator of macrophage activation, and *mx (mxa)* expression as an indicator of *ifnα* and *ifnβ* production (Haller, Staeheli et al. 2007). *Nitr9* is an NK cell receptor, so *nitr9* expression indicates NK cell activation. *T-bet* is a T cell and NK cell transcription factor, so increased *t-bet* expression in *rag1*$^{-/-}$mutant zebrafish (which lack T cells) indicates NK cell development.

The second objective of our study was to determine the NK cell specific gene expression associated with the protective/memory to bacteria (*E. ictaluri*) infection. We injected *rag1*$^{-/-}$ mutant zebrafish with a combination of live attenuated *E. ictaluri* vaccine-RE33 or TLR ligands β-glucan, Poly I:C and R848 or a combination of RE33 and TLR ligands. After 4 weeks, the fish were challenged with a virulent wild type *E. ictaluri* strain and protection evaluated. NK cell activation by gene expression of NK cell specific markers *nitr9*, *t-bet*, *nkla*, *nklb*, *nklc* and *nkld* was determined in tissues.

The third objective of our study involved the administration of two NK cell stimulators: R848 and VTX, to elucidate NK cell activation by gene expression of NK cell specific markers *nitr9*, *t-bet*, *nkla*, *nklb*, *nklc* and *nkld* and protein expression of Nitr9 in liver, kidney and spleen tissues. Protein expression of Nitr9 in liver, kidney and spleen was correlated to gene expression. Flow cytometric analyses determined tissue locations of Nitr9^{+} cells and the cellular location of the Nitr9 receptor.

The fourth objective used isolated kidney leukocytes for flow cytometry analyses. Monoclonal antibodies for Nitr9, non-specific cytotoxic cell receptor protein -1 (Nccrp-1) and macrophage protein expressing gene (Mpeg) were used. Cytospins were also

prepared from kidney, liver and spleen leukocytes. The cells were stained, viewed and photographed.

The fifth objective of this study was to develop transgenic and knock zebrafish models to be able to study the function of NK cells. The results from this study reveal the role of NK cells in teleost innate immune responses.

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CHAPTER II

THE CHARACTERIZATION OF LYMPHOCYTE-LIKE CELL POPULATIONS IN *RAG1*$^{-/-}$ MUTANT ZEBRAFISH

Abstract

This study morphologically characterized lymphocyte-like cells (LLCs) with mammalian NK cell morphology from liver, kidney and spleen tissues of *rag1*$^{-/-}$ mutant zebrafish. The LLC population included Natural Killer (NK) cells and non-specific cytotoxic cells (NCCs). The LLC populations could be discriminated by size, and further by the presence of cytoplasmic granules. These cell populations were also discriminated by flow cytometric side scatter (SSC) and forward scatter (FSC) characteristics. The monoclonal antibodies anti-NITR9, anti-NCCRP-1 (5C6) and polyclonal antibody anti-MPEG-1 were also used to discriminate and evaluate these cell populations by flow cytometry.

2.1 Introduction

The zebrafish model is well established for studying vertebrate hematopoiesis and immune cell interactions (Trede, Langenau et al. 2004). NK cells possess the most primitive and advanced biochemical and physiological process of cellular cytotoxicity of tumor and transformed cells. NK cells from other species appear to share some of these target specificities, but NK cells in fish are not well described.

Non-specific cytotoxic cells (NCCs) were first described in catfish (Evans, Carlson et al. 1984). The cells were described as monocyte like cells with a reniform nucleus (with peripheral chromatin), non-phagocytic, prominent Golgi apparatus, low nucleus to cytoplasm ratio and no NK cell like cytoplasmic granules but contain osmiophilic granules in the cytoplasm. NCCs have been well characterized in fish. NCCs are morphologically variable ranging from agranular small monocyte-like cells in catfish to a mix of lymphocytes, acidophilic granulocytes and monocyte-macrophages in seabream (Evans, Carlson et al. 1984), (Cuesta, Esteban et al. 2002).

NCCs form close contact with the target without fusion or fragmentation. They form a bond with the target cell by extending their moderate surface filament-like surface projections to the target cell membranes. NCCs account for 5-7% of leukocyte population in the teleosts. Nonspecific cytotoxic cells (NCC) (Evans, Jaso-Friedmann et al. 1988, Evans, Leary et al. 2001) are found in the anterior kidney, liver, spleen, and peripheral blood in teleosts including channel catfish (Evans and Jaso-Friedmann 1992), tilapia (Jaso-Friedmann, Ruiz et al. 2000) and carp (Markkula, Salo et al. 2005), (Salo, Jokinen et al. 2000), (Jokinen, Salo et al. 2000). Though NK cells and NCCs are known to kill the same type of target cells (reviewed by (Ristow, Evans et al. 2000), the cytotoxic effect of NCCs is not as robust as NK cells because of NCCs incapability to recycle or the long lag period they require to replenish the cytotoxic capability following prior contact with target cell (Graves, Evans et al. 1984). Further, large numbers of NCCs are required to kill an individual transformed target cell. NK cells and NCCs also differ based on density, killing kinetics, target antigen specificities and radiation sensitivity (Mazia, Schatten et al. 1975). NCCs were considered to be a possible phylogenic precursor of NK

cell (Evans, Hogan et al. 1984) because of their physiological characteristics during contact with target cell. NCCs display characteristics such as margination of nuclear chromatin, polarization of the golgi toward the target cell contact area and movement of the bulk of cytoplasm at the concave aspects of the nucleus towards the target cell contact area. NCCs also possess anti-parasitic cellular immunity functions (Graves, Evans et al. 1985). Moss et al describe NCCs to have a smooth ruffled outer membrane surface and containing small cytoplasmic granules (Moss, Monette et al. 2009).

Monoclonal antibodies anti- NITR9 (non-specific immune type receptor 9), anti-NCCRP1 (non-specific cytotoxic cell receptor protein-1), anti-MPEG-1 (macrophage protein expressing gene) and anti-IgZ (polyclonal antibody) can be used to label zebrafish NK cells, NCCs, macrophages and IgZ^+ cells, respectively. Anti-NITR9 recognizes novel immune type receptor 9 (NITR9) that is believed to be present on zebrafish NK cells. NITR9 is the homologue of human killer like immunoglobulin (KIR) and mouse Ly49 activating inhibitors on NK cells. Novel immune type receptors (NITRs) are unique to multiple bony fish species including zebrafish. They are encoded by large multi gene families and share structural and signaling similarities to mammalian natural killer receptors Ly49 in mice (Ortaldo and Young 2005, Mason, Willette-Brown et al. 2006) and KIRs (Farag and Caligiuri 2006) in humans. NITRs can be classified as: inhibitory, activating and functionally ambiguous based on peptide signaling motifs (Yoder 2009). Bony fish possess cytotoxic NK-like cells ($TCR\alpha^-\beta^-\gamma^-IgM^-$) as well as cytotoxic T cells ($TCR\alpha^+\beta^+IgM^-$) (Shen, Stuge et al. 2002) and inhibitory and activating NITRs may play significant roles in the functional regulation of both of these lymphocyte populations. NITR expression is observed in spleen, kidney and intestine, and is more abundant than

in muscle and liver (Strong, Mueller et al. 1999, Hawke, Yoder et al. 2001, Yoder, Mueller et al. 2002, Evenhuis, Bengtén et al. 2007). There are 14 families of NITRs that are expressed in hematopoietic kidney, spleen and intestine. NITR9 functions as either an activating or inhibitory receptor. NITR9 structurally consists of an extracellular Ig-type domain, transmembrane region, an activating region that possesses a positively charged amino acid and a cytoplasmic tail, or an inhibitory receptor that possesses 1 or 2 ITIMs or ITIM-like sequences (Yoder 2009). The activating receptor associates with ITAM containing adaptor protein, zebrafish Dap12, to initiate downstream signaling (Wei, Zhou et al. 2007, Yoder, Cannon et al. 2008). The inhibitory receptor mediates ITIM dependent inhibition of MAPK activation by activating phosphatases which dephosphorylate any active MAPKs (Wei, Zhou et al. 2007).

Anti-NCCRP-1 labels the non-specific cytotoxic cell receptor protein on non-specific cytotoxic cells (NCCs). This receptor was isolated and characterized in channel catfish, tilapia and zebrafish (Jaso-Friedmann, Leary et al. 1997). NCCRP-1 is a membrane protein on NCCs and binds to antigens present on target cells or to cognate synthetic peptides. This binding initiates a biochemical signaling response leading to increased cytotoxicity. NCCRP-1 is a member of the lectin-type subfamily of F box proteins. It is proline rich (9%), has two glycosylation sites and 18% of all amino acids are potential phosphorylation sites (serine, threonine, tyrosine). The NCCRP-1 proteins have no signal peptides or transmembrane domain (Kallio, Tolvanen et al. 2011). Structurally, NCCRP-1 has one extracellular proline-rich domain, a transmembrane portion of 15-18 aa and a cytoplasmic tail composed of a high frequency of phosphorylation sites. In teleost, NCCRP-1 is mainly responsible for innate immune

responses (Evans, Kaur et al. 2005), target cell recognition and cytolytic function of NCCs (Moss, Monette et al. 2009). Reports suggest that the zebrafish NCCRP-1 protein may function as an antigen recognition molecule and, as such, may participate in innate immunity in teleost. NCCs are a lymphocyte like population and are specific to teleost (Jaso-Friedmann, Peterson et al. 2002). The anti-NCCRP-1 antibody (5C6) is mouse monoclonal antibody that is specific for teleost NCCRP-1(Evans, Jaso-Friedmann et al. 1988).

Anti-mpeg-1 binds to the protein encoded by the macrophage protein expressing gene (mpeg) in zebrafish macrophages (Ellett, Pase et al. 2011). Mpeg is expressed in macrophage-lineage cells. The purpose of this study was to isolate and describe the cellular morphology of LLCs in the kidney, liver and spleen tissues of r*ag1*$^{-/-}$ mutant zebrafish. These cells were also evaluated for the presence of NCCRP-1, NITR9, MPEG-1 and IgZ using flow cytometry.

2.2 Materials and methods

2.2.1 Zebrafish care

All zebrafish used in this study were bred from a homozygous colony of *rag1*$^{-/-}$ mutant zebrafish previously established in the specific pathogen free hatchery in the College of Veterinary Medicine, Mississippi State University (MSU) (Petrie-Hanson, Hohn et al. 2009). The MSU Institutional Animal Care and Use Committee (IACUC) approved the propagation and experimental protocols. Both adult males and females of 6 months age and weighing on an average 0.5g, were used in this study.

2.2.2 Flow cytometry

2.2.2.1 Cell isolations and cytospins

Liver, kidney and spleen tissues were removed from 3 *rag1*$^{-/-}$ mutant zebrafish and individually weighed. Each tissue type from 3 fish were pooled and comprised one replicate. The weight of each tissue was recorded. Tissues were collected in cold FACS buffer (2% BSA in Hank's buffer) and disrupted on ice with a teflon homogenizer. The homogenate was passed through a sterile 40µm nylon cell strainer to make single cell suspensions. The liver sample was passed through the cell strainer twice to remove cell clumps. Filtered cells were placed on a histopaque 1119 gradient (Sigma –Aldrich) and the cells at the gradient interface layer were collected. This suspension was centrifuged at 400 x g for 20 minutes, and the supernatant decanted off. Pellets from the kidney and liver were resuspended in 2.5 ml and the spleen pellet was resuspended in 1 ml of cold Hank's buffer without Ca^{2+} and Mg^{2+}. Cells were counted with a BioRad TC20™ Automated Cell Counter and viability was assessed by Trypan blue (Invitrogen) exclusion.

The cytospin cartridges were filled with 400µl of cell suspension containing 10^4 to 10^6 cells/ml and centrifuged in a Cyto-tech® centrifuge at 500 x g for 1 minute. The cytospin slides were air dried for 20 minutes and stained with Wrights-Giemsa stain (Fisher Scientific Company) following the manufacturer's instructions. Slides were viewed on an Olympus BX43 at 1000x magnification. Differential leukocyte counts were performed in each tissue. Cell classifications were based on morphology. Lymphocyte-like cells (LLCs) were classified as small, large, and agranular or granular. Briefly, each cell preparation was transferred into 3 mL tube containing 2 mL cold Hank's buffer

without Ca^{2+} and Mg^{2+} with 1% fetal bovine serum, Sigma-Aldrich, St. Louis, MI. Zombie green cell viability dye (Biolegend) was used to determine cell survival. Cells were kept on ice until analyzed by forward scatter and side scatter on a FACS Calibur flow cytometer (Becton Dickinson). One hundred thousand events were analyzed per tissue. Forward scatter (FSC) represents cell diameter. The running parameters were amp gain 3.0 and the threshold 80. Side scatter (SSC) represents cell granularity or complexity. The amp gain was 1.0 and the threshold was 80.

2.2.2.2 Preparing, staining and fixing cells for viability assay

Rag1$^{-/-}$ mutant zebrafish (n=15) kidney cells were used in this experiment. Kidney cells were sampled and pooled in HBSS w/o Ca^{2+}- Mg^{2+} or HBSS w/ Ca^{2+}- Mg^{2+} or RPMI w/ 5% channel catfish serum. The steps were as follows: (1) directly from fish; (2) 1st rinse before gradient; (3) after gradient; (4) post 1h incubation, rinsed; (5) post 30 min incubation, not rinsed; (6) post 30 min, rinsed. During each step cells were stained with a zombie green viability dye (BioLegend) and immediately fixed in 4% paraformaldehyde and kept at 4C in dark. The positive control for this experiment was cells treated with 0.01% triton X 100, stained with viability dye and fixed. The cells were stained after separation on the gradient. Negative control was a “cells only” tube i.e., cells not stained with the viability dye zombie green but fixed. This experiment was done in triplicate.

2.2.2.3 Flow cytometry

Kidney cells were sampled from *rag1*$^{-/-}$ mutant zebrafish. Tissues were macerated and strained through a 40micron filter in HBSS buffer w/o $Ca2^{+}$ and $Mg2^{+}$ (Sigma, H4891-10x1L). Cells were washed and separated on a histopaque 1119 gradient (11191).

Buffy coat layer was aspirated and washed, fixed with 4% Paraformaldehyde and permeablized in BD Perm/Wash buffer (BD, 51-2091KZ). We analyzed two non-commercial/in-house monoclonal antibodies (mAb): anti-NCCRP-1 (5C6) and anti-NITR9, as well as a commercial anti-NCCRP-1 (5C6) mAb. Cells were incubated with primary antibodies: IgG mouse anti-NITR9$^{90.10.1}$ monoclonal antibody (gift from Dr. Jeffrey Yoder), anti-MPEG polyclonal antibody (Anaspec), anti-NCCRP-1 monoclonal antibody (gift from Dr. Jaso-Friedmann) and anti-IgZ polyclonal antibody 9 (Anaspec) for 1 hour at 4°C. This was followed by incubation with secondary antibodies: goat anti-mouse IgG-PE (Biolegend), goat anti-rabbit IgG-FITC (Thermo fisher), goat anti-mouse IgM-APC, goat anti-rabbit IgG-FITC (Thermo fisher) respectively, for 30 minutes at 4°C. Cells were also incubated with monoclonal antibody: Rat IgG2b (commercial 5C6/NCCRP-1) conjugated to FITC. Cells were washed after each step twice with 1ml FACS buffer at 500 x g. Labelled cells were analysed on the FACS NovoCyte with NovoExpress software.

2.2.2.4 Cellular location

We developed a working protocol for intracellular staining of anti-NITR9 mAb (a gift from Dr. Jeffrey Yoder, NCSU) using the *rag1*$^{-/-}$ mutant zebrafish model. Cells (from kidney or liver) were fixed in 4% paraformaldehyde and incubated with commercial 5C6 (rat anti-mouse CD11b FITC) (Imgenex), in-house anti-NCCRP1 (5C6) or in-house anti-NITR9 monoclonal antibodies in 1X permeation buffer (BD Bioscience) for 1h, washed and incubated for 30 min with an phycoerythrin (PE) conjugated anti-mouse IgG

secondary antibody (BD Bioscience). Labeled cells were washed and resuspended in FACS buffer (HBSS w/o Ca^{2+}- Mg^{2+}) and subjected to flow cytometric analysis.

2.2.3 Data analysis and statistical evaluation

All slides were viewed on an Olympus BX43 microscope and photographed using an Infinity Luminera camera and Infinity Analyze software. The different cell sizes were averaged and a Student's t-test statistical analyses was preformed to compare the cell size within each tissue. ANOVA was performed to compare cell sizes between tissues. An alpha level of 0.05 was used to determine the significance of all analyses. Flow cytometry analyzed cells by forward scatter (FSC) and side scatter (SSC) properties. The data from FACS caliber was analyzed using Flowjo software. The data was statistically analyzed by followed by student t-test using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

2.3 Results

2.3.1 Morphological descriptions of lymphocyte-like cells (LCCs) in the liver, kidney and spleen of T and B cell deficient *rag1*$^{-/-}$ mutant zebrafish

The average weights of liver, kidney and spleen tissues were 43.33mg, 15mg and 2.66mg respectively. The average number of cells isolated from the histopaque 1119 gradients were 1x 10^6 cells from liver tissue, 6.75 x10^5 cells from kidney tissue, and 1.4x10^5 cells from spleen tissue. Leukocyte differentials revealed different predominant cell populations in liver, kidney and spleen tissues (Fig 2.1). Liver preparations were 36% hepatocytes, 34% small agranular LLCs, 20% large agranular LLCs and 12% macrophages/monocytes. Kidney preparations were 10% small agranular and granular LLCs, 28% agranular and granular large LLCs, 23% megakaryocytes, 15% granulocytes,

4% dendritic cells, 9% monocytes and 9% macrophages. Spleen preparations were 27% small agranular and granular LLCs, 50% large granular LLCs, 16% macrophages and 7% monocytes. We found 90% cell survival with HBSS w/o Ca^{2+}- Mg^{2+}, 86.1% with RPMI and 80.1% HBSS w/ Ca^{2+}- Mg^{2+}. The 90% live cell count is an acceptable cell count to proceed with primary-mAb staining procedures and HBSS w/o Ca^{2+} and Mg^{2+} were used in subsequent assays.

Examination of *rag1*$^{-/-}$ mutant zebrafish kidney, liver and spleen cells by flow cytometry demonstrated different cell populations (Fig 2.1). On the basis of forward scatter (FSC) and side scatter (SSC) properties and location in a FSC vs. SCC plot, liver small agranular LCCs were included in gate 1 and large agranular LCCs were included in gate 2 (Fig. 2.1A), the kidney small agranular and granular LCCs were included in gate 1 and large agranular and granular LCCs were included in gate 2 (Fig. 2.1B), and macrophages, large granulocytes (NK like cells) and megakaryocytes were included in gate 3. Spleen small agranular and granular LCCs were included in gate 1 and large granular LCCs were included in gate 2 (Fig. 5.1C).

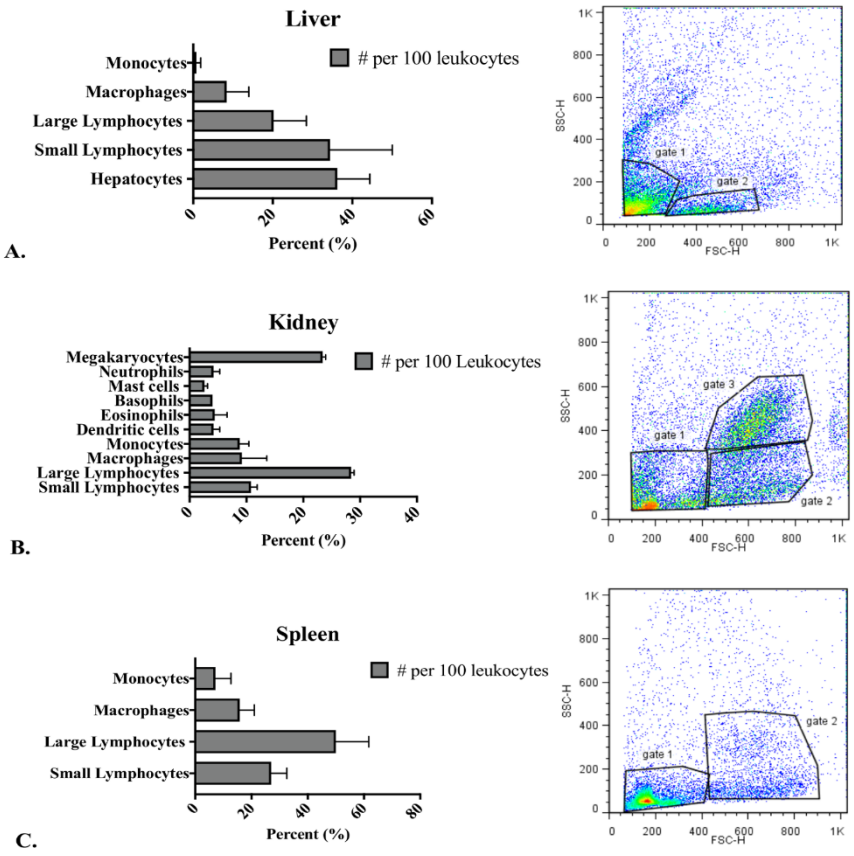

Figure 2.1 Leukocytes from liver (A), kidney (B) and spleen (C) tissues, based on morphological appearance and FACS forward scatter (FSC) and side scatter (SSC) characteristics.

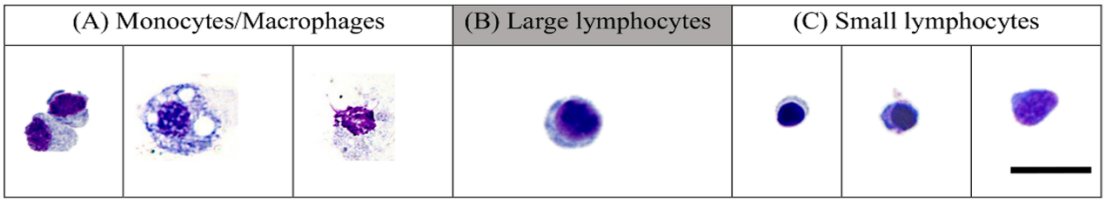

Figure 2.2 Leukocytes in *rag1*$^{-/-}$ mutant zebrafish liver included: (A) monocytes/macrophages, (B) large LLCs, (C) small LLCs.

Cells were stained with Wright Giemsa and examined under oil immersion by light microscopy and viewed at 1000x magnification. The size bar represents 10µm.

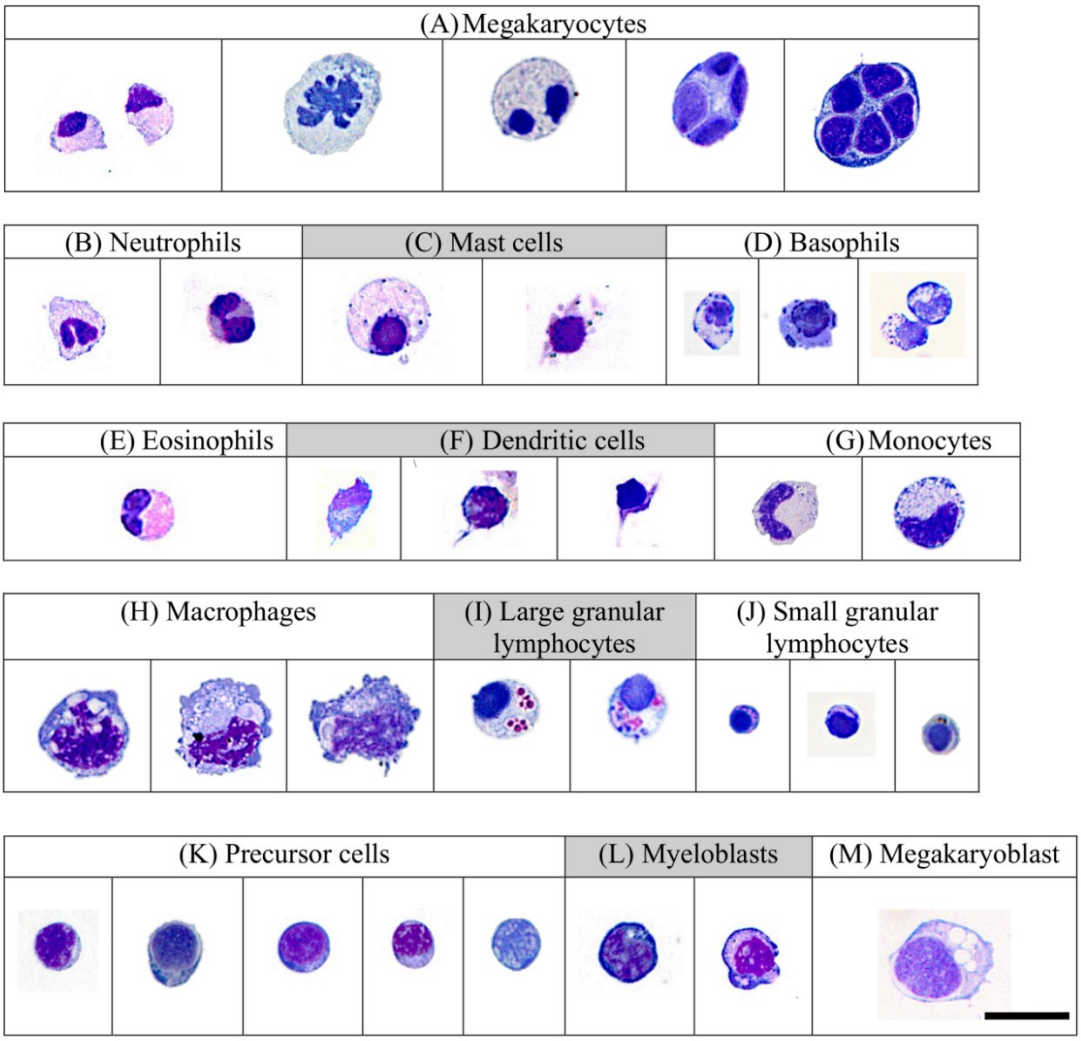

Figure 2.3 Leukocytes in *rag1*$^{-/-}$ mutant zebrafish kidney included: (A) megakaryocytes, (B) neutrophils, (C) mast cells, (D) basophils, (E) eosinophils, (F) dendritic cells, (G) monocytes, (H) macrophages, (I) large LLCs, (J) small LLCs, (K) precursor cell, (L) myeloblasts and (M) megakaryoblast.

Cells were stained with Wright Giemsa and examined under oil immersion by light microscopy and viewed at 1000x magnification. The size bar represents 10µm.

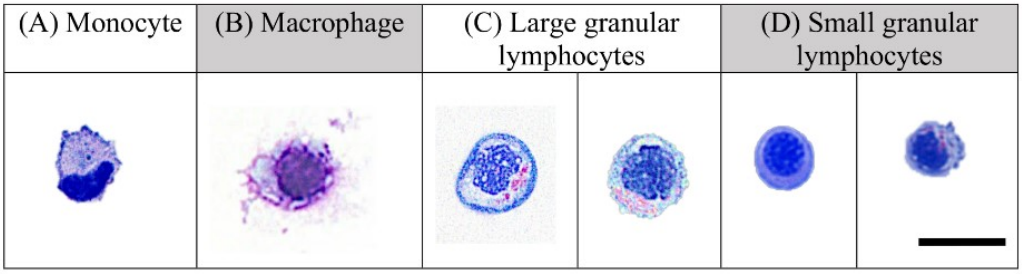

Figure 2.4 Leukocytes in *rag1*$^{-/-}$ mutant zebrafish spleen included: (A) monocytes, (B) macrophages, (C) large granular and small granular and agranular LCCs.

Cells were stained with Wright Giemsa and examined under oil immersion by light microscopy and viewed at 1000x magnification. The size bar represents 10µm.

Table 2.1 Size analyses of lymphocyte-like cells from liver, kidney and spleen tissues in *rag1*$^{-/-}$ mutant zebrafish.

Tissues	Cells	Size ± Sd	t-test p value	ANOVA
Liver	Large	7.4µm±1.1µm	0.0039*	A
	Small	4.8µm±0.8µm		B
Kidney	Large	8.8µm±1.1µm	0.0096*	A
	Small	6.2µm±1.3µm		B
Spleen	Large	9.8µm±1.1µm	0.0005*	A
	Small	6.45µm±0.5µm		B

Cells with the same letter are not significantly different from each other. *significant when alpha value is <p value. (p=0.05).

2.3.2 Cell membrane integrity and cell viability

The results from this experiment provide interesting information about the different range of cellular viability as an effect of the 3 buffers used for the cell isolations and sample preparations. The cells form the HBSS w/o Ca^{2+}- Mg^{2+} treatment (Fig. 2.4) had better survival (90%) when compared with the other buffers (80.1%) and (86.1%) i.e., HBSS w/ Ca^{2+}- Mg^{2+} (Fig. 2.5) and RPMI (Fig. 2.6) respectively. Samples/tubes 1

and 2 had less live cells compared to 3. This can be justified by the fact that we gated out the lymphocyte cells from forward and side scatter plot for analysis and there were less cells in the gate before enrichment/gradient separation. The cells in tubes 1 and 2 were aliquoted from the cell suspension before the gradient separation and these cell suspensions had more red blood cells and less LLCs. The cells separated on the gradient (tube 3) (Fig. 2.5) had an enriched lymphocyte population and showed more live cells compared to buffers 1 and 2. The flow cytometry results were consistent with the cytospin observations. The cytospins for the HBSS w/o Ca^{2+}- Mg^{2+} had morphologically healthier looking cells (data not shown) with distinct nucleus and cytoplasm as well as intact cell membrane when viewed at 1000X magnification on a light microscope. The cell membranes in the cells in RPMI were intact, but the cells did not stain well with Wrights-Geimsa stain (data not shown). The cell cytoplasm did not take up the stain and were very lightly stained. This could be a result of the serum in the RPMI media. The cells for HBSS w/ Ca^{2+}-Mg^{2+} did not have an intact cell membrane and most of the cells looked stressed and damaged.

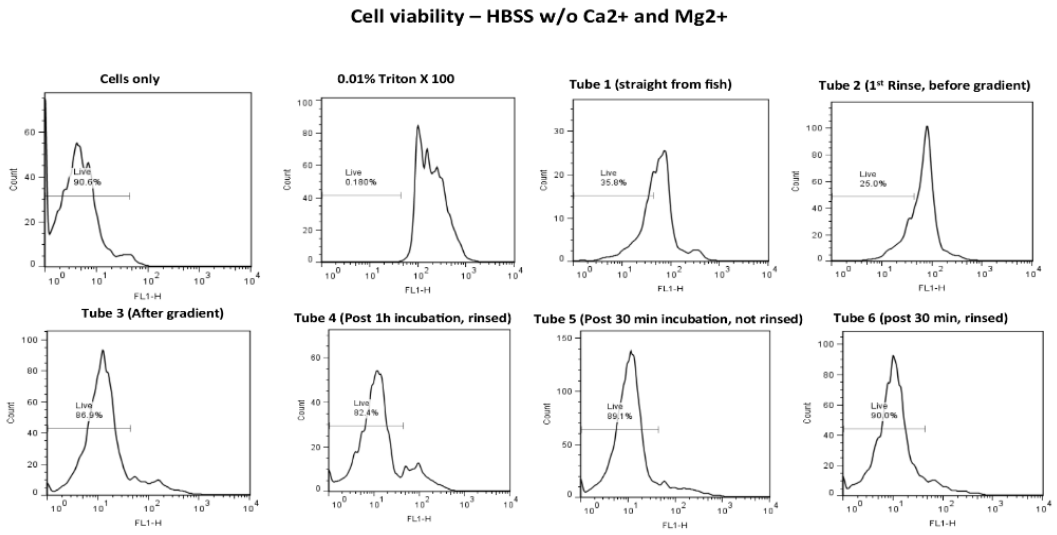

Figure 2.5 Create a short, concise figure title and place all detailed caption, notes, reference, legend information, etc in the notes secti *Rag1*$^{-/-}$ mutant zebrafish head-kidney (hematopoietic) cells were sampled in HBSS w/o Ca^{2+}- Mg^{2+} buffer.

At every step during the cell prep, aliquots were taken aside and stained with a viability dye and fixed.

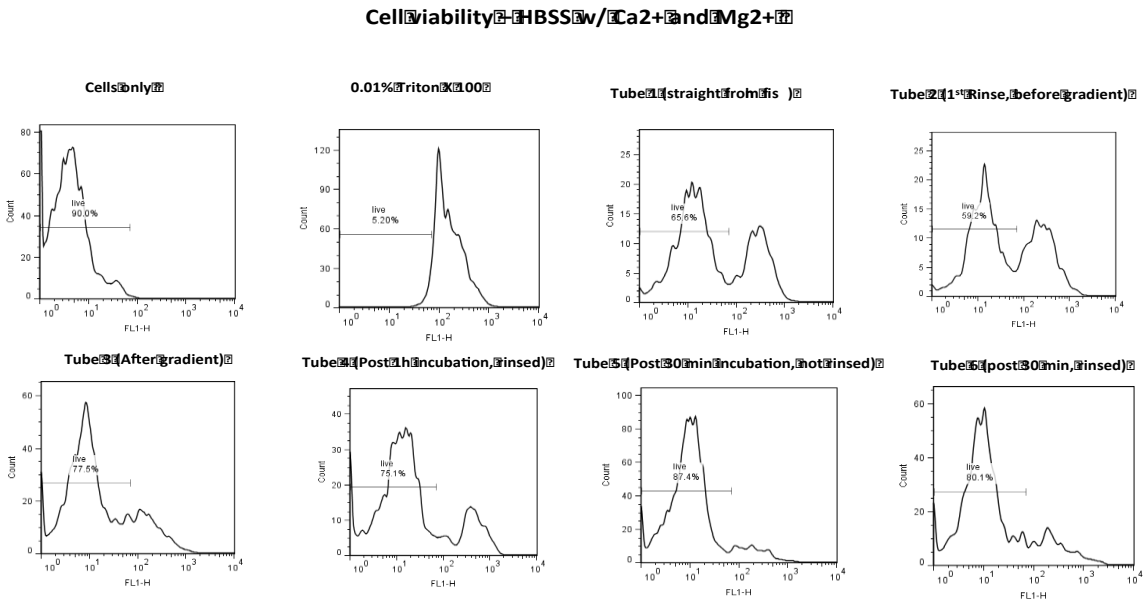

Figure 2.6 *Rag1*$^{-/-}$ mutant zebrafish head-kidney (hematopoietic) cells were sampled in HBSS w/ Ca^{2+}- Mg^{2+} buffer.

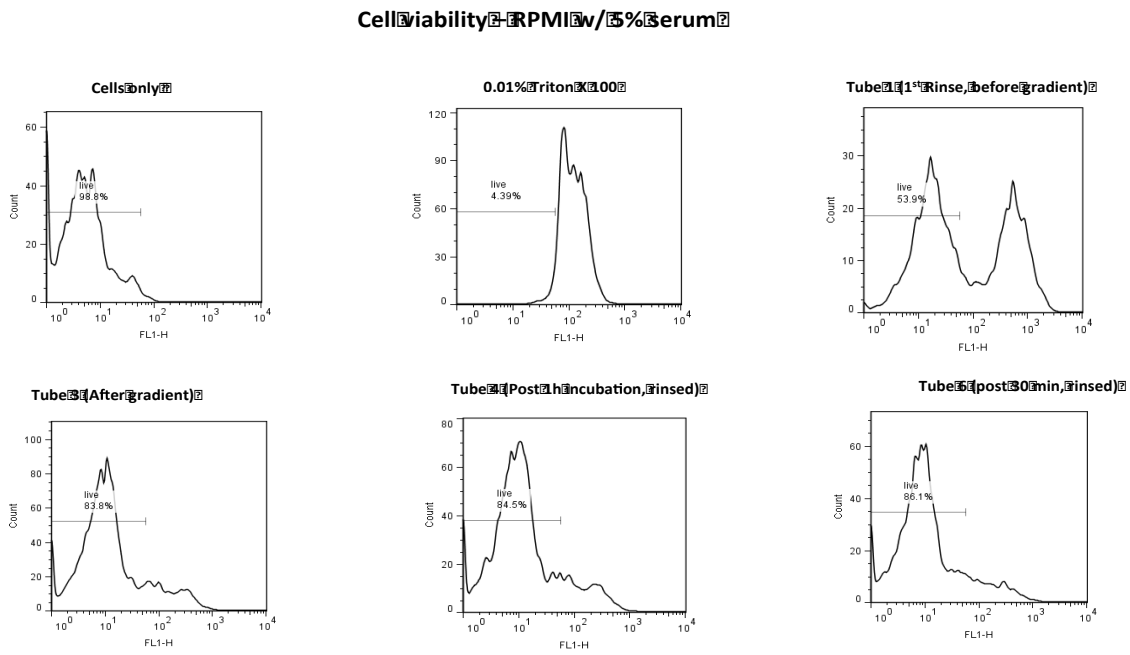

Figure 2.7 *Rag1*$^{-/-}$ mutant zebrafish head-kidney (hematopoietic) cells were sampled in RPMI media with 5% catfish serum.

At every step during the cell prep, aliquots were taken aside and stained with a viability dye and fixed.

2.3.3 Cell staining with anti-NITR9 and anti-NCCRP-1 antibodies

To evaluate the Fc blocking ability, *rag1*$^{-/-}$ mutant zebrafish cells were blocked with either mouse Fc block (Fig. 2.7) or zebrafish serum (Fig. 2.8) and stained with primary antibodies: Commercial 5C6 (anti-NCCRP-1), In-house 5C6 (anti-NCCRP-1) or anti-NITR9 antibody. There was no discernible staining difference between the cells blocked with zebrafish serum or the mouse Fc block.

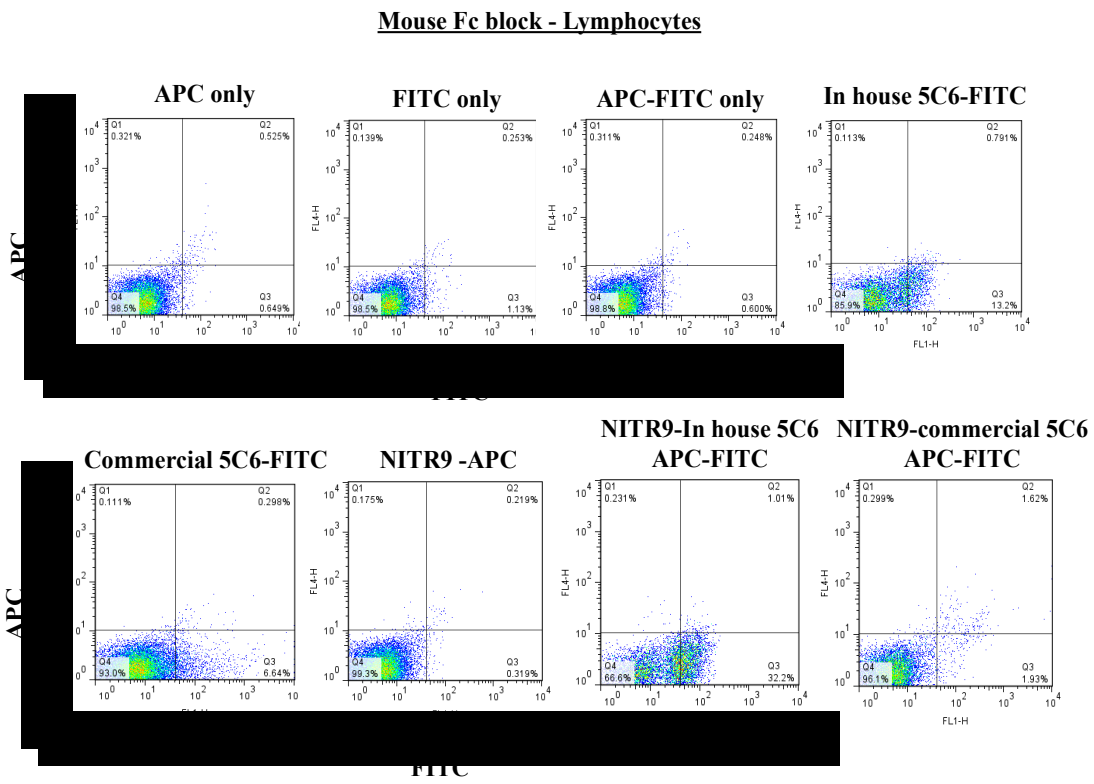

Figure 2.8 *Rag1*$^{-/-}$ mutant zebrafish head-kidney (hematopoietic) cells were blocked with mouse Fc block and stained with primary antibodies: Commercial 5C6 (anti-NCCRP-1), In-house 5C6 (anti-NCCRP-1) or anti-NITR9.

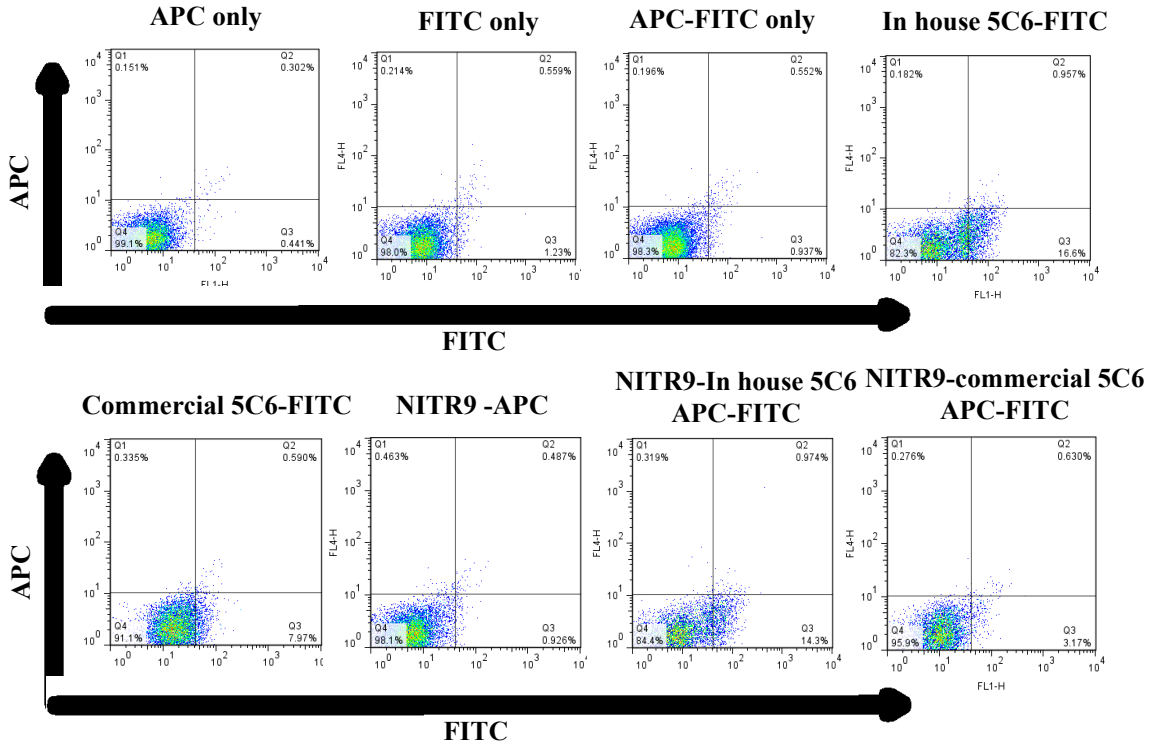

Figure 2.9 Rag1$^{-/-}$ mutant zebrafish head-kidney (hematopoietic) cells were blocked with zebrafish serum and stained with primary antibodies: Commercial 5C6 (anti-NCCRP-1), In-house 5C6 (anti-NCCRP-1) or anti-NITR9.

The experiment was repeated with antibody staining in triplicate and blocked with zebrafish serum. The fixed samples were analyzed on two different days. In the second run (Fig. 2.9), the cells in the FSC and SSC showed a slight shift downwards (but still had separated clear population) compared to the first run. Again, this shift in the cell population could be due to the fixed cells sitting for 5 days at 4C. However, this shift in the population did not affect the staining patterns of antibodies. By using the Flowjo software, cells were gated into granulocyte and LLC populations. After comparing the data from the two days, there is not much difference in the staining patterns. Anti-NITR9 did not stain any cells LLC gate (0.26 ± 0.12) and granulocyte gate (0.11 ± 0.09). In-

house (IH) 5C6 did stain the cells LLC gate (7.35 ± 1.76) but not any in the granulocyte gate (0.57 ± 0.71). However, the percentage of staining reduced to half following storing fixed cells at 4C for 5 days (Fig. 2.10). It could infer that the intensity of fluorescence wears off with time in-spite of fixing the cells. The commercial (new) 5C6 stained the LLC population (1.52 ± 0.84) and less of the granulocyte population (1.43 ± 0.88). Staining with the commercial 5C6 is not as good as the in-house 5C6. The dual staining of anti-NITR9 w/ In-house (IH) 5C6 or commercial (new) 5C6 did not give any double stained cells; anti-nitr9 w/ In-house (IH) 5C6: LLC = 0.27 ± 0.07; granulocyte = 0.03 ± 0.01; anti-NITR9 w/ commercial (new) 5C6: LLC = 0.77 ± 0.56; granulocyte = 0.29 ± 0.24.

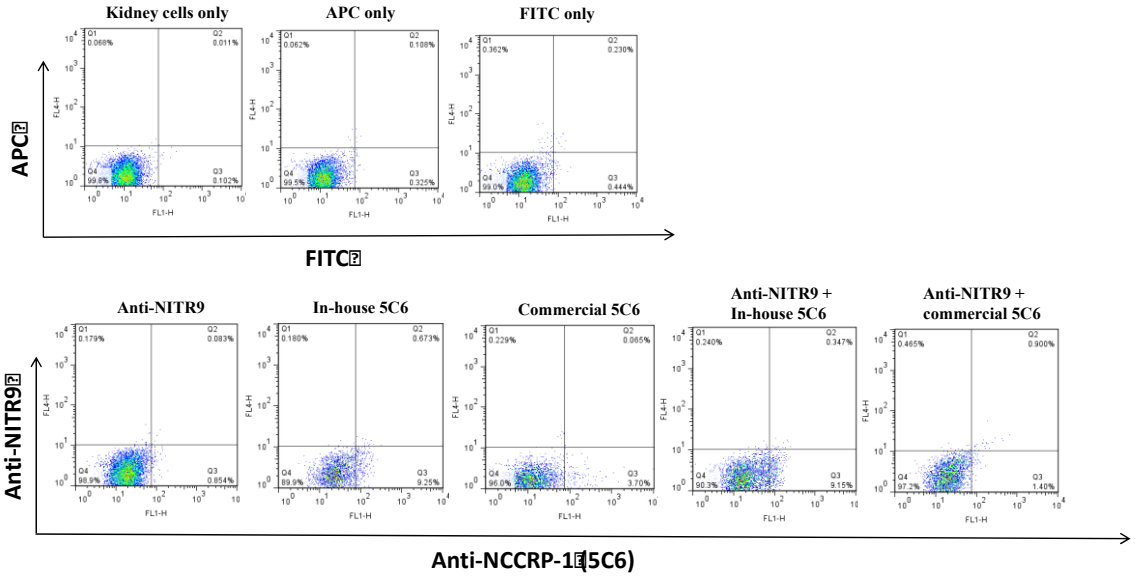

Figure 2.10 Rag1$^{-/-}$ mutant zebrafish head-kidney (hematopoietic) cells were blocked with zebrafish serum and stained with primary antibodies: Commercial 5C6 (anti-NCCRP-1), In-house 5C6 (anti-NCCRP-1) or anti-NITR9.

2.3.4 Extracellular and intracellular staining of NITR9

Extracellular and intracellular staining of NITR9 on WT and *rag1*$^{-/-}$ mutant zebrafish kidney cells demonstrated extracellular staining in both WT (63.4±7.72; p=0.0079) and mutant zebrafish (43.12±3.04; p=0.111), staining was not statistically significantly different in mutant kidney cells (Fig. 2.11). This could be explained by the absence of cytotoxic T cells in the *rag1*$^{-/-}$ mutant zebrafish. The extracellular staining in the WT is due to the presence of cytotoxic T cells and NK cells/NITR9^{+} cells. However, following intracellular staining, there was a shift in the histogram peak with a significant rise in NITR9 (93.32±1.98; p=0.0079) for WT and (88.86±5.02; p=0.0079) for *rag1*$^{-/-}$ mutant zebrafish staining intensity. This NITR9^{+} staining is similar in the WT and mutant. This could be due to the increase of NK cells in the absence of T and B cells in the mutant fish.

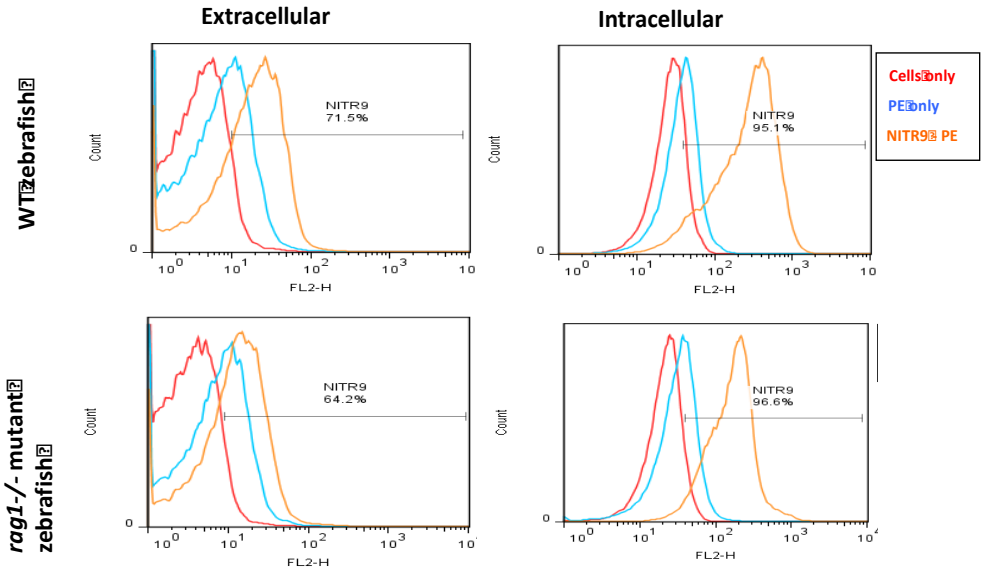

Figure 2.11 Extracellular and intracellular NITR9 expression of lymphocyte cell population, analyzed by flow cytometry after cells were stained with PE-labeled secondary antibody.

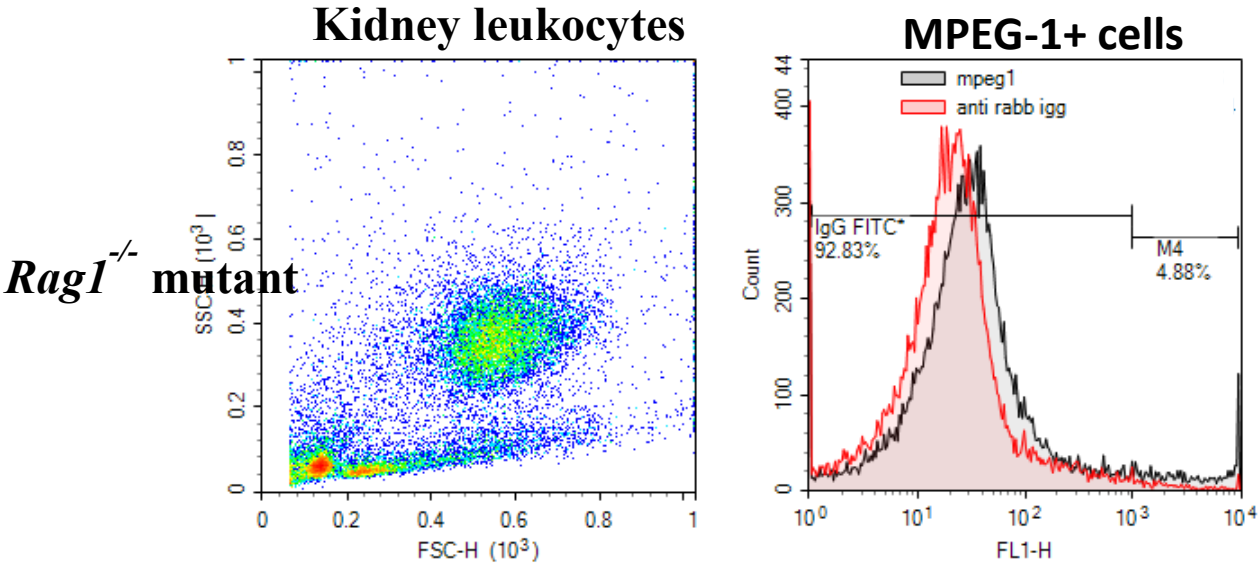

Figure 2.12 Extracellular MPEG-1 expression of kidney cell population, analyzed by flow cytometry after cells were stained with FITC-labeled secondary antibody.

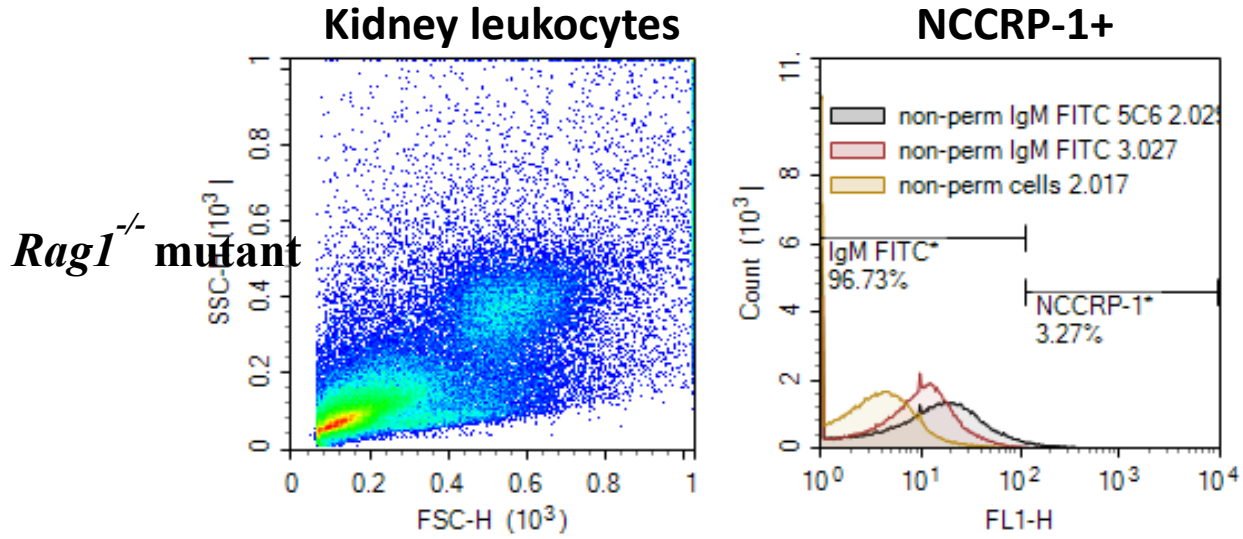

Figure 2.13 Extracellular NCCRP-1 expression of kidney cell population, analyzed by flow cytometry after cells were stained with APC-labeled secondary antibody.

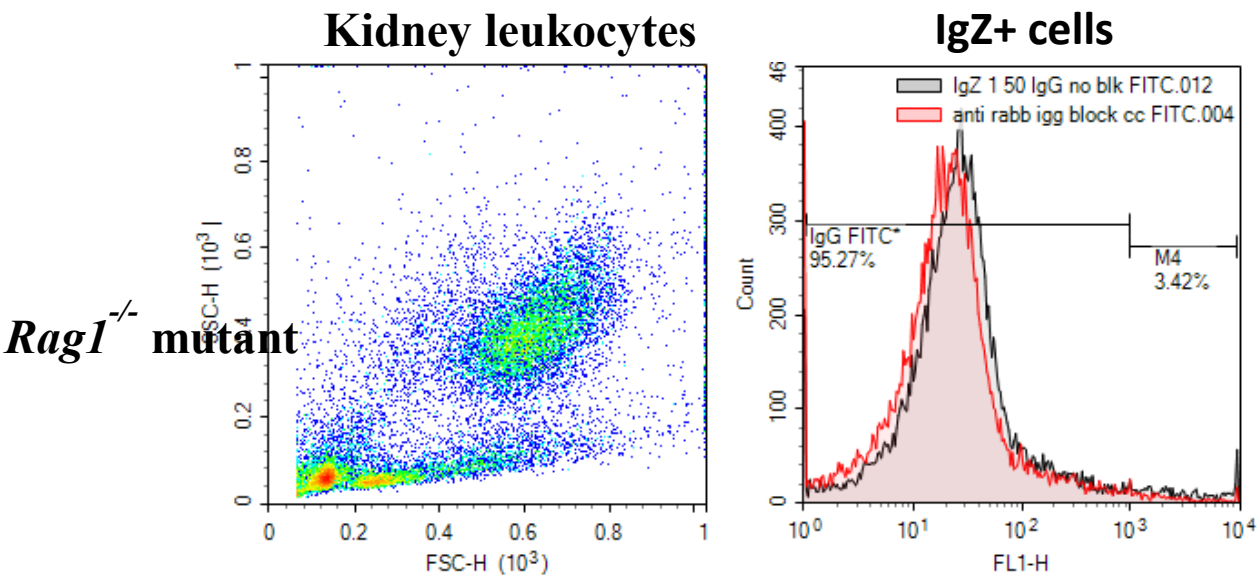

Figure 2.14 Extracellular IgZ expression of kidney cell population, analyzed by flow cytometry after cells were stained with FITC-labeled secondary antibody.

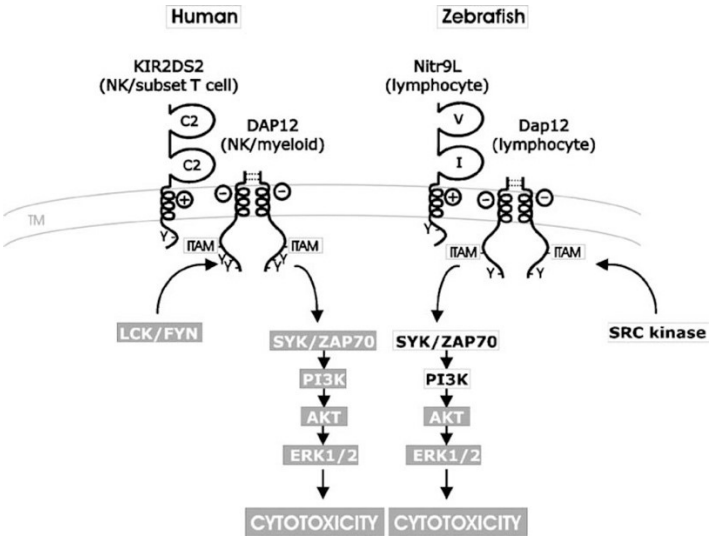

Figure 2.15 Adopted from Wei et al 2007, In this study, the authors have shown that Nitr9L and Dap12 can physically associate and that cross-linking Nitr9L results in increased phosphorylation of AKT and ERK (Wei, Zhou et al. 2007).

2.4 Discussion

Cytology demonstrated the presence of LLCs with NCC and NK cell morphology.

The size of zebrafish LLCs varied in size depending on their tissue locations, and similar

variations in NK cell populations have also been observed in mammals (Trinchieri 1989). In this study, we identified small agranular and large agranular LLCs in the livers, small agranular and granular and large agranular and granular LLCs in the kidneys and small agranular and granular and large granular LLCs in spleens of *rag1*$^{-/-}$ mutant zebrafish (Table 2.1). Two NK cell homologues have been described in teleosts: NCCs and NK-like cells. Based on RT-PCR, lymphocyte-like cells from the *rag1*$^{-/-}$ mutant zebrafish express Nk-lysin and NCCRP-1 (Petrie-Hanson, Hohn et al. 2009). NK lysin is expressed by NK cells (Andersson, Gunne et al. 1996), and NCCRP-1 is expressed by NCC cells (Harris, Kapur et al. 1992), suggesting that NCCs and NK cells are included in the LCC populations we observed. The morphology of the larger zebrafish LLCs was similar to mammalian NK cells. The smaller LCCs we observed in our *rag1*$^{-/-}$ mutant zebrafish had granules and were not morphologically similar to zebrafish NCCs described by Moss *et al.* (Moss, Monette et al. 2009). Flow cytometry findings were similar to similar analyses in zebrafish (Pereiro, Varela et al. 2015).

Circulating lymphocytes with morpho-functional similarities to mammalian NK cells were also reported in catfish (Shen et al 2004). We recently reported the presence of NK cells in zebrafish that are morphologically similar to mammalian NK cells. They are described as large lymphocyte like cells (LLCs), with cytoplasmic granules and reniform nucleus (Muire et al 2017). NK cells are commonly found in peripheral blood and were indentified in liver (Fig 2.2), kidney (Fig 2.3), spleen (Fig 2.4) and intestine (data not shown).

We saw large LLCs in the mutant zebrafish liver. However, these cells were not granulated but had the morphology of a lymphocyte cell. Normal fish non-parenchyma

liver cells contain NCC-like cells that have little to no cytolytic activity (Evans, Jaso-Friedmann et al. 1988). NK cells or large granular lymphocytes or "pit cells" have also been described in the liver of rats (Bouwens, Remels et al. 1987) (Connor, Jaso-Friedmann et al. 2009) and as such may be an important effector protein of innate immune responses (Kaur, Kamboj et al. 2005) of phylogenetically diverse vertebrates.

The use of flow cytometry in zebrafish research has increased over the last decade. However, there are a very few protocols available which describe detailed procedures for flow cytometry. Most protocols are not clear about the type of buffer (with or without Ca^{2+} & Mg^{2+}) they use for the cell preparation. For a successful flow cytometry experiment, it is essential to attain >90% cell survival for antibody staining and FACS analysis. We determined that HBSS w/o $Ca2^{+}$ and $Mg2^{+}$was the best buffer to attain zebrafish cell membrane integrity and cell survival for flow cytometry.

There was no statistical difference between the background staining of cells blocked with zebrafish serum or mouse-Fc block. However, the disadvantage of using zebrafish serum is 30 wild type zebrafish provide 50 µl of serum. The mouse Fc-block is commercially available and a small amount is required for blocking cells. Fixing cells in 4% paraformaldehyde is a standard procedure in flow cytometry. However, in zebrafish cells we noticed a shift in the cell population, although the staining patterns had the same trend as the first run. Due to the shift in the population, fixing the cells for use within 24h is acceptable, but it is not advisable to analyze cells that have been fixed for more than a day.

The putative NK cell activating receptor NITR9 in zebrafish has shown to be signaling via DAP12 intracellularly (Wei, Zhou et al. 2007) (Fig 2.15). An anti-NITR9

zebrafish antibody is available (Shah, Rodriguez-Nunez et al. 2012). However, a working protocol for this antibody was lacking, and extracellular staining did not result in efficient staining of the NITR9 positive cells in zebrafish (Shah, Rodriguez-Nunez et al. 2012). The intracellular labeling of zebrafish cells with anti-NITR9 antibody resulted in efficient staining of NK cells. We hypothesis that NCCs are NCCRP-1^{+} and $NITR9^{-}$ whereas the NK cells are NCCRP-1^{-} and $NITR9^{+}$. But were unable to prove it in this study.

There was a positive staining with in-house and commercial anti-NCCRP-1 (5C6) mAb. However, the staining with anti-NITR9 mAb was not significantly different compared to the secondary isotype control antibody. Next, we performed extracellular and intracellular (non-permeated and permeated) assay to compare the anti-NITR9 labeling patterns between WT and mutant zebrafish. There was a substantial difference in the staining patterns between intracellular an extracellular staining. However, there was little extracellular staining involved, which could suggest that the antibody is able to recognize the NITR9's extracellular region as well but to a lesser degree.

2.5 Conclusion

We identified LCCs that have characteristic NK cell morphology. Based on size, two LLC populations were found in the liver, kidney and spleen. Gene expression data from a previous study (Muire et al 2017) supported the observations of resident LCC populations in the liver, kidney and spleen. We also optimized flow cytometric procedures to attain a high percentage of viable cells (>90%) for FACS analysis and also optimized protocols of anti-NITR9 and anti-NCCRP-1 monoclonal and anti-MPEG polyclonal antibodies. In order to achieve a reliable result following the use of a secondary antibody, it is very important to rule out any background staining patterns.

Here, we compared two blocking agents: mouse Fc block and zebrafish serum. The main challenge behind using zebrafish serum is use of the several (n=30) wild type fish to attain a tiny amount of serum. Since there was no significant staining percentage difference between the cells blocked with the mouse Fc block or zebrafish serum, we continued further experiment using mouse Fc block. Finally, we confirm that anti-NITR9 mAb labels zebrafish NK cells intracellularly.

Acknowledgements

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CHAPTER III

DIFFERENTIAL GENE EXPRESSION FOLLOWING TLR STIMULATION IN *RAG1*$^{-/-}$ MUTANT ZEBRAFISH TISSUES

Abstract

Rag1$^{-/-}$ mutant zebrafish mediate protective immunity in the absence of T and B cells. Differential expression of immune response genes suggest how tissue environments induce changes in innate immune cells that can result in these cells having the capacity to mediate protective immunity on subsequent pathogen exposures. We determined tissue expression levels of *mx, tnfα, ifnγ, t-bet* and *nitr9* in response to β glucan (TLR2/6 ligand), Poly I:C (TLR3 ligand) and resiquimod (R848) (TLR7/8 ligand) intra-coelomic injections. β glucan up-regulated *tnfα* and *ifnγ* in liver; *tnfα, ifnγ, t-bet* in kidney; *ifnγ* in spleen tissues and down-regulated *nitr9* in kidney tissues. Poly I:C up-regulated *mx* in liver; *mx*, *ifnγ, t-bet, nitr9* in kidney; *mx* in spleen tissues and down-regulated *tnfα* in kidney and spleen tissues. R848 up-regulated *mx*, *ifnγ*, *t-bet, nitr9* in liver and kidney; *mx, ifnγ* in spleen tissues. These findings suggest the presence and tissue location of cytotoxic cells in zebrafish.

3.1 Introduction

In fish, the innate immune system is critical in mediating a protective role against pathogens during early life stages. At this time, fish have not yet developed adaptive immunity (Petrie-Hanson and Ainsworth 1999, Mulero, García-Ayala et al. 2007).

Zebrafish develop adaptive immunity 3 weeks post fertilization (Trede, Langenau et al. 2004). *Rag1*$^{-/-}$ mutant zebrafish lack T cell receptor (TCR) and immunoglobulin (Ig) but have non-specific cytotoxic cells (NCCs), NK like cells, monocytes/macrophages and neutrophils (Petrie-Hanson, Hohn et al. 2009). These fish are a model for studying immune responses in young fish. *Rag1*$^{-/-}$ zebrafish demonstrate protective immunity to bacteria after vaccination that is at least partially specific, unlike typical innate responses mediated only by PRRs (Hohn and Petrie-Hanson 2012). However, the specific cell population(s) mediating this protection has not been identified.

TLRs are present on macrophages, B cells, dendritic cells and NK cells. They are the most widely studied pattern recognition receptors (PRRs) because of their importance for recognizing conserved pathogen associated molecular patterns (PAMPs) and their important roles in mediating innate as well as helping initiate adaptive immune responses mediated by B and T lymphocytes. There are 10 TLRs in humans (Lee, Kok et al. 2014, Pandey, Kawai et al. 2015), 13 TLRs in mice (Kawai and Akira 2010) and 22 TLRs in zebrafish (Meijer, Gabby Krens et al. 2004).

β glucan, a yeast cell wall derivative, specifically binds to TLR2/6 (Di Renzo, Yefenof et al. 1991, Brown and Gordon 2001, Brown and Gordon 2003, Brown, Herre et al. 2003, Chan, Chan et al. 2009, Vaclav Vetvicka Oct 2013). Recognition of β glucan requires the TLR2/6 heterodimer and the co-receptor Dectin-1 (Janeway, Murphy et al. 2008). Previous studies have shown that β glucan triggers the activation of macrophages, neutrophils, monocytes, NK cells and dendritic cells and can enhance the function of human NK cell cytotoxicity (Di Renzo, Yefenof et al. 1991). Poly I:C, an endosomal TLR3 ligand, is a dsRNA synthetic molecule and is one of the most commonly used TLR

ligands (Falco, Miest et al. 2014). It stimulates type I interferon and *mx* production (Falco, Miest et al. 2014) and is known to activate mammalian NK cells in the absence of antigen presenting cells (Dong, Wei et al. 2004, Pisegna, Pirozzi et al. 2004, Schmidt, Leung et al. 2004, McCartney, Vermi et al. 2009). Poly I:C induced *mx* expression and demonstrated anti-viral activity in flatfish (Fernandez-Trujillo, Ferro et al. 2008). Further, in mice, Poly I:C treatment induced the activation and accumulation of hepatic NK cells (Dong, Wei et al. 2004, Wang, Xu et al. 2005). R848, an endosomal TLR7/8 ligand, is a synthetic anti-viral compound that mimics viral ssRNA, and belongs to the family of imidazoquinolines, that are known to induce *ifn* genes (Hemmi, Kaisho et al. 2002, Jurk, Heil et al. 2002, Caron, Duluc et al. 2005, Kileng, Albuquerque et al. 2008), especially *ifnγ* expression from activated human NK cells (Hart, Athie-Morales et al. 2005).

Zebrafish *mx* is a GTPase, and belongs to the dynamin family (Obar, Collins et al. 1990) and includes highly conserved motifs common to mammalian *mx* (Staeheli, Haller et al. 1986, Aebi, Fäh et al. 1989). The *mx* genes in teleost are highly polymorphic and can vary between species. Seven *mx* isoforms have been identified in zebrafish (Altmann, Mellon et al. 2004). *Mx* is an indirect indicator of type I interferon expression in teleost (Leong, Fryer et al. 1988, Purcell, Kurath et al. 2004, Overturf and LaPatra 2006, Robertsen 2006, Fischer, Koppang et al. 2013, Falco, Miest et al. 2014) and can inhibit viral replication at various stages of the virus life cycle (Fernandez-Trujillo, Ferro et al. 2008).

Tnfα is a pro-inflammatory cytokine critical to the host during bacterial and viral infections (Phelan, Pressley et al. 2005, Pressley, Phelan et al. 2005), and is an indicator of NK cell and macrophage stimulation. In teleost, the predominant role of *tnfα* is

endothelial cell activation, and it is partly responsible for the expression of type 1 interferons (Roca, Mulero et al. 2008).

In bony fish, *ifnγ* is one of the pro-inflammatory cytokines (class II cytokines family) required for protection against bacterial and viral attacks (Zou, Carrington et al. 2005, Milev-Milovanovic, Long et al. 2006, Robertsen 2006, Stolte, Savelkoul et al. 2008, Sieger, Stein et al. 2009). Zebrafish *ifnγ* has two isoforms, *ifnγ-1* and *ifnγ-2* (Altmann, Mellon et al. 2003), that are functionally similar to mammalian IFNγ (Stein, Caccamo et al. 2007). Teleost NK and T cell produce *ifnγ* (Robertsen 2006).

Tbox-21 (*t-bet*) is a transcription factor required for development of mammalian NK and Th1 cells (Szabo, Kim et al. 2000), and has been characterized in ginbuna crucian carp (Takizawa, Araki et al. 2008), zebrafish (Mitra, Alnabulsi et al. 2010), rainbow trout (Wang, Holland et al. 2010), grass carp (Wang, Shang et al. 2013) and Atlantic salmon (Kumari, Zhang et al. 2015). Tissue expression of *t-bet* following pathogen exposure has been investigated in several fish species (Takizawa, Araki et al. 2008, (Wang, Shang et al. 2013, Kumari, Zhang et al. 2015).

Novel Immune Type-Receptors (NITRs) are unique to bony fish, and are homologous to mammalian NK KIRs (killer like immunoglobulin receptors) (Wei, Zhou et al. 2007). The NITR structure is similar to the TCR variable arm. All *nitr* genes encode V domains and some J regions. Part of the *nitr* gene is very similar to the variable region genes of antibodies and the T-cell receptor, but it does not undergo genetic rearrangement. Phylogenetic studies suggest *nitrs* might represent the primordial gene(s) that gave rise to adaptive immune receptors (Litman, Hawke et al. 2001, Litman, Rast et al. 2010). NITR genes have been identified in puffer fish (Rast, Haire et al. 1995),

zebrafish (*Danio rerio*) (Yoder, Mueller et al. 2001, Yoder, Litman et al. 2004, Yoder, Cannon et al. 2008), rainbow trout (*Oncorhynchus mykiss*) (Yoder, Mueller et al. 2002), channel catfish (*Ictalurus punctatus*) (Hawke, Yoder et al. 2001, Evenhuis, Bengtén et al. 2007), Japanese flounder (*Paralichthys olivaceus*) (Piyaviriyakul, Kondo et al. 2007), medaka (*Oryzias latipes*) (Desai, Heffelfinger et al. 2008), Fugu (*Takifugu rubripes*) and *Tetraodon nigroviridis* (Strong, Mueller et al. 1999). Of 39 NITR genes, only *nitr9* is a putative activating receptor with immunoreceptor tyrosine-based activation motifs (ITAMs). Similar to mammalian activating NK receptors (KIRs and Ly49s), NITR9L (NITR9 isoform) signals via Dap12 adaptor protein (Wei, Zhou et al. 2007). The remaining *nitrs* are inhibitory receptors, and possess cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) (Wei, Zhou et al. 2007).

T and B cell deficient zebrafish exhibit a specific memory-like response when re-exposed to bacteria (Hohn and Petrie-Hanson 2012). Adoptive transfer of leukocytes from vaccinated T and B cell deficient mutants demonstrated that one or more of the cells present in the kidney tissue mediated the protection. The cells present in the kidney are macrophages, neutrophils, NCCs and NK cells (Petrie-Hanson et al 2009).

Microarray analysis of kidney tissue following secondary exposure suggested increased cell receptor activation and expansion of a cell population (Krishnavajhala et al 2017). These changes followed exposure to attenuated bacteria. Targeted activation of specific TLRs could suggest the cell population(s) mediating the protective response in our model.

Few tools are available for immune cell studies in zebrafish. In this study, TLR2/6, TLR 3 and TLR 7/8 ligands were administered. Tissue specific expression of

immune cell specific genes *mx, tnfα, ifnγ, t-bet* and *nitr9* were evaluated to gain a better understanding of the responsive cell population underlying protective immunity in *rag1*$^{-/-}$ mutant zebrafish. Data presented in this chapter were included in Muire et al. 2017.

3.2 Materials and methods

3.2.1 Zebrafish care

Zebrafish were mated, raised and maintained at 28°C in the specific pathogen free hatchery in the College of Veterinary Medicine, Mississippi State University (MSU) following standard lab protocols. Experimental protocols were approved by the MSU Institutional Animal Care and Use Committee (IACUC). A homozygous breeding colony of *rag1*$^{-/-}$ mutant zebrafish was previously established (Petrie-Hanson, Hohn et al. 2009) and bred at the CVM-SPF hatchery and the experimental fish are progeny from that colony.

3.2.2 TLR ligand injection rates

Rag1$^{-/-}$ zebrafish were intra-coelomically (IC) injected with β glucan (50µg/0.5g of fish), Poly I:C (50µg/0.5g of fish) and R848 (0.08µl/0.5g of fish) or endotoxin free PBS (10µl/fish).

3.2.3 RNA extraction and cDNA preparation

Fish were euthanized in buffered 0.02% Tricaine Methanesulfonate solution (Finquel MS-222; Argent Chemical Laboratories, Redmond, WA) and liver, kidney and spleen tissues from fish were surgically removed at 0h (non-injected fish (n=5) for basal expression), 1, 6, 12 and 24 hours post injection (hpi) (n=3) for each TLR ligand. Whole tissues were immediately transferred to 400µl Trizol reagent (Zymo Research, USA) and

homogenized following standard procedures in our lab (Elibol-Fleming 2006). Total RNA was extracted individually from each liver, kidney and spleen homogenized sample according to manufacturer's protocol using RNA extraction kit (Zymo Research, USA). The quantity of extracted total RNA was determined by NanoDrop ND-1000 and ND-8000 8-Sample Spectrophotometer and stored at -80°C until used. 100ng cDNA was prepared from RNA by using Super script III VILO^{TM} cDNA Synthesis Kit (Invitrogen).

3.2.4 Quantifying gene expression

Mx, tnfα, ifnγ, t-bet and *nitr9* were measured using real time quantitative PCR (Takara). The *mx* and *t-bet* primers and probes (Table 3.1) were designed by Beacon Design software (BioRad) and Primer3 plus (GraphPad) software, respectively. All primers and probes were purchased from Eurofins MWG, Operon, Huntsville, Alabama, USA. Amplification of the ubiquitously expressed acidic ribosomal phosphoprotein (*arp*) gene was used for the internal control (Ju, Xu et al. 1999). The amplification was performed in a 25µl volume containing 10 µl target cDNA and 15 µl master mix containing: 8.8 µl Nuclease free water (GIBCO, Ultra Pure^{TM}), 1.5 µl $MgCl_2$ (5mM), 2.5 µl 10x buffer, 0.5 µl dNTPs, 0.2µl Taq Polymerase HS enzyme (Hot Start PCR Kit, TAKARA, Japan), 0.5 µl forward primer (20µM), 0.5 µl reverse primer (20µM) and 0.5 µl probe (10µM). Thermal cycler parameters for the qPCR program were set as follows: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 61°C for 1 minute. All samples (biological reps) were run in triplicates i.e., 3 technical reps/sample.

Table 3.1 Oligonucleotide primers and probes used for qRT-PCR to quantify gene expression levels of *mx, tnfα, ifnγ, t-bet* and *nitr9*.

Gene	Oligonucleotide sequences (5'–3')	GenBank Accession No.
arp	**Fwd:** CTGCAAAGATGCCCAGGGA **Rev:** TTGGAGCCGACATTGTCTGC **Probe:**[6~FAM]TTCTGAAAATCATCCAACTGCTGGATGACTACC [BHQ1a~ Q] (Vojtech, Sanders et al. 2009)	NM_131580
mx	**Fwd:**GCATCATTAGTTCAGACAGTCG **Rev:**AAATTATCGATAGTGTCGATACAAG **Probe:**[6~FAM]TGCTGACTGAACGTGTAACTCAACT [BHQ1a~ Q] *	NM_182942.4
tnfα	**Fwd:**TCGCATTTCACAAGGCAATTT **Rev:** GGCCTGGTCCTGGTCATCTC **Probe:**[6~FAM]AGGCTGCCATCCATTTAACAGG[BHQ1a~Q] (Vojtech, Sanders et al. 2009)	NM_212859
ifnγ	**Fwd:** CTTTCCAGGCAAGAGTGCAGA **Rev:** TCAGCTCAAACAAAGCCTTTCG **Probe:**[6~FAM]AACGCTATGGGCGATCAAGGAAAACGAC[BHQ1a~ Q] (Vojtech, Sanders et al. 2009)	NM_212864
t-bet	**Fwd:**GATCAAGCTCTCTCTGTGATAG **Rev:**GCTAAAGTCACACAGGTCT **Probe:**[6~FAM]TTCTGAAGGTCACGGTCACA[BHQ1a~Q] *	NM_001170599.1
nitr9	**Fwd:** GTCAAAGGGACAAGGCTGATAGTT **Rev:**GTTCAAAACAGTGCATGTAAGACTCA **Probe:** [6~FAM]CAAGGTTTGGAAAAGCAC[BHQ1a~Q] (Shah, Rodriguez-Nunez et al. 2012)	AY570237.1

Housekeeping gene *arp* was used as a reference gene*. The *mx* and *t-bet* primers and probes were designed by Beacon Design software (BioRad) and Primer3 plus (GraphPad) software, respectively.

3.2.5 Data analysis and statistical evaluation

Relative gene expression was determined using the Pfaffl method (Pfaffl 2004). Data obtained from qRT-PCR were expressed as fold change and were converted to log2 values. Data were analyzed by two-way analysis of variance using PROC MIXED (SAS for Windows 9.4, SAS Institute, Inc., Cary, NC). Separate models were used for each gene and tissue combination. The explanatory variables for all models were treatment, time post injection, and the treatment x time interaction. The effect of treatment was reported only if the treatment and treatment x time interaction was significant. If the interaction was significant, treatment to control comparisons were made at each time

point with p values corrected for multiple comparisons using the SIMULATE option in a LSMESTIMATE statement. Similarly, the 0 hour (non injected fish) data were transformed to log2 values and analyzed by analysis of variance using PROC MIXED (SAS for Windows 9.4, SAS Institute, Inc., Cary, NC). Separate models were used for *mx, tnfα, ifnγ, t-bet* and *nitr9* to compare their basal expression within the liver, kidney and spleen. An alpha level of 0.05 was used to determine the significance of all analyses.

3.3 Results

3.3.1 Gene expression in control tissues

No significant differences were observed in the gene expressions of *mx, tnfα*, *ifnγ, t-bet* and *nitr9* between different tissues prior to injecting *rag1*$^{-/-}$ mutant zebrafish with TLR ligands (Fig. A1).

3.3.2 Effect of β glucan on *mx, tnfα, ifnγ, t-bet* and *nitr9* expression in liver, kidney and spleen

Mx expression in liver, kidney and spleen

β glucan did not affect *mx* expression in the liver, kidney or spleen. Statistical values for tissue gene expression studies were summarized (Table A2).

Tnfα expression in liver, kidney and spleen

Liver *tnfα* expression was affected by β glucan and expression was significantly different between time points (Fig. 3.1 A). β glucan significantly increased the expression of *tnfα* at 1 hpi and 12 hpi. However, β glucan did not affect *tnfα* expression at 6 hpi and 24 hpi. In the kidney, *tnfα* expression was significantly different between time points (Fig. 3.1 B). β glucan caused significantly greater *tnfα* expression at 1 hpi and 6 hpi. β

glucan did not affect 12 hpi or 24 hpi *tnfα* expression. In the spleen, *tnfα* expression was not significantly affected.

***Ifnγ* expression in liver, kidney and spleen**

In the liver, *ifnγ* expression was significantly affected by β glucan at 1 hpi, 6 hpi, 12 hpi and 24 hpi (Fig. 3.1 C). In the kidney, expression was significantly different between time points (Fig. 3.1 D). Kidney *ifnγ* expression was significantly greater at 1 hpi, 6 hpi and 12 hpi. β glucan did not significantly effect *ifnγ* expression at 24 hpi. In the spleen, *ifnγ* expression was significantly affected by β glucan at 1 hpi, 6 hpi, 12 hpi and 24 hpi (Fig. 3.1 E).

***T-bet* expression in liver, kidney and spleen**

T-bet expression in the liver was not significantly affected by β glucan. In the kidney, expression was significantly different between time points (Fig. 3.1 F), with *t-bet* expression significantly greater only at 6 hpi. β glucan did not significantly affect *t-bet* expression at 1 hpi, 12 hpi or 24 hpi. In the spleen, expression of *t-bet* was not significantly affected by β glucan.

***Nitr9* expression in liver, kidney and spleen**

β glucan had no effect on *nitr9* expression in the liver. In the kidney, expression of *nitr9* was significantly different between time points (Fig. 3.1 G), with *nitr9* expression significantly down-regulated at 12 hpi and 24 hpi. β glucan had no effect on *nitr9* expression when compared to PBS treatment in the spleen.

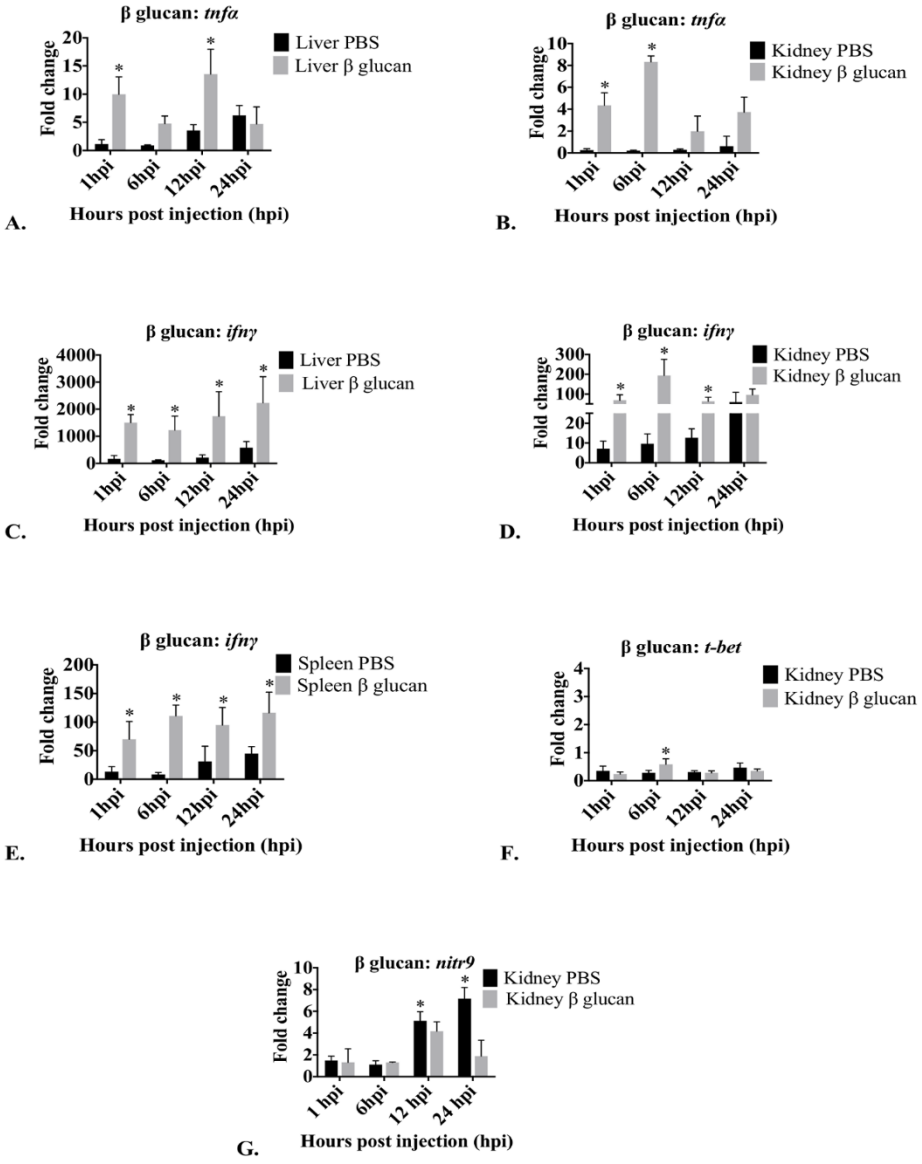

Figure 3.1 Graphs depicting changes in mRNA expression over time of *tnfα, ifnγ, t-bet* and *nitr9* in liver, kidney and spleen after treatment with β glucan.

Only tissues and genes that demonstrated significant changes in expression compared to PBS injected controls are presented. Fold changes in *tnfα* in liver (A) and kidney (B), *ifnγ* in liver (C), kidney (D) and spleen (E), *t-bet* in kidney (F) and *nitr9* in kidney (G) are presented as mean fold change relative to the time zero group ± standard deviation as measured by quantitative RT-PCR. *Arp* was used as a housekeeping gene. hpi= hours post injection; control= PBS (endotoxin-free); Treated= β glucan. *Significant ($p<0.05$) difference in expression of treated compared to control. No significant changes in expression were observed in *tnfα* in spleen, in *t-bet* in liver and spleen and in *nitr9* in liver and spleen (Table A1).

3.3.3 Effect of Poly I:C on *mx, tnfα, ifnγ, t-bet* and *nitr9* in liver, kidney and spleen.

_Mx_ expression in liver, kidney and spleen

Poly I:C significantly affected *mx* expression in liver at 1 hpi, 6 hpi, 12 hpi and 24 hpi (Fig. 3.2 A). In the kidney, expression was significantly different between time points (Fig. 3.2 B). There was increased *mx* expression at 6 hpi, 12 hpi and 24 hpi. The expression of kidney *mx* was not significantly affected by Poly I:C at 1 hpi. In the spleen, expression was significantly different between time points (Fig. 3.2 C). The expression of *mx* was significantly greater at 6 hpi and 12 hpi. Poly I:C did not significantly affect *mx* expression at 1 hpi and 24 hpi.

***Tnfα* expression in liver, kidney and spleen**

Liver *tnfα* expression was not significantly affected by Poly I:C. In the kidney, expression was significantly different between time points (Fig. 3.2 D). The expression of *tnfα* was significantly down-regulated at 24 hpi. In the spleen, the expression between different time points was significantly different, and *tnfα* expression was significantly down-regulated at 24 hpi (Fig. 3.2 E).

***Ifnγ* expression in liver, kidney and spleen**

Poly I:C did not affect *ifnγ* expression in the liver. In the kidney, expression was significantly different between time points (Fig. 3.2 F), and *ifnγ* expression was significantly greater at 12 hpi. Poly I:C did not significantly affect kidney *ifnγ* expression at 1 hpi, 6 hpi and 24 hpi. Poly I:C did not significantly affect splenic expression of *ifnγ* (Data not shown).

T-bet expression in liver, kidney and spleen

Poly I:C had no effect on *t-bet* expression in the liver. In the kidney, expression was significantly different between time points (Fig. 3.2 G), and Poly I:C significantly increased *t-bet* expression at 6 hpi. However, Poly I:C did not affect *t-bet* expression at 1 hpi, 12 hpi and 24 hpi. Poly I:C had no effect on *t-bet* expression in the spleen.

Nitr9 expression in liver, kidney and spleen

Poly I:C did not significantly effect *nitr9* expression in liver. In the kidney, *nirt9* expression was significantly different between time points (Fig. 3.2 H), and *nitr9* expression was significantly greater at 1 hpi. Poly I:C had no effect on *nitr9* expression at 6 hpi, 12 hpi and 24 hpi. Poly I:C did not significantly affect splenic *nitr9* expression.

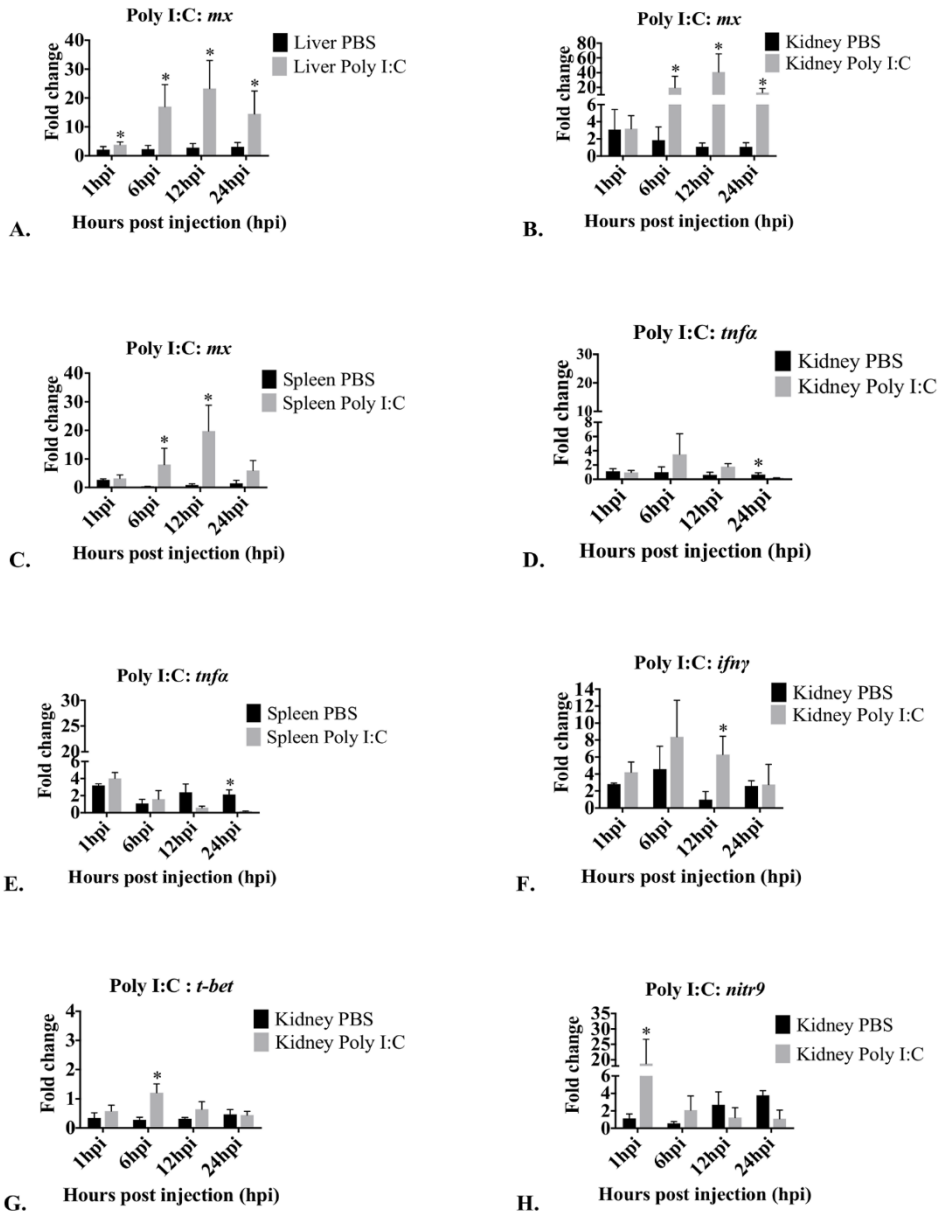

Figure 3.2 Graphs depicting changes in mRNA expression over time of *mx, tnfα, ifnγ, t-bet* and *nitr9* in liver, kidney and spleen after treatment with Poly I:C.

Only tissues and genes that demonstrated significant changes in expression compared to PBS injected controls are presented. Fold changes in *mx* in liver (A), kidney (B) and spleen (C), *tnfα* in kidney (D) and spleen (E), *ifnγ* in kidney (F), *t-bet* in kidney (G) and *nitr9* in kidney (H) are presented as mean fold change relative to the time zero group ± standard deviation as measured by quantitative RT-PCR. *Arp* was used as a housekeeping gene. hpi= hours post injection; control= PBS (endotoxin-free); Treated= Poly I:C. *Significant ($p<0.05$) difference in expression of treated compared to control. No

significant changes in expression were observed in *tnfα* in liver, in *ifnγ*, *t-bet* and in *nitr9* in liver and spleen (Table A1).

3.3.4 Effect of R-848 on *mx, tnfα, ifnγ, t-bet* and *nitr9* in liver, kidney and spleen

Mx expression in liver, kidney and spleen

Mx expression was significantly affected by R848 in liver at 1 hpi, 6 hpi, 12 hpi and 24 hpi (Fig. 3.3 A). *Mx* expression was significantly affected by R848 in kidney at 1 hpi, 6 hpi, 12 hpi and 24 hpi (Fig. 3.3 B). Splenic *mx* expression was affected by R848 and expression was significantly different between time points (Fig.3.3 C). R848 significantly increased the splenic *mx* expression at 6 hpi and 12 hpi. R848 did not affect *mx* expression at 1 hpi and 24 hpi.

Tnfα expression in liver, kidney and spleen

R848 did not affect *tnfα* expression in the liver, kidney or spleen.

Ifnγ expression in liver, kidney and spleen

Liver *ifnγ* expression was effected by R848, and expression was significantly different between time points (Fig. 3.3 D). R848 significantly increased the expression of *ifnγ* at 6 hpi, 12 hpi and 24 hpi. However, R848 did not affect *ifnγ* expression at 1 hpi. In the kidney, *ifnγ* expression was effected by R848 at 1 hpi, 6 hpi, 12 hpi and 24 hpi (Fig. 3.3 E). Splenic *ifnγ* expression was effected by R848 and expression was significantly different between time points (Fig. 3.3 F). R848 significantly increased the expression of *ifnγ* at 6 hpi, 12 hpi and 24 hpi. However, R848 did not affect *ifnγ* expression at 1 hpi.

T-bet expression in liver, kidney and spleen

Liver *t-bet* expression was significantly different between time points (Fig. 3.3 G) and was increased at 1 hpi, 6 hpi and 24 hpi. R848 did not affect *t-bet* expression at 12

hpi. Kidney *t-bet* expression was significantly different between time points (Fig. 3.3 H) and was increased at 1 hpi and 6 hpi. R848 did not affect *t-bet* expression at 12 hpi and 24 hpi. In the spleen, expression of *t-bet* was not significantly affected by R848.

***Nitr9* expression in liver, kidney and spleen**

Liver *nitr9* expression was significantly different between time points (Fig. 3.3 I). R848 significantly increased *nitr9* expression at 1 hpi and 6 hpi. R848 did not affect *nitr9* expression at 12 hpi or 24 hpi. In the kidney, *nitr9* expression was significantly different between time points (Fig. 3.3 J), and was increased at 6 hpi. R848 did not affect *nitr9* expression at 1 hpi, 12 hpi and 24 hpi. In the spleen, *nitr9* expression was not significantly affected by R848.

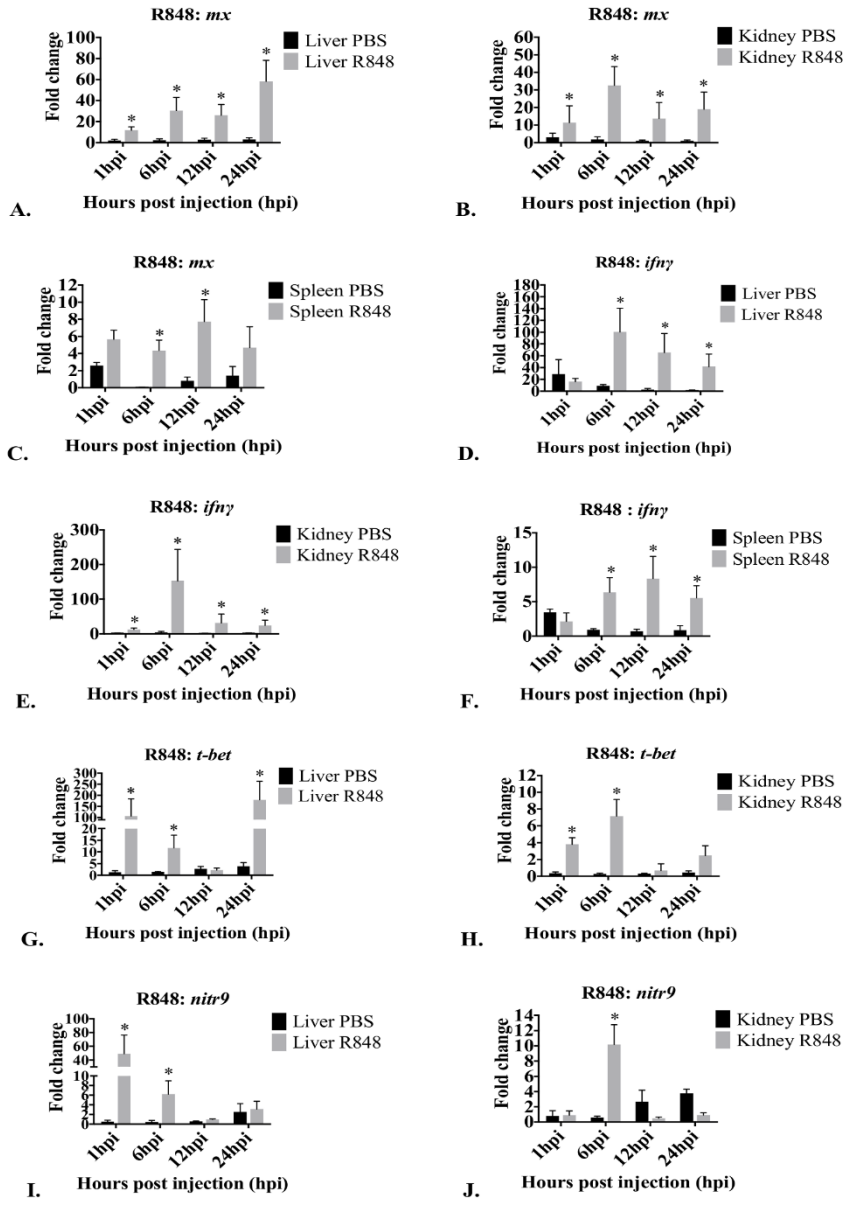

Figure 3.3 Graphs depicting changes in mRNA expression over time of *mx, tnfα, ifnγ, t-bet,* and *nitr9* in liver, kidney and spleen after treatment with R848.

Only tissues and genes that demonstrated significant changes in expression compared to PBS injected controls are presented. Fold changes in *mx* in liver (A), kidney (B), and spleen (C), *ifnγ* in liver (D), kidney (E) and spleen, (F), *t-bet* in liver (G), and kidney (H) and *nitr9* in liver (I) and kidney (J) are presented as mean fold change relative to the time zero group ± standard deviations measured by quantitative RT-PCR. *Arp* was used as a housekeeping gene. hpi= hours post injection; control= PBS (endotoxin-free); Treated= R848. *Significant ($p<0.05$) difference in expression of treated compared to control. No significant changes in expression were observed in *t-bet* and *nitr9* in spleen (Table A1).

Table 3.2 Summary of expression changes of significantly up-regulated and down-regulated genes following immune stimulation

Treatment	Tissues	<10 fold change	10-100 fold change	>100 fold change	Down regulated genes
β glucan	**Liver**	*tnfα* (1 hpi) *tnfα* (12 hpi)		*ifnγ* (1 hpi) *ifnγ* (6 hpi) *ifnγ* (12 hpi) *ifnγ* (24 hpi)	
β glucan	**Kidney**	*tnfα* (1 hpi) *tnfα* (6 hpi) *t-bet* (6 hpi)	*ifnγ* (1 hpi) *ifnγ* (12 hpi)	*ifnγ* (6 hpi)	*nitr9* (12 hpi) *nitr9* (24 hpi)
β glucan	**Spleen**		*ifnγ* (1 hpi) *ifnγ* (12 hpi)	*ifnγ* (6 hpi) *ifnγ* (24 hpi)	
Poly I:C	**Liver**	*mx* (1 hpi) *mx* (6 hpi) *mx* (12 hpi) *mx* (24 hpi)			
Poly I:C	**Kidney**	*ifnγ* (12 hpi) *t-bet* (6 hpi)	*mx* (6 hpi) *mx* (12 hpi) *mx* (24 hpi) *nitr9* (1 hpi)		*tnfα* (24 hpi)
Poly I:C	**Spleen**	*mx* (6 hpi)	*mx* (12 hpi)		*tnfα* (24 hpi)
R848	**Liver**	*nitr9* (6 hpi)	*ifnγ* (12 hpi) *ifnγ* (24 hpi) *nitr9* (1 hpi) *t-bet* (6 hpi) *mx* (1 hpi) *mx* (6 hpi) *mx* (12 hpi) *mx* (24 hpi)	*ifnγ* (6 hpi) *t-bet* (1 hpi) *t-bet* (24 hpi)	
R848	**Kidney**	*t-bet* (1 hpi) *t-bet* (6 hpi)	*ifnγ* (1 hpi) *ifnγ* (12 hpi) *ifnγ* (24 hpi) *nitr9* (6 hpi) *mx* (1 hpi) *mx* (6 hpi) *mx* (12 hpi) *mx* (24 hpi)	*ifnγ* (6 hpi)	
R848	**Spleen**	*mx* (6 hpi) *mx* (12 hpi) *ifnγ* (6 hpi) *ifnγ* (12 hpi) *ifnγ* (24 hpi)			

3.4 Discussion

In this study, targeted activation of specific TLRs could suggest the cell population(s) mediating the protective response in our model. Fold changes of significantly up-regulated or down-regulated genes are summarized in Table 3.2.

Mx is produced by many leukocyte types and increased expression suggests immune system stimulation. *Tnfα* is produced by NK cells and macrophages, and increased *tnfα* expression suggests NK cell and macrophage stimulation. *Ifnγ* is produced by NK cells and macrophages, but primarily by NK cells, and up-regulated *ifnγ* suggests NK cell stimulation in our lymphocyte deficient fish. T-bet is a transcription factor of NK cells and T cells and up regulation suggests NK cell expansion in a T cell deficient model. Increased *nitr9* suggests increased NK cell activity.

β glucan did not affect *mx* expression in any *rag1*$^{-/-}$ mutant zebrafish tissues. In contrast, β glucan + Poly I:C co-administration up-regulated *mx* expression in kidney and spleen tissue of common carp (Falco, Miest et al. 2014).

β glucan down-regulated *tnfα* expression in common carp (Falco, Miest et al. 2014). β glucan induced less than 10 fold increases (4 and 8 fold change) in kidney *tnfα* expression at 1 and 6 hpi in our *rag1*$^{-/-}$ mutant zebrafish. β glucan administered with *A. hydrophila* up-regulated *tnfα* expression in zebrafish kidney cells (Rodríguez, Chamorro et al. 2009), while zebrafish infected with live *A. hydrophila* had increased kidney *tnfα* expression that later declined (Rodríguez, Novoa et al. 2008). However, in fish injected with heat killed *A. hydrophila* there was a low but consistent expression of kidney *tnfα.* Following *E. tarda* infection of zebrafish, there was a 14 fold increase of *tnfα* in pooled liver and spleen tissues, and these levels later declined (Pressley, Phelan et al. 2005).

Elevated expression of *tnfα* in zebrafish larvae followed exposures of *Listonella anguillarum* (Rojo, de Ilárduya et al. 2007) and *Vibrio anguillarum* (Oyarbide, Rainieri et al. 2012).

These cumulative responses resulted in a 1500 fold increase of liver *ifnγ* expression at 1, 6, 12 and 24 hpi in our mutant zebrafish. The overall greatest change was in *ifnγ* expression following β glucan exposure. β glucan stimulates all immune cells and this resulted in increased production of a broad range of cytokines that further stimulated immune cells, resulting in an expanding immune response and accumulation of cytokines. Stimulated accessory immune cells such as macrophages and dendritic cells further stimulate NK cells (Adib-Conquy, Scott-Algara et al. 2014). In mice treated with soluble β glucan, changes in cytokine expression correlated with changes in cell populations (Hida, Ishibashi et al. 2009). At 6 hpi, *rag1*$^{-/-}$ kidney *ifnγ* expression increased to 193 fold change, while 10 to 100 fold increases (68 and 63 fold change) in kidney *ifnγ* expression occurred at 1 and 12 hpi. Similarly increased *ifnγ* expression levels were found in WT zebrafish kidney tissue at 6 hpi following β glucan treatment (Rodríguez, Chamorro et al. 2009). In the *rag1*$^{-/-}$ mutant zebrafish spleen, β glucan induced greater than 100 fold increases in *ifnγ* expression at 6 and 24 hpi (111 and 116 fold change respectively) and between 10 and 100 fold increase in splenic *ifnγ* expression at 1 and 12 hpi (70.24 and 95 fold change respectively). *Ifnγ* was also up-regulated in Atlantic salmon kidney, spleen, skin and intestine following *Aeromonas salmonicida* infection (Kumari, Zhang et al. 2015). In contrast, administration of β glucan alone induced *ifnγ* expression only at 6 hpi in kidney, and when injected with *A. hydrophila,* induced kidney *ifnγ* expression at 4 hpi and later declined (Rodríguez, Novoa et al. 2008).

In our *rag1*$^{-/-}$ mutant zebrafish, β glucan induced a <10 fold increase (0.6 fold change) in kidney *t-bet* expression at 6 hpi, but did not induce changes in *t-bet* expression in any other tissues. In healthy grass carp, *t-bet* expression was higher in head kidney and spleen than in gill, kidney, intestine and liver. Further, following infection with grass carp reovirus, elevated expression of *ifnγ* co-related with elevated *t-bet* expression in kidney tissue (Wang, Shang et al. 2013). In Atlantic salmon infected with *A. salmonicida*, there was an increased expression of *t-bet* in kidney tissue and moderate to low expression in spleen tissue (Kumari, Zhang et al. 2015). *T-bet* is a transcription factor for T cells and NK cells, and these findings suggest that in *rag1*$^{-/-}$ mutant zebrafish, β glucan stimulated NK cell development in the kidney hematopoietic tissue, resulting in *t-bet* up-regulation at 6 hpi. The decrease in *t-bet* expression after 6 hpi in our study could result from NK cells trafficking out of the kidney hematopoietic tissue at that time.

In our study *rag1*$^{-/-}$ mutant zebrafish kidney tissue, β glucan treatment resulted in down-regulated *nitr9* expression at 12 and 24 hpi. After WT zebrafish were infected with *Listonella anguillarum*, there was a 2 fold and 2.6 fold increased *nitr9* expression at 4 hpi and 6 hpi, respectively, followed by a decline in expression at 22 hpi (Rojo, de Ilárduya et al. 2007). However, in that study, *nitr9* expression was quantified from individual whole fish, and we analyzed individual tissues from separate fish. The downregulation of *nitr9* expression in the *rag1*$^{-/-}$ mutant kidney tissue (Table 2.2) probably resulted from *nitr9* positive NK cells trafficking out of the kidney and into other tissues in the zebrafish, similar to how changes in tissue cytokine expression were correlated to changes in cell populations following β glucan exposure in mice (Falco, Miest et al. 2014). *A. salmonicida* infection strongly induced *t-bet* expression in Atlantic salmon

kidney (Kumari, Zhang et al. 2015). The trends of up and down regulation of gene expression of NK cell specific *t-bet* and *nitr9* (in our T and B cell deficient zebrafish) suggest the stimulation of NK cells by TLR 2/6 and the resulting production of *ifns*, and the development of NK cells reflected by the up-regulation of the *t-bet* transcription factor at 6 hpi. At 12 and 24 hpi, NK cells trafficked out of the kidney, and *nitr9* expression decreased below control levels.

Poly I:C induced *ifn* and *ifn*-inducible genes (Robertsen 2006), and *mx* expression is an indicator of increased type I interferon in fish (Saint-Jean and Pérez-Prieto 2007) and mice (Roers, Hochkeppel et al. 1994). In the liver, Poly I:C induced a <10 fold up-regulation of *mx* at 1, 6, 12 and 24 hpi in our *rag1*$^{-/-}$ mutant zebrafish. Liver *mx* expression was also up-regulated in yellow perch and directly correlated to viral load of viral hemorrhagic septicemia virus (Olson, Emmenegger et al. 2013). *Mx* expression was elevated 10 fold in liver of Atlantic salmon one week following treatment with Poly I:C (Das, Ellis et al. 2009). Following Poly I:C treatment, there was a <10 fold increased expression of *ifna* and *ifnc* (type 1 interferon) in the liver of Atlantic salmon, suggesting *mx* up-regulation (Svingerud, Solstad et al. 2012).

In the kidney, we saw the greatest fold increases (10 to 100 fold) in *mx* expression at 6, 12 and 24 hpi following Poly I:C treatment in *rag1*$^{-/-}$ mutant zebrafish. Type 1 interferon was up-regulated in rainbow trout head kidney tissue following treatment with Poly I:C (Palti, Gahr et al. 2010), suggesting subsequent up-regulation of *mx* expression. *Mx* expression was elevated 35 fold and 10 fold in head kidney of Atlantic salmon at 2 and 5 days, respectively, following treatment with Poly I:C (Das, Ellis et al. 2009). When WT zebrafish kidney cells were exposed to Poly I:C, *mx* expression was up-regulated

(Mitra, Alnabulsi et al. 2010). In another study, Poly I:C induced *mx* expression in zebrafish within 48 hours after injection (Kavaliauskis, Arnemo et al. 2015). Following Poly I:C treatment in Atlantic salmon hepatic *mx* expression was elevated 10 fold (Das, Ellis et al. 2009) and a <10 fold increased hepatic *mx* expression of type I interferon (Svingerud, Solstad et al. 2012) and an up-regulation of type 1 and 2 interferon in rainbow trout head kidney tissue (Palti, Gahr et al. 2010), suggesting *mx* up-regulation in these fish.

Rag1$^{-/-}$ mutant zebrafish splenic *mx* expression was up-regulated <10 fold at 6 hpi and 10 to 100 fold at 12 hpi. Poly I:C treatment also induced *mx* up-regulation in WT zebrafish spleen cells (Mitra, Alnabulsi et al. 2010), and rainbow trout spleen (Purcell, Kurath et al. 2004). Poly I:C induced a <10 fold increased expression of *ifna* and *ifnc,* suggesting *mx* expression (Svingerud, Solstad et al. 2012) and 30 and 10 fold *mx* expression at day 2 and 5, respectively, (Strandskog, Skjæveland et al. 2008) in the spleen of Atlantic salmon. Further, following Poly I:C treatment in rock bream, *mx* expression increased and peaked at day 2 (4.2 fold) and then gradually decreased (Kim, Oh et al. 2012). In contrast to these findings, Poly I:C did not increase *mx* expression in carp spleen tissues (Falco, Miest et al. 2014).

In our study, Poly I:C did not affect liver *tnfα* in *rag1*$^{-/-}$ mutant zebrafish, and this agreed with findings in common carp (Falco, Miest et al. 2014). Poly I:C down-regulated *tnfα* in *rag1*$^{-/-}$ mutant kidney tissues. In gilthead seabream, at 0.5 and 1.5 hpi, Poly I:C significantly up-regulated *tnfa* in acidophilic granulocytes and macrophages (Sepulcre, López-Castejón et al. 2007), in the head kidney (García-Castillo, Pelegrín et al. 2002, Roca, Mulero et al. 2008) and rainbow trout anterior kidney leukocytes (Purcell, Smith et

al. 2006). Interestingly, we found kidney *tnfα* was down-regulated following Poly I:C treatment. These decreases in our mutant zebrafish could result from macrophages and/or NK cells trafficking out of the kidney hematopoietic tissue. Another study reported that Poly I:C did not have an effect on *tnfα1* and *tnfα2* in common carp kidney tissue (Falco, Miest et al. 2014). Poly I:C also down-regulated splenic *tnfα* in our mutant zebrafish. Poly I:C had no effect on splenic *tnfα* expression in rainbow trout (Purcell, Kurath et al. 2004) and common carp (Falco, Miest et al. 2014).

In *rag1*$^{-/-}$ mutant zebrafish, Poly I:C treatment had no effect on liver *ifnγ* expression. Kidney *ifnγ* expression was up-regulated less than 10 fold at 12 hpi in *rag1*$^{-/-}$ zebrafish kidney. In Atlantic salmon following Poly I:C treatment, there was 10 fold increased expression of *ifnγ* on day 2 and no effect on day 5 (Strandskog, Skjæveland et al. 2008). *Ifnγ* was up-regulated following Poly I:C treated kidney tissues in rainbow trout (Zou, Carrington et al. 2005), in grass carp (Wang, Shang et al. 2013) and in kidney cells of *Labeo rohita* (Parhi, Sahoo et al. 2015). Poly I:C exposure up-regulated *ifnγ* expression in WT zebrafish kidney cells (Mitra, Alnabulsi et al. 2010) and in head kidney tissue of grass carp (Wang, Shang et al. 2013). In *rag1*$^{-/-}$ mutant zebrafish, Poly I:C treatment had no effect on splenic *ifnγ* expression. Similarly, in Atlantic salmon following Poly I:C treatment, there was no effect on *ifnγ* expression in the spleen on day 2 and 5 (Strandskog, Skjæveland et al. 2008). In contrast, *ifnγ* was up-regulated in Poly I:C treated spleen tissues in rainbow trout (Zou, Carrington et al. 2005).

Poly I:C did not affect liver *t-bet* expression of *rag1*$^{-/-}$ mutant zebrafish. *T-bet* expression was up-regulated 1.2 fold at 6 hpi in kidney tissue suggesting Poly I:C induced NCC and NK cell development. Poly I:C exposure also up-regulated kidney *t-bet*

in WT zebrafish (Mitra, Alnabulsi et al. 2010) and grass carp (Wang, Shang et al. 2013). Poly I:C did not affect splenic *t-bet* expression in *rag1*$^{-/-}$ mutant zebrafish, but up-regulated splenic *t-bet* in WT zebrafish (Mitra, Alnabulsi et al. 2010).

At 1 hpi of Poly I:C, mutant zebrafish kidney tissue demonstrated a 19 fold increase in *nitr9* expression suggesting stimulation of NK cells. This was the only Poly I:C induced up-regulation of *nitr9* in our study. Following SHRV infection, there was no change in *nitr9* expression between *rag1*$^{-/-}$ and WT zebrafish kidney and spleen (Shah 2009). Since *nitr9* is a putative receptor of zebrafish NK cells (Wei, Zhou et al. 2007, Shah, Rodriguez-Nunez et al. 2012), our findings suggest that Poly I:C induced kidney NK cell stimulation, followed by an expansion of NK cells (demonstrated by kidney *t-bet* up-regulation at 6 hpi).

R848 has immune modulating roles in mammals (Tomai, Imbertson et al. 2000, Wu, Huang et al. 2004, Gorski, Waller et al. 2006), Japanese flounder (Zhou and Sun 2015), Atlantic salmon (Svingerud, Solstad et al. 2012), and rainbow trout (Purcell, Kurath et al. 2004, Purcell, Smith et al. 2006). The TLR7/8 NF-κB–My-d88 signaling pathway has been demonstrated in Japanese flounder (Zhou and Sun 2015). R848 significantly up-regulated hepatic *mx* expression at 1, 6, 12 and 24 hpi (50 fold increase), suggesting a steady rise in type I interferon expression in our mutant zebrafish, and we also saw a 35 fold up-regulation of *mx* in kidney tissue. Increases in type 1 interferon were observed in rainbow trout (Palti, Luo et al. 2009) and Atlantic salmon kidney (Svingerud, Solstad et al. 2012), and fluorescent *in* situ hybridization further demonstrated that a small population of head kidney cells produced type 1 interferon (Svingerud, Solstad et al. 2012). We also saw a 6 fold increase in splenic *mx* gene

expression at 6 hpi and 8 fold increase at 12 hpi. In Atlantic salmon, type 1 interferon was significantly up-regulated by a small population of splenic cells (Svingerud, Solstad et al. 2012).

In our study, R848 did not induce differential *tnfα* expression in any tissues at any time. R848 induced up-regulation of *tnfα* in rainbow trout kidney (Purcell, Kurath et al. 2004), when used higher at levels than in our study.

In *rag1*$^{-/-}$ mutant zebrafish, we noted that liver *ifnγ* gene expression increased 100 fold at 6 hpi, 70 fold at 12 hpi and 40 fold at 24 hpi. Kidney *ifnγ* increased 10 to 100 fold at 1, 12 and 24 hpi (13, 31 and 24, respectively). The highest expression for kidney *ifnγ* following R848 treatment was 153 fold change at 6 hpi. Splenic *ifnγ* gene expression increased 7 fold at 6 hpi, 8 fold at 12 hpi and 6 fold at 24 hpi. The effects of R848 on *ifnγ* expression are not documented in other fish.

In our mutant zebrafish, R848 induced increased *t-bet* expression in the liver, (100 fold at 1 hpi, 12 fold at 6 hpi and 200 fold at 24 hpi), and kidney (1 hpi and a 7 fold increase at 6 hpi). Hepatic *nitr9* expression increased (50 fold at 1 hpi, and 6 fold at 6 hpi), as did kidney *nitr9* gene expression (increased 10 fold at 6 hpi). The effects of R848 on *t-bet* or *nitr9* tissue expression in other fish are not documented. Gene expression of transcription factors does not necessarily correlate to protein expression. However, we feel the significant increases seen in *t-bet* following TLR ligand treatments suggests an increased number of non-specific cytotoxic cells (NCCs) and NK cells, while *nitr9* expression more specifically represents an NK cell population.

3.5 Conclusion

Our findings reveal important insight into the way fish that have limited lymphocyte based immunity can respond to infections. T and B cell deficient *rag1*$^{-/-}$ mutant zebrafish demonstrate specific protection (Hohn and Petrie-Hanson 2012), and our current study was performed to elucidate the cell population(s) involved in that response by interpreting transcript level changes in immune tissues. Differential expressions of immune response genes suggest how tissue environments induce changes in innate immune cells that can result in these cells having the capacity to mediate protective immunity at subsequent pathogen exposure.

Following β glucan treatment the up-regulated *tnfα* predominately resulted from stimulated macrophages. The lack of NK cell specific *nitr9* up-regulation suggests that the hepatic NK cell population is small, and below detectable levels in the liver, or that there is a hepatic population of *nitr*$^{-}$ NK cells. β glucan induced hepatic *ifnγ* expression could have resulted from stimulated macrophages and dendritic cells, or *nitr9*$^{-}$ cytotoxic cells. Up-regulation of kidney *t*-bet expression at 6 hpi suggests NK cells are developing. Down-regulation of kidney *nitr9* expression at 12 hpi and 24 hpi suggests *nitr9* expressing NK cells may be trafficking out of the kidney at those times. The finding that β glucan did not stimulate *nitr9* expression and induced stimulation of *tnfα* expression, suggests a potential monocyte/macrophage role as well as possibly NK cells during protective immunity in *rag1*$^{-/-}$ mutant zebrafish. This protective response could involve 'trained' monocytes/macrophages, using functions previously described (Bowdish, Loffredo et al. 2007, Quintin, Saeed et al. 2012).

Poly I:C induced significant *mx* up-regulation, which is indicative of type 1 interferons. Type 1 interferons induce multiple immune changes, and undoubtedly induced the kidney tissue *tnfα* down-regulation observed in our fish. Significant increases in *t*-bet and *nitr9* indicate NK cell development and stimulation. In lymphocyte deficient animals, *ifnγ* is primarily produced by stimulated NK cells and by macrophages to a lesser extent.

Following R848 treatment the greatest up-regulation of *nitr9* and *t*-bet occurred in the liver, but the expression profiles indicate that R848 also induces NK cell development and stimulation in the kidney. Our studies suggest R848 stimulates NK cells better than Poly I:C, inducing *t-bet*, *nitr9* and *ifnγ*. R848 is a better NK cell inducer than Poly I:C in humans as well (Hart, Athie-Morales et al. 2005). It is interesting to note that the strong induction of liver NK cell gene expression occurred with R848 and studies with lymphocyte deficient mice demonstrated that protection against cytomegalovirus infection involved a population of $Ly49H^{+}$ NK cells in the liver (Sun et al. 2009) and this population demonstrated proliferation and contraction, forming a memory NK cell pool that can respond rapidly and provide protection following secondary exposure.

Down-regulated gene expression levels did not occur in the liver, suggesting cells do not exit from this tissue. In the kidney, down-regulated expression of *nitr9* may be the result of NK cells exiting the kidney, and indicative of the bone-marrow equivalent role of this tissue. The spleen has largely antigen presentation and processing roles, and in our mutant zebrafish, changes in splenic immune gene expressions are not predictable and are much lower than in the liver and kidney. *T-bet* and *nitr9* levels did not change, suggesting

that under the conditions of this study, splenic NK cells were not stimulated to differentiate, respond immunologically, or proliferate.

We saw a pattern of NK cell associated gene expression. *T-bet* up-regulation either preceded or occurred simultaneously with *nitr9*, and *nitr9* expression changes never preceded those of *t-bet*, suggesting NK cell development and differentiation occur before *nitr9* expression. Another possibility is *t-bet* up-regulation represents *nitr9*$^{-}$ NK cells and *nitr9*$^{+}$ NK cells. Our findings of strong *ifnγ* up-regulation and a small *t-bet* up-regulation with no *nitr9* up-regulation (in the β glucan/vaccine group) suggest that there may be a *nitr9*$^{-}$ NK cell subpopulation and this population was stimulated. Intracoelomic injection of β glucan and R848 caused >100 fold increase in *ifnγ* expression in liver and kidney. *Rag1*$^{-/-}$ mutant zebrafish can develop protective immunity (Hohn and Petrie-Hanson 2012) and these findings suggest protective immunity can be induced and/or enhanced by NK cells when lymphocyte based immunity is compromised.

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CHAPTER IV

DIFFERENTIAL EXPRESSION OF NK CELL SPECIFIC MARKERS FOLLOWING R848 AND VTX TREATMENTS IN *RAG1*$^{-/-}$ MUTANT ZEBRAFISH

Abstract

Previously we used targeted activation of specific TLRs to determine the presence and tissue location of Natural Killer (NK) cells in *rag1*$^{-/-}$ mutant zebrafish. We also identified the NK cell genes associated with protection and enhanced protection from bacterial infection. In this study, we analyzed differential expression of NK cell specific genes *ifnγ, nitr9, t-bet*, *Nk-lysin*(*nkl)a, nklb, nklc* and *nkld* in liver, kidney and spleen tissues after treatment with two mammalian NK cell stimulators. The expression level of the genes was tissue specific and varied between the two concentrations of R848 and VTX that are TLR7/8 and TLR8 ligands, respectively. Overall, the highest responses were from R848, and predominant Nk-lysins were *nklc* in the liver and spleen, and *nkld* in the kidney. The Nk-lysins most affected by VTX were *nklc* in the spleen and *nkld* in the kidney. VTX did not significantly increase NK-lysins in hepatic NK cells. We also evaluated novel immune type receptor 9 (NITR9) protein expression in liver and kidney tissues, and by flow cytometry. Results generated from the selected NK cell genes support the role of NK cells during immune stimulation in teleosts.

4.1 Introduction

Teleost have a well-developed innate and adaptive immune system with cells morphologically similar to their mammalian counterparts. These features provide the fish with a capacity to mediate immune responses to infections similar to mammals. In teleost, the adaptive immune system is developed between two to eight weeks post hatch. Fish larvae and young survivors depend mostly on their innate immune defense system for protection against pathogens. Zebrafish innate immune cells include monocytes, macrophages, basophils, eosinophils, mast cells, neutrophils, non-specific cytotoxic cells (NCCs) (Moss, Monette et al. 2009) and natural killer (NK) cells (Muire, Hanson et al. 2017). The NK cells were recently identified as large granular lymphocyte like cells (LLCs) in zebrafish (Muire, Hanson et al. 2017). Cytokine release is a by-product of a cellular encounter with infected cells, tumor induced cells or indirect activation by cytokines produced by toll like receptor (TLR) activation on other innate immune cells.

Zebrafish NK cells are morphologically similar to mammalian NK cells. Zebrafish NK cells contain granules that resemble the cytotoxic granules of mammalian NK cells. NK cells contain Nk-lysins that are anti-microbial peptides (AMPs) present in the cytotoxic granules. The primary function of Nk-lysins is to provide defense against pathogens. Nk-lysins are the largest AMPs with 74-78 amino acids (Liepinsh, Andersson et al. 1997). They were first identified as anti-bacterial peptides in porcine NK cells and cytotoxic T lymphocytes (Andersson, Gunne et al. 1996). Four Nk-lysins paralogs have been characterized in zebrafish (*nkla, nklb, nklc* and *nkld*) (Pereiro, Varela et al. 2015).

Nk-lysins are mainly found in T cells and NK cells. They mediate antimicrobial activity in three ways (i) by activating dendritic cells, monocytes and macrophages via

chemotaxis and triggering the release of IL-1, IL-8, cytokines and other proinflammatory cytokines (Zhang, Long et al. 2013) (ii) by binding to intracellular and (iii) extracellular bacterial membranes and causing lysis. Junfeng Chen et al in 2016 have shown pore formation and lysis of bacterial cells by NK1, a hydrophobic domain containing Nk-lysin (Chen, Yang et al. 2016).

Until now, Nk-lysin genes have been identified in mammalian species such as cow (Endsley, Furrer et al. 2004), pig (Andersson, Gunne et al. 1996), horse (Davis, Sang et al. 2005), water buffalo (Kandasamy and Mitra 2009), in birds such as chicken (Lee, Jang et al. 2014) and quail (Ishige, Hara et al. 2016). In fish, eight NK-lysin genes have been identified from five species, i.e. Japanese flounder (*Paralichthys olivaceus*) (Hirono, Kondo et al. 2007), pufferfish (Takifugu rubripes) (GenBank accession number XP_003962755), Atlantic salmon (Salmo salar) (GenBank accession number NP_001134582), zebrafish (Danio rerio) (GenBank accession number AY184216), channel catfish (Ictalurus punctatus) (Wang, Wang et al. 2006) and *Cynoglossus semilaevis* (Zhang, Li et al. 2014). In 2004, Yoder et al 2004 sequenced *nkld* cDNA from zebrafish (Yoder 2004). Recently, Pereiro et al 2015 identified 4 paralogs of NK lysins (*nkla, nklb, nklc* and *nkld*) in tandem on chromosome 17 in zebrafish (Pereiro, Varela et al. 2015). Zebrafish have two tripled clusters of Nk-lysin genes on chromosome 17 and one on chromosome 5 (Wang, Wang et al. 2006). The reason for these multiple repeats on the same gene is due to the fact that in fish, during evolution, the Nk-lysin gene underwent a teleost-specific duplication event. There is 44.6% similarity between the Nk-lysin genes in channel catfish and zebrafish (Wang, Wang et al. 2006). NK lysins' highest synteny was found between zebrafish and cave fish (*A. mexicanus*) (Pereiro,

Varela et al. 2015). Zebrafish *nkla* and *nklb* were found to be more homologous to catfish *Nkl3*, while *nklc* and *nkld* were more related to *Nkl2* catfish (Pereiro, Varela et al. 2015). The zebrafish *nkld* showed the highest scores with regard to synteny with Nk-lysins from teleost species (Pereiro, Varela et al. 2015). So far three distinct Nk-lysin transcripts have been identified in channel catfish respectively Nk-lysin 1, Nk-lysin 2, Nk-lysin 3 (Wang, Wang et al. 2006), (Chen, Yang et al. 2016). The catfish NK-lysin shared less than 25% amino acid identity with any mammalian sequence. Catfish NK-lysin exhibits a conserved expression pattern with mammalian orthologues suggesting conservation of gene function (Wang, Wang et al. 2006).

Granulysin and Nk-lysins are intracellularly synthesized and are stored in the cytoplasmic granules of NK cells and cytotoxic T lymphocytes (Andersson, Curstedt et al. 1995), (Jongstra, Schall et al. 1987) , (Yabe, Mcsherry et al. 1990).

R848 and VTX enhance NK cell function in mammals (Hart, Athie-Morales et al. 2005) (Lu, Dietsch et al. 2012). R848 is a synthetic TLR7/8 ligand that mimics ssRNA. VTX is a novel, highly potent and selective TLR8 ligand and an immunetherapeutic chemical. NK cells are major producers of interferon gamma (*ifnγ*) (Arase, Arase et al. 1996). Both these ligands are known to induce *ifnγ* expression through activation of NK cells, increase NK cell lytic activity and enhance NK cell ability to lyse tumor cells through antibody-dependent cell-mediated cytotoxicity (ADCC) (Lu, Dietsch et al. 2012). TLR7 and 8 when activated by their ligands, have the ability to upregulate other TLRs and further modulate the immune response to bacterial pathogens. VTX activates monocytes, DCs and NK cells and increase *ifnγ* production, cytolytic activity, and enhance rituximab-mediated ADCC (Lu, Dietsch et al. 2012). The use of R848

(Resiquimod) is effective at activating local immune responses and NK cells (Tomai, Miller et al. 2007). Yet, Hart et al 2012 report that despite the presence of TLR8 in NK cells, most of the time NK cell cytotoxicity and cytokine production is primarily mediated through accessory cells. However, Hailing Lu 2012 et al in their study have demonstrated that purified NK cells were directly activated by VTX. They reported an increased ADCC activity of NK cell as well as increase in *ifnγ* production (Lu, Dietsch et al. 2012).

TLR 7 and 8 are phylogenetically similar and are expressed on intracellular vesicles. They are capable of sensing different viral pathogens by recognizing ssRNA and short dsRNA that are released from phagosomes to the endolysosomal compartments (Gringhuis, Van Der Vlist et al. 2010). TLR-mediated recognition of PAMPS in the phagosome promotes selection of antigens for optimal presentation on MHC class II. TLR8 responds not only to viral pathogens but also gets activated upon binding of bacterial RNA (in humans) which leads to the production of a variety of nuclear factor (NF)-kB-mediated cytokines (i.e. tumor necrosis factor) and type I IFNs necessary for activation and differentiation of lymphocytes (Davila, Hibberd et al. 2008) (Davila, Hibberd et al. 2008, Cervantes, Dunham-Ems et al. 2011, Kawai and Akira 2011). Recent studies have shown that stimulation of TLR 7 and 8 can play a role in mediating NK cell activation (Jurk, Heil et al. 2002, Hart, Athie-Morales et al. 2005).

NK cells are major producers of interferon gamma (*ifnγ*) (Arase, Arase et al. 1996). The novel type immune receptor 9 (*nitr9*) has been characterized in zebrafish (Wei, Zhou et al. 2007). *Nitr9* is a putative activating receptor present on NK cells similar to human KIRs and murine Ly49 (activating NK cell receptors) and it mediates its signal

via the adaptor molecule DAP12 (Wei, Zhou et al. 2007) similar to mammalian activating receptors. *T-bet* is a NK cell and T cell transcription factor (Gordon, Chaix et al. 2012) and has been characterized in zebrafish (Mitra, Alnabulsi et al. 2010). *T-bet* or Tbox-21 is a transcription factor for NK cells and Th1 cells and is required for cellular development. *T-bet* gene was recently characterized in zebrafish (Mitra, Alnabulsi et al. 2010).

A few reports are available on the anti-bacterial and anti-viral activity of Nk-lysins from aquatic species but no work has been published showing the different expression levels of the Nk-lysins following exposure to different NK cell specific TLR ligands. The *rag1*$^{-/-}$ mutant zebrafish are devoid of mature T and B cells (Tokunaga, Shirouzu et al. 2017), (Petrie-Hanson, Hohn et al. 2009) and is a suitable model to study the functions and characteristics of NK cells.

In our study, we use the *rag1*$^{-/-}$ mutant zebrafish model and TLR ligands R848 (TLR7/8 ligand) and VTX (TLR8 ligand) to discern NK cell subpopulations in zebrafish tissues. We used genes that are expressed in T call and NK cells: *nkla, nklb, nklc, nkld, ifnγ, nitr9* and *t-bet* as bio-markers for NK cells (in *rag1*$^{-/-}$ mutant zebrafish). We compared the differential effect of the TLR ligands on the expression of NK cell expressed genes and estimated NITR9 protein expression. We also used flow cytometry to identify cell populations positive for NITR9.

4.2 Materials and methods

4.2.1 Zebrafish care

Zebrafish were raised and maintained at 28°C in the specific pathogen free hatchery in the College of Veterinary Medicine, Mississippi State University (MSU) following standard lab protocols. The MSU Institutional Animal Care and Use

Committee (IACUC) approved experimental protocols. A homozygous breeding colony of *rag1*$^{-/-}$ mutant zebrafish was previously established (Petrie-Hanson, Hohn et al. 2009) and bred at the CVM-SPF hatchery and the experimental fish are progeny from that colony. Both adult males and females of 6 months age and weighing on an average 0.5g, were used in this study.

4.2.2 TLR ligand injection rates

Rag1$^{-/-}$ zebrafish were IC injected with VTX (0.25µl or 2.5µl/0.5g of fish) and R848 (0.08µl or 0.8µl)/0.5g of fish) or endotoxin free saline. TLR ligands were reconstituted in endotoxin free saline and a final volume of 10µl was injected per fish.

4.2.3 RNA extraction and cDNA preparation

Fish were euthanized in buffered 0.02% Tricaine Methanesulfonate solution (Finquel MS-222; Argent Chemical Laboratories, Redmond, WA) and liver, kidney and spleen tissues were surgically removed at 0h (non-injected fish (n=5) for basal expression), 6, 12 and 24 hours post injection (hpi) (n=5) for each TLR ligand. Whole tissues were immediately transferred to 400µl Trizol reagent (Zymo Research, USA) and homogenized following standard procedures in our lab (Elibol-Fleming 2006). Total RNA was extracted individually from each liver, kidney and spleen homogenized sample according to manufacturer's protocol using RNA extraction kit (Zymo Research, USA). The quantity of extracted total RNA was determined by NanoDrop ND-1000 spectrophotometer and stored at -80°C until use. cDNA (100ng) was prepared from RNA by using Super script III VILO^{TM} cDNA Synthesis Kit (Invitrogen).

4.2.4 Quantifying gene expression

Ifnγ, t-bet, *nitr9, nk lysin a, b, c* and *d* were measured using real time quantitative PCR (Takara). The *t-bet, nk lysin a, b, c* and *d* primers and probes (Table 1) were designed by Primer3 plus (GraphPad) software and primers and probes of *ifnγ, nitr9* (Table 1) were adopted from previous publications. All primers and probes were purchased from Eurofins MWG, Operon, Huntsville, Alabama, USA. Amplification of the ubiquitously expressed acidic ribosomal phosphoprotein (*arp*) gene was used for the internal control (Ju, Xu et al. 1999). The amplification was performed in a 25µl volume containing 10 µl target cDNA and 15 µl master mix containing: 8.8 µl Nuclease free water (GIBCO, Ultra Pure^{TM}), 1.5 µl $MgCl_2$ (5mM), 2.5 µl 10x buffer, 0.5 µl dNTPs, 0.2µl Taq Polymerase HS enzyme (Hot Start PCR Kit, TAKARA, Japan), 0.5 µl forward primer (20µM), 0.5 µl reverse primer (20µM) and 0.5 µl probe (10µM). Thermal cycler parameters for the PRC program were set as follows: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 61°C for 1 minute. All samples (biological reps) were run in triplicate.

Table 4.1 Oligonucleotide primers and probes used for qRT-PCR to quantify gene expression levels of *mx, tnfα, ifnγ, t-bet* and *nitr9*.

Gene	Oligonucleotide sequences (5'–3')	GenBank Accession No.
arp	**Fwd:** CTGCAAAGATGCCCAGGGA **Rev:** TTGGAGCCGACATTGTCTGC **Probe:**[6~FAM]TTCTGAAAATCATCCAACTGCTGGATGA CTACC [BHQ1a~ Q] (Vojtech, Sanders et al. 2009)	NM_131580
ifnγ	**Fwd:** CTTTCCAGGCAAGAGTGCAGA **Rev:** TCAGCTCAAACAAAGCCTTTCG **Probe:**[6~FAM]AACGCTATGGGCGATCAAGGAAAACGAC [BHQ1a~ Q] (Vojtech, Sanders et al. 2009)	NM_212864
t-bet	**Fwd:**GATCAAGCTCTCTCTGTGATAG **Rev:**GCTAAAGTCACACAGGTCT **Probe:**[6~FAM]TTCTGAAGGTCACGGTCACA[BHQ1a~Q] *	NM_001170599.1

Table 4.1 (continued)

nitr9	**Fwd:** GTCAAAGGGACAAGGCTGATAGTT **Rev:**GTTCAAAACAGTGCATGTAAGACTCA **Probe:** [6~FAM]CAAGGTTTGGAAAAGCAC[BHQ1a~Q] (Shah, Rodriguez-Nunez et al. 2012)	AY570237.1
nkla	**Fwd:** TTTCTGGTCGGCTTGCTCAT **Rev:** TTCTCATTCACAGCCCGGTC **Probe:**[6~FAM]TCTGCAGCTCACTGGGAGGTTCGTGA[BHQ1a~Q]	NM_001311794
nklb	**Fwd:** TCCGCAACATCTTTCTGGTCA **Rev:** AGCCTGCTCATGAATGAAAATGA **Probe:**[6~FAM]CACGCCTGCAAATCTGAACCACCCA[BHQ1a~Q]	NM_001311792
nklc	**Fwd:** CTGCTTGTGCTGCTCACTTG **Rev:** AGCACACATGGAGATGAGAACA **Probe:** [6~FAM]GGGCTTGCAAGTGGGCCATGGGAA[BHQ1a~Q]	NM_001311793.1
nkld	**Fwd:** ACCCTGCTCATCTCCTCTGT **Rev:** CCCCAGCTAAAGCAAAACCC **Probe:**[6~FAM]TGCCTGGGATGTGCTGGGCTTGCAA[BHQ1a~Q]	NM_212741.1

Housekeeping gene arp (house-keeping gene) was used as a reference gene. * The *nkla, nklb, nklc, nkld* and *t-bet* primers and probes were designed by Primer3 plus (GraphPad) software.

4.2.5 Protein expression

Rag1$^{-/-}$ mutant zebrafish were injected with R848 (0.08µl/fish) and the liver, kidney and spleen were sampled at 6 hpi. Tissues were homogenized in tissue protein extraction buffer (T-PER) (ThermoScientific) and supernatant was collected. Protein concentration was estimated in the supernatant by Bradford's assay (Sigma-Aldrich). 30µg of protein from liver, kidney and spleen cell lysate was resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad) for western blot analysis. PVDF membranes were incubated in blocking buffer (3% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T)) overnight. Membranes were washed in TBS-T and TBS twice for 5 minutes and once for 5 minutes respectively and were incubated with primary antibody: IgG mouse anti zebrafish Nitr9$^{90.10.5}$ mono-

clonal antibody (1:500), a gift from J. Yoder (Shah, Rodriguez-Nunez et al. 2012). The membrane was washed in TBST and TBS twice for 5 minutes and once for 5 minutes respectively and incubated with secondary antibody: goat anti-mouse IgG/HRP conjugated (1:4000) (ThermoScientific) for 1h at 4°C. The PVDF blot was stripped for 7 minutes at room temperature by Restore^{TM} PLUS Western Blot Stripping buffer (ThermoScientific) followed by one wash in TBS for 5 minutes. Membrane was incubated in blocking buffer overnight at 4°C followed by incubation with anti-GAPDH rabbit polyclonal antibody (1:500) (AnaSpec, Fremont, CA) overnight at 4°C. Membrane was washed in TBST and TBS twice for 5 minutes and once for 5 minutes respectively and incubated with secondary antibody goat anti-rabbit IgG-HRP conjugated (1:2000) (ThermoScientific) for 1h at 4°C. To visualize the bands, PVDF membrane was washed in TBST and stained with Pierce ECL western blotting substrate (ThermoScientific) and developed by using clear blue X-Ray Film (ThermoScientific). Band densities were determined using Studio Lite Software (Li-Cor).

4.2.6 Flow cytometry

Rag1$^{-/-}$ mutant zebrafish were IP injected with Saline (control), R848 (0.08µl) and VTX (2.5 mM). Kidney and liver cells were sampled 12 hours post injections (hpi). Tissues were macerated and strained through a 40micron filter in HBSS buffer w/o $Ca2^{+}$ and $Mg2^{+}$ (Sigma, H4891-10x1L). Cells were washed and separated on a histopaque 1119 gradient (11191). The layer at the interface between the cell suspension and the gradient was aspirated and washed, fixed with 4% paraformaldehyde and permeablized in BD Perm/Wash buffer (BD, 51-2091KZ). Cells were incubated with IgG mouse Anti-NITR9$^{90.10.1}$ monoclonal antibody for 1 hour at 4°C. This was followed by incubation

with goat anti-mouse IgG-PE (Biolegend, 405307) for 30 minutes at 4°C. Cells were washed after each step twice with 1ml FACS buffer at 500 x g. Labelled cells were analysed on the FACS NovoCyte with NovoExpress software.

4.2.7 Data analysis and statistical evaluation

Relative gene expression was determined using the Pfaffl method (Pfaffl 2004). Data obtained from qRT-PCR were expressed as fold change and were converted to log2 values. Two-way ANOVA followed by Dunnett's multiple comparisons test was performed to TLR ligand with PBS control. Tukey's test was performed to compare R848 to VTX treatments. Flow cytometry data was analyzed by two way ANOVA multiple comparison test. Statistics software used was GraphPad Prism version 7 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com. An alpha level of 0.05 was used to determine the significance of all analyses.

4.3 Results

4.3.1 Gene expression of *ifnγ*

In liver tissues, 0.8µl R848 caused significantly increased *ifnγ* at 6 hpi. There was no difference in expression at any time points in 2.5µl VTX treated fish. In kidney tissues, *ifnγ* expression was up-regulated at 12 hpi after treatment with 0.8µl R848. *Ifnγ* significantly increased at 6 hpi and 12 hpi in spleen tissues (Table 4.2).

In liver, there was no increase of *ifnγ* at any time points after 0.08µl of R848 or 0.25µl of VTX exposure (Table 4.2). In kidney and spleen tissues, *ifnγ* expression increased at 12 hpi following R848 injections. There was no difference in *ifnγ* expression

at any time points after VTX exposure in kidney or spleen tissues at either concentration (Table 4.2).

4.3.2 Gene expression of *t-bet*

There were no increases of *t-bet* at any time point in liver tissues after 0.8µl R848 or 2.5µl VTX. In kidney tissues, *t-bet* expression increased 24 hpi of 2.5µl VTX. In spleen tissues, *t-bet* increased 12 hpi of 2.5µlR848 (Table 4.2).

After 0.08µl R848 injection, *t-bet* increased at 24 hpi in the liver. VTX had no effect on *t-bet* in the liver. 0.08µl R848 caused increased kidney *t-bet* at 12 and 24 hpi. 0.25µl VTX increased kidney *t-bet* at 6 and 12 hpi. Injection of 0.08µl R848 increased spleen *t-bet* at 12 hpi (Table 4.2).

4.3.3 Gene expression of *nitr9*

In liver, there was a significant increase of *nitr9* expression on fish treated with 0.8µl R848 at 6 hpi (p=0.0001) and 24 hpi (p=0.0453). There was no difference in the level of expression for 2.5µl VTX (Table 2). In kidney, the expression level of *nitr9* up-regulated at 6 hpi (p=0.0046) and 24 hpi (p=0.0001) following VTX injections and at 24 hpi (p=0.0001) following R848 treatment. In spleen, there was no difference in the level of expression of *nitr9* at any time points after R848 and VTX injections (Table 4.2).

In liver, there was a significant increase of *nitr9* when treated with 0.08µl R848 at 24 hpi (p=0.0453). There was no difference in the level of expression at any other time points (Table 4.2). In kidney, the expression level of *nitr9* up-regulated at 6 hpi (p=0.0099) and 24 hpi (p=0.0064) following 0.25µl VTX injections. In spleen, the

expression level of *nitr9* up-regulated at 12 hpi (p=0.0003) and 24 hpi (p=0.0001) following R848 injections (Table 4.3).

4.3.4 Gene expression of *nkla*

In liver and kidney tissues, there were no difference in the levels of *nkla* at any time points after 0.8µl R848 and 2.5µl VTX injections (Table 4.2). In spleen tissues, the only increase in *nkla* was at 12 hpi, following 2.5 mM R848.

In liver tissues, there were no difference in the levels of *nkla* at any time points after 0.08µl R848 and 0.25µl VTX injections (Table 4.2). In kidney tissues, the only increase was at 24 hpi R848. In spleen, the expression level of *nkla* up-regulated at 12 hpi and 24 hpi after 0.08µl R848.

4.3.5 Gene expression of *nklb*

In liver, there was a significant increase of *nklb* when treated with 0.8µl R848 at 6 hpi (p=0.0001). There was no difference in liver *nklb* at any other time points, or any treatment of VTX (Table 4.2). In kidney, there was a significant *nklb* increase at 24 hpi 0.8µl R848. In spleen, there was no difference in the level of expression of *nklb* between PBS treated with R848 and VTX at 6 hpi, 12 hpi and 24 hpi (Table 4.2). In spleen tissues there were no changes in *nklb* at any time point for any treatment.

In liver tissue there were no changes in *nklb* at any time point for any 0.08µl R848 or 0.25µl VTX treatments (Table 4.2). In kidney, there was a significant increase of *nklb* at 24 hpi when treated with 0.25µl VTX. There were no differences in spleen *nklb* expression any other time points or 0.08µl R848 treatments. In spleen, there were

significant increases of *nklb* when treated with 0.08µl R848 at 12 hpi (p=0.004) and 24 hpi (p=0.0107). There were no differences for the VTX treatments (Table 4.2).

4.3.6 Gene expression of *nklc*

In liver, there was a significant increase of *nklc* when treated with 0.8µl R848 at 12 hpi. There was no difference in the level of expression of liver *nklc* for any other time points or treatments (Table 4.2). In kidney, there was a significant increase of *nklc* at 6 hpi and 24 hpi when treated with 0.8µl R848. There was no difference in the level of expression of kidney *nklc* for any other time points or treatments. In spleen, there were significant increases of *nklc* at 24 hpi following administration of 0.8µl R848 and 2.5µl VTX. There were no differences in *nklc* expression at any other time points or treatments in spleen tissues (Table 4.2).

In liver, there was a significant increase of *nklc* when treated with 0.08µl R848 at 12 hpi. There were no other differences in *nklc* expression in liver tissues for 0.08µl R848 or 0.25µl VTX (Table 4.3). In kidney, there were significant increases of *nklc* at 6 hpi and 12 hpi when treated with 0.25µl VTX, and at 12 hpi when treated with 0.08µl R848. In spleen, there was a significant increase of *nklc* at 12 hpi when treated with 0.08µl R848. There were no other differences in splenic *nklc* for 0.08µl R848 and 0.25µl VTX (Table 4.3).

4.3.7 Gene expression of *nkld*

In liver, there was a significant increase of *nkld* when treated with 2.5µl VTX at 12 hpi. There were no other differences in liver tissue (Table 4.2). In kidney, there was a

significant increase of *nkld* when treated with 2.5µl VTX at 6 hpi. There were no other differences in kidney tissues (Table 4.2). In spleen, there were significant increases of *nkld* at 24 hpi following administration of 0.8µl R848 and VTX. There were no differences in *nkld* expression at any other time points or treatments in spleen tissues (Table 4.2).

In liver, there were no differences in *nkld* expression after 0.08µl R848 and 0.8µl R848 for any time points (Table 4.3). In kidney tissues, there were significant increases of *nkld* at 6 hpi and 12 hpi when treated with 0.25µl VTX, and at 12 hpi and 24 hpi when treated with 0.08µl R848. There were no other differences for kidney tissues (Table 4.3). In spleen tissues, there were significant increases of *nkld* at 12 hpi and 24 hpi when treated with 0.08µl R848. There were no other differences in splenic *nkld* (Table 4.3).

Table 4.2 ANOVA of gene expression data comparing 0.8µl R84 with 2.5µl VTX treatments in liver, kidney and spleen tissues at 6 hpi, 12 hpi and 24 hpi.

	6 hpi Mean±SD (p values)		**12 hpi Mean±SD (p values)**		**24 hpi Mean±SD (p values)**	
Liver	**2.5µl VTX**	**0.8µl R848**	**2.5µl VTX**	**0.8µl R848**	**2.5µl VTX**	**0.8µl R848**
ifnγ	234.98±206.65 (0.5261)	713.37±681.31 (0.0001)	97.02±49.34 (0.9990)	177.11±173.71 (0.8689)	54.58±15.80 (0.9995)	110.95±95.03 (0.9909)
t-bet	2.92±1.99 (0.9999)	2.97±1.01 (0.9999)	5.25±3.85 (0.6645)	2.24±1.25 (0.2510)	4.73±1.88 (0.9996)	7.85±10.73 (0.9995)
nitr9	16.35±11.12 (0.9997)	280.971±271.8 (0.0001)	47.62±37.41 (0.9991)	40.84±39.20 (0.9993)	11.08±7.20 (0.9958)	68.21±43.26 (0.0453)
nkla	15.58±10.92 (0.7336)	25.24±14.47 (0.9904)	9.51±6.42 (0.9999)	6.74±8.12 (0.9998)	85.01±74.98 (0.9999)	16.05±26.24 (0.9993)
nklb	2.27±1.71 (0.9999)	44.93±32.82 (0.0001)	1.55±1.40 (0.9997)	5.22±3.95 (0.9916)	1.63±0.19 (0.9991)	7.64±4.48 (0.9995)
nklc	16.90±10.07 (0.9999)	21.19±18.41 (0.9999)	5.14±8.20 (0.9999)	2415.03±2396 (0.0001)	6.32±1.87 (0.9999)	62.12±61.93 (0.9998)

Table 4.2 (continued)

nkld	64.38±32.85 (0.1353)	5.95±2.69 (0.9999)	1.44±0.92 (0.0002)	118.61±109.48 (0.9999)	43.85±89.67 (0.6587)	7.80±4.88 (0.9999)
Kidney	**2.5µl VTX**	**0.8µl R848**	**2.5µl VTX**	**0.8µl R848**	**2.5µl VTX**	**0.8µl R848**
ifnγ	500.86±243.47 (0.9790)	1515.2±686.09 (0.0598)	383.51±187.2 (0.9958)	1598.5±1344.1 (0.0414)	1105.3±157.01 (0.6941)	555.99±506.81 (0.9993)
t-bet	2392.14±357.6 (0.7275)	1821.5±1138.8 (0.9815)	1517.8±791.1 (0.9954)	3390.1±1622.5 (0.1333)	5027.6±2627.3 (0.0270)	1152.4±588.64 (0.9993)
nitr9	1.04±0.40 (0.0046)	0.23±0.17 (0.9897)	0.33±0.28 (0.9131)	0.321±0.55 (0.9276)	0.05±0.03 (0.0001)	0.11±0.07 (0.0001)
nkla	682.86±126.80 (0.9999)	1900±1712.47 (0.9996)	204.74±120.4 (0.9999)	1207.72±11.49 (0.9997)	209.73±119.59 (0.9999)	263.98±220.98 (0.9999)
nklb	76.50±45.83 (0.9994)	508.26±420.65 (0.0518)	9.55±12.84 (0.9990)	630.30±432.85 (0.9997)	978.63±486.72 (0.7209)	188.35±91.04 (0.0295)
nklc	88.25±16.91 (0.9858)	276.28±203.20 (0.0101)	20.46±8.09 (0.9999)	1.43±1.34 (0.9996)	350.65±192.18 (0.6362)	169.25±118.20((0.0017)
nkld	3068±568.47 (0.0001)	18.51±19.78 (0.9999)	218.42±109.4 (0.9998)	36.13±32.63 (0.9997)	1091.93±494.8 (0.9668)	764.01±558.6 (0.9995)
Spleen	**2.5µl VTX**	**0.8µl R848**	**2.5µl VTX**	**0.8µl R848**	**2.5µl VTX**	**0.8µl R848**
ifnγ	51.40±23.90 (0.1728)	72.98±56.07 (0.0261)	7.45±3.42 (0.9963)	106.49±90.69 (0.0006)	99.47±62.76 (0.0678)	19.85±17.65 (0.9973)
t-bet	0.75±0.42 (0.9999)	2.00±1.39 (0.9999)	0.69±0.56 (0.9999)	29.40±39.29 (0.0001)	17.00±10.48 (0.5286)	3.64±3.09 (0.9995)
nitr9	0.12±0.22 (0.9998)	0.55±1.05 (0.9306)	0.004±0.002 (0.9999)	0.07±0.10 (0.9998)	0.52±0.55 (0.9994)	0.23±0.16 (0.9997)
nkla	5.86±6.64 0.9994	18.20±6.45 (0.8475)	3.55±5.78 (0.9997)	49.46±38.38 (0.0103)	10.42±3.25 (0.9996)	32.20±26.05 (0.3604)
nklb	4.30±8.27 (0.9991)	19.36±16.74 (0.1124)	1.59±1.54 (0.9997)	12.20±17.24 (0.6057)	25.11±28.77 (0.9993)	28.83±16.19 (0.1724)
nklc	50.97±69.48 (0.9998)	156.80±126.7 (0.9993)	15.76± 15.99 (0.9999)	262.17±198.07 (0.9882)	928.32±309.32 (0.0001)	2067.86±1695 (0.0001)
nkld	8.10±6.67 (0.9959)	5.85±3.96 (0.9994)	1.26±1.55 (0.9999)	40.82±26.42 (0.0706)	55.82±23.50 (0.0395)	62.38±33.16 (0.0006)

4.3.8 Comparative effect of TLR ligands

The effect of immune stimulation was compared between 0.08µl R848 and 0.25µl VTX; and 0.8µl R848 and 2.5µl VTX using Tukey's multiple comparison test (Table 4.3). 0.08µl R848 concentration up-regulated at 6 hpi: *t-bet* in liver and kidney; at 12 hpi: *ifnγ, t-bet, nklc* and *nkld* in kidney; *ifnγ*, *nitr9, nkla, nklb* and *nklc* in spleen; at 24 hpi: *nkla, nklb, nkld* in kidney; *nitr9*, *nklb* in spleen. 0.25µl VTX up-regulated at 6 hpi: *nklc* and *nkld* in kidney. 0.8µl R848 up-regulated at 6 hpi: *ifnγ*, *nitr9, nklb* in liver; *nitr9* in kidney; at 12 hpi: *nklc, nkld* in liver; *nklb* in kidney; *ifnγ, t-bet, nkla* and *nkld* in spleen; at 24 hpi: *nklc* in spleen. 2.5µl VTX up-regulated at 6 hpi: *nkld* in kidney and at 24 hpi: *nklb* in kidney.

Table 4.3 Summary of significant differences between treatments R848 vs VTX.

Tissue	**R848**		**VTX**	
	0.08µl	**0.8**µl	**0.25**µl	**2.5**µl
Liver	*t-bet* (24 hpi)	*ifnγ* (6 hpi), *nitr9* (6 hpi), *nklb* (6 hpi), *nklc* (12 hpi) nkld (12 hpi)		
Kidney	*ifnγ* (12 hpi), *t-bet* (6 hpi), *t-bet* (12 hpi), *nkla* (24 hpi), *nklb* (24 hpi), *nklc* (12 hpi), *nkld* (12 hpi), *nkld* (24 hpi)	*nitr9* (6 hpi), *nklb* (12 hpi)	*nklc* (6 hpi), *nkld* (6 hpi)	*nklb* (24 hpi), *nkld* (6 hpi)
Spleen	*ifnγ* (12 hpi), *nitr9* (12 hpi), *nitr9* (24 hpi), *nkla* (12 hpi), *nklb* (12 hpi), *nklb* (24 hpi), *nklc* (12 hpi), *nkld* (12 hpi), *nkld* (24 hpi)	*ifnγ* (12 hpi), *t-bet* (12 hpi), *nkla* (12 hpi), *nklc* (24 hpi), *nkld* (12 hpi)		

4.3.9 Protein expression by Western blots

The NITR9 expression levels increased in R848 and VTX treated tissue protein samples from liver and spleen when compared to saline treated samples at 6 hpi (Fig 4.1). In the case of liver, there was no difference in the expression of NITR9 between R848 and VTX. NITR9 was down-regulated in kidney with R848 and VTX compared to PBS treatment. Splenic NITR9 increase following R848 treatment when compared with PBS treatment. In the case of VTX treatment in spleen, NITR9 protein up-regulated when compared to PBS, but not as much as R848 treatment (Fig 4.1).

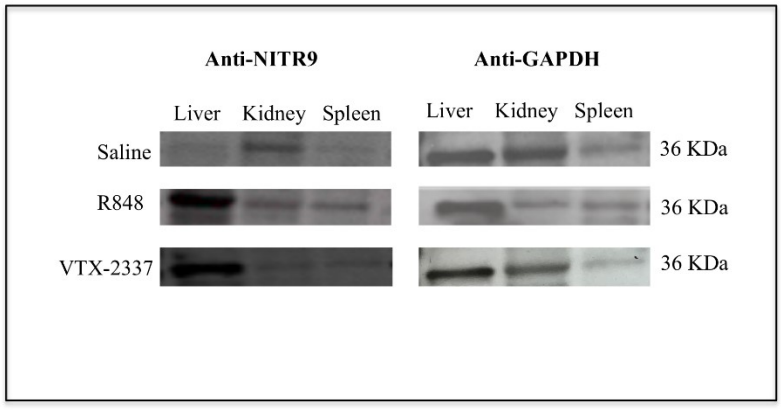

Figure 4.1 NITR9 protein expression in liver, kidney and spleen of *rag1*$^{-/-}$ mutant zebrafish treated with PBS (control), 0.08µl R848 and 2.5µl VTX.

4.3.10 Flow cytometry

NITR9 expression was evaluated in *rag1*$^{-/-}$ mutant zebrafish gated lymphocyte like cells (LLCs) following 0.8µl R848, 2.5µl VTX and saline (control) treatments at 12 hpi. In liver, the NITR9^{+} cells decreased as a percentage of total cells following VTX ($p=0.0311$) treatment. However, there was no significant difference between control and R848 ($p=0.3908$) or R848 vs VTX ($p=0.1824$) treatment (Fig 4.2). There was no

statistical difference in kidney NITR9^{+} cells between the R848 (p=0.3790) and VTX (p=0.9705) compared to control treatments as well as R848 compared to VTX (p=0.4898) treatment (Fig 4.3).

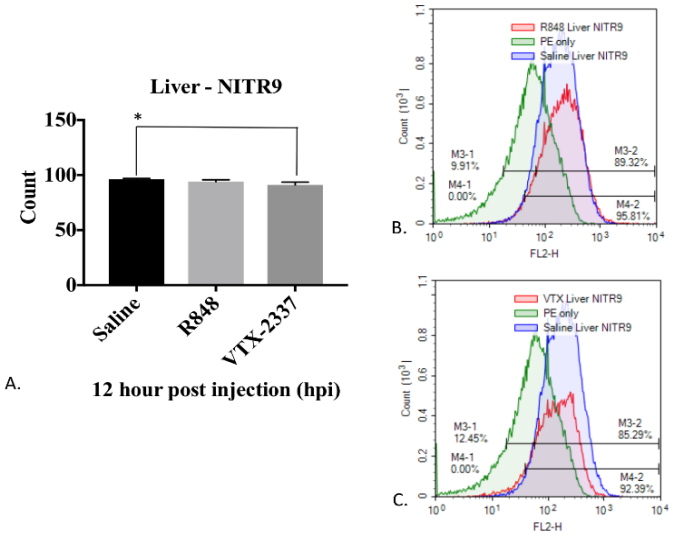

Figure 4.2 NITR9 expression in *rag1*$^{-/-}$ mutant zebrafish liver lymphocyte like cells (LLCs).

(A) Graphical representation of NITR9 labeled liver LLCs from fish IP injected with Saline (control), R848 and VTX. (B) Intracellular NITR9 expression of lymphocyte cell population from R848 injected fish, analyzed by flow cytometry at 12 hpi. (C) Intracellular NITR9 expression of lymphocyte cell population from VTX injected fish, analyzed by flow cytometry at 12 hpi. NITR9 expression was compared between treatments and control by one-way ANOVA. *Significant ($p<0.05$) difference in expression of treated compared to control. The green peak represents anti-mouse IgG: PE conjugated secondary antibody (control) (NITR9^{-} cells); the blue peak represents anti-NITR9 monoclonal antibody + anti-mouse IgG: PE conjugated secondary antibody (NITR9^{+} cells following saline treatment), and the red peak represents anti-NITR9 monoclonal antibody + anti-mouse IgG: PE conjugated secondary antibody (NITR9^{+} cells following R848 (B) or VTX (C) treatments).

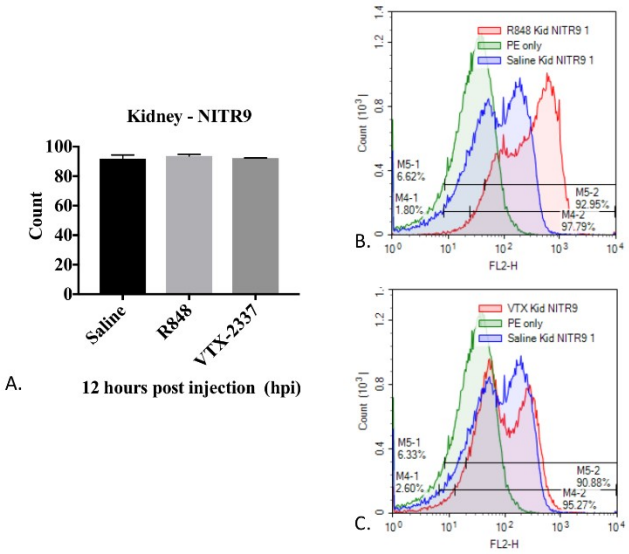

Figure 4.3 NITR9 expression in *rag1*$^{-/-}$ mutant zebrafish kidney lymphocyte like cells (LLCs).

(A) Graphical representation of NITR9 labeled kidney LLCs from fish IP injected with Saline (control), R848 and VTX. (B) Intracellular NITR9 expression of lymphocyte cell population from R848 injected fish, analyzed by flow cytometry at 12 hpi. (C) Intracellular NITR9 expression of lymphocyte cell population from VTX injected fish, analyzed by flow cytometry 12 hpi. NITR9 expression was compared between treatments and control by one-way ANOVA. *Significant ($p<0.05$) difference in expression of treated compared to control. The green peak represents anti-mouse IgG: PE conjugated secondary antibody (control) (NITR9^{-} cells); the blue peak represents anti-NITR9 monoconal antibody + anti-mouse IgG: PE conjugated secondary antibody (NITR9^{+} cells following saline treatment), and the red peak represents anti-NITR9 monoclonal antibody + anti-mouse IgG: PE conjugated secondary antibody (NITR9^{+} cells following R848 (B) or VTX (C) treatments).

4.4 Discussion

Nk-lysins are important agents in the fish immune response against pathogens. The response can vary by pathogen, but generally Nk-lysins increase significantly as an initial immune response (Pridgeon, Mu et al. 2012) (Zhou, Wang et al. 2016) (Pereiro, Varela et al. 2015) (Murji 2015) (Hirono, Kondo et al. 2007) (Liang, Ji et al. 2013, Zhang, Li et al. 2014). In chickens, Nk-lysin transcript levels coincide with parasite

oocyst production, suggesting that transcription levels increase when more pathogens are present (Hong, Lillehoj et al. 2006).

In our study, the overall greatest response was from R848, a TLR7 and TLR8 ligand. Generally, there was less response to VTX, a TLR8 ligand. Our findings suggest that TLR7 may be more specific to NK cell activation than TLR8 in zebrafish, or TLR7 and 8 are both required for NK activation. Following R848 administration, predominate responses were from *nklc* in the liver and spleen, and *nkld* in the kidney. Specifically, in the liver, the greatest increase was 2415 fold in *nklc* at 12 hpi of 0.8µl R848. In the spleen, the greatest increase was 2067 fold in *nklc* at 24 hpi of 0.8µl R848, and 1553 fold increase in *nklc* at 12 hpi of 0.08µl R848. In the kidney, the greatest fold increase was 4368 fold in *nkld* at 12 hpi 0.08µl R848 and 3517 fold increase of *nkld* at 24 hpi 0.08µl R848. In the kidney, *nklc* increases were much lower and ranged from 169 to 525 fold following R848 administration. With VTX administration and TLR8 stimulation, the only significant fold increase in the liver was 1.44 fold in *nkld*. In the spleen, VTX caused a 928 fold increase of *nklc* at 24 hpi and a 55.8 fold increase of *nkld* at 24 hpi. In the kidney, 2.5µl VTX caused a 3068 fold increase in in *nkld* at 6 hpi, and 2583 and 2130 fold increases in *nkld* at 6 and 12 hpi (0.25 mM VTX), respectively.

Spring Viremia of Carp virus caused predominant *nkla* and *nkld* responses in zebrafish kidney tissues, but the highest expressions were from *nklc* and *nkld* in the spleen (Pereiro, Varela et al. 2015). Expression profiles of Lc-Nk-lysin also varied significantly in gill, head kidney, spleen and liver tissues of *Larimichthys crocea* (Zhou, Wang et al. 2016). Lc-Nk-lysin was differentially expressed in tissues from *Cynoglossus semilaevis* (Zhang, Long et al. 2013). The catfish Nk-lysin gene exhibited strong tissue-

specific expression in tissues with lymphocyte populations. In another study, expression profiles of Channel catfish NK lysins showed that these AMPs are highly, but differentially expressed in gill, spleen, anterior and posterior kidney (Kocabas, Li et al. 2002). A different catfish study showed high levels of NK-lysin expression was detected in gill, head kidney, intestine, and spleen, and no expression was detected in muscle (Wang, Wang et al. 2006). *Nkla* was mainly expressed in muscle, whereas *nklb* and *nklc* showed highest expression in intestine and spleen, respectively. High differential expressions of Nk-lysins was limited to lymphoid tissues that house immune cell populations. This was also observed with porcine Nk-lysin that was predominately found in spleen, bone marrow, colon, and small intestine (Andersson, Gunne et al. 1995), (Bonetto, Andersson et al. 1999).

In a study that compared total Nk-lysin amounts between wild-type (WT) and *rag1*$^{-/-}$ mutant zebrafish, there was a higher absolute expression of the Nk-lysins in the *rag1*$^{-/-}$ when compared to the WT zebrafish. This is understandable, because in the absence of the T and B cells, the NK cells tend to fill in the void and would lead to higher expression of NK cell specific Nk-lysins (Pereiro, Varela et al. 2015). This same study also showed tissue specific differential expression of Nk-lysin in WT and *rag1*$^{-/-}$ mutant zebrafish.

It has been suggested that *nkla* could be primarily produced by CTLs and the NK cells mainly express *nklb*, *nklc* and *nkld* (Pereiro, Varela et al. 2015). In another study, zebrafish *nkl4*$^{+}$(*nkld*$^{+}$) cell types were reported and were suggested to represent putative CTLs. This same population declined in the T cells in a *rag2* mutant zebrafish, and *nkld*$^{+}$ expressing cells and myeloid cells substantially increased (Moore, Garcia et al. 2016).

Nklc and *nkld* have been shown to have a closer synteny between other Nk-lysins and mammalian Nk-lysins/granulysin, indicating a higher evolutionary conservation of *nklc* and *nkld* (Pereiro, Varela et al. 2015). The precursor cell population in whole kidney marrow was the highest producer of the 4 NK lysins in general and mainly showed expression of *nkla* and *nkld* mRNA, followed by next highest in lymphoid and least in myeloid populations showed highest expression of *nklb* and *nkld* mRNA (Pereiro, Varela et al. 2015).

Data detailing the transcriptional responses of zebrafish Nk-lysins to viral and bacterial infections are now available. Therefore, it will be important to conduct further studies testing the natural functions of NK-lysins in immune responses and their potential therapeutic value. In order to provide a more comprehensive survey of which TLR ligand leads to changes in transcript levels of zebrafish NK-lysins, similar studies could evaluate the transcriptional responses of NK-lysins to additional bacteria, viruses, parasites and fungi. These observations could lead to findings of new NK cell markers and also study NK cell biology in zebrafish to use it as a potential host model for infectious diseases.

Our flow cytometry and protein expression analyses were inconclusive. It is important to note that unlike the qPCR where we estimated the gene expression in tissues from individual fish, for western blot and flow cytometry we pooled tissues from fish in order to get an optimal amount of protein or cells for our analysis. For this reason, it is not easy to correlate gene expression with protein expression in tissue. Individual fish mount responses in varying levels that make interpretation challenging.

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CHAPTER V

THE EFFECT OF IMMUNOMODULATION BY TLR LIGANDS UPON *EDWARDSIELLA ICTALURI* EXPOSURE IN IMMUNOCOMPROMISED *RAG1*$^{-/-}$ MUTANT ZEBRAFISH (*Danio rerio*)

Abstract

Rag1$^{-/-}$ mutant zebrafish were intra coelomically injected with RE-33®, (a live attenuated *Edwardsiella ictaluri* vaccine), or one of the TLR ligands β glucan, Poly I:C or R848. The TLR ligands were also co-administered with RE-33®. Fish were challenged with WT *E. ictaluri* 4 weeks later and survival evaluated. RE-33® alone significantly increased survival. β glucan alone significantly increased survival more than above that of RE-33®. Survival after co-administration of β glucan and RE-33® was significantly higher than observed after treatment with RE-33®. Survival from administration of R848 alone was not significantly higher than with RE33® alone. Co-administration of R848 and RE-33® resulted in survival significantly higher than RE-33® or R848 alone. Treatment with R848 potentiated the protective effect of RE-33®. Poly I:C did not significantly increase survival more than RE-33®, but co-administration of Poly I:C and RE-33® resulted in survival significantly higher than observed with RE-33® or Poly I:C alone. Poly I:C potentiated RE-33®. Treatment with Poly I:C potentiated the protective effect of RE33®. Tissue specific gene expression of *ifnγ, t-bet, nitr9, nkla, nklb, nklc and nkld* were determined and correlated to survival. After the primary exposure in the liver,

all of these genes were up regulated over 100 fold in fish with enhanced protection 4 weeks later. After the WT *E. ictaluri* exposure, all of the genes evaluated were up regulated including *ifnγ* in the fish group was associated with the highest survival rates. After primary exposure in kidney tissue, the greatest differentially expressed genes were *ifnγ, t-bet, nkla, nklc,* and *nkld*. After the WT *E. ictaluri* exposure, up-regulated gene expressions of kidney *ifnγ*, *nklb*, and *nkld* was associated with the highest survival.

5.1 Introduction

Rag1$^{-/-}$ mutant zebrafish lack T cells and B cells but still exhibit specific protection and a memory-like response (Hohn and Petrie-Hanson 2012). The cellular basis for this protection has not been determined, but NK cells have been identified and characterized in this fish model (Muire, Hanson et al. 2017), and NK cells have been implicated in specific immunity in mammalian models (reference).. Immune stimulants are agents that stimulate an immune response. Such a response could be beneficial during the outbreak of a disease. Toll like receptors (TLRs) are pattern recognition receptors and are activated by specific pathogen associated molecular patterns (PAMPs). PAMPs facilitate the role of NK cells as effector cells and are also involved in NK cell mediated adaptive immune responses (Guo and Zhang 2012). TLRs activate NK cells to trigger an induction of interferons (IFNs), tumor necrosis factor (TNF) and several interleukins, at variable levels of expression, via different signaling pathways (Hart, Athie-Morales et al. 2005, Gorski, Waller et al. 2006).

The use of immune stimulants in aquaculture has increased over the past decades, to protect fish from several pathogenic infections. Petit et al. 2016 have reviewed the long-lived effects of one such immune stimulant, β glucan in different species of fish,

including zebrafish. T and B cell deficient zebrafish exhibit mount specific protection when re-exposed to bacteria (Hohn and Petrie-Hanson 2012). The cellular basis for this protection has not been determined in fish. In this study, TLR ligands were co-administered with vaccination, and their ability to enhance survival evaluated. After enhanced survival was demonstrated, the TLR ligands were administered alone to determine their effect on innate immune cells in *rag1*$^{-/-}$ zebrafish. Tissue specific macrophage and NK cell gene expression were evaluated to determine the potential contribution of these cells in initiating protective immunity in *rag1*$^{-/-}$ mutant zebrafish.

Leukocytes are generally categorized as being involved in either innate or adaptive immunity. Innate immune responses were believed to not be modified by repeated exposure to the same pathogen. Over the past decade that definition has dramatically changed. Natural Killer (NK) cells are innate immune cells but are now known to mediate adaptive immune responses to chemical haptens and cytomegalovirus in lymphocyte deficient mice (Sun and Lanier 2011). T and B cell deficient zebrafish (*rag1*$^{-/-}$ mutants) can mediate specific protection from bacteria, and NK cells are believed to mediate that response (Hohn and Petrie-Hanson 2012). The use of *rag1*$^{-/-}$ mutant zebrafish in immunological analyses has provided new insights into our understanding of teleost immunity. However, a limited availability of monoclonal antibodies for zebrafish immune cells has made it very challenging. We believe that a population of zebrafish NK cells mediates a protective memory response, and that these cells have a mechanism for enhanced discrimination of specific bacterial targets following primary exposure. This study was performed to determine if specific TLR ligands could enhance protective

memory and which NK cell specific genes were differentially regulated during this protective response.

5.2 Material and methods

5.2.1 Zebrafish care

Zebrafish were raised and maintained at 28°C in the specific pathogen free hatchery in the College of Veterinary Medicine, Mississippi State University (MSU) following standard lab protocols. Experimental protocols were approved by the MSU Institutional Animal Care and Use Committee (IACUC). A homozygous breeding colony of *rag1*$^{-/-}$ mutant zebrafish was previously established (Petrie-Hanson, Hohn et al. 2009) and bred at the CVM-SPF hatchery and the experimental fish are progeny from that colony.

5.2.2 Survival trials

Rag1$^{-/-}$ adult zebrafish (n=10) were intra-coelomically (IC) injected with either a TLR ligand alone or a combination of 1x10^4 CFU live attenuated vaccine strain of *Edwardsiella ictaluri* (RE-33®) (Klesius and Shoemaker 2000) and each ligand (β glucan 50µg/0.5g of fish; Poly I:C 50µg/0.5g of fish and R848 0.08µl/0.5g of fish or endotoxin free PBS 10µl/fish). The following treatments were administered: RE-33® only; β glucan only; Poly I:C only; R848 only; β glucan + RE-33®; Poly I:C + RE-33®; R848 + RE-33®; and control (PBS injected). Two tanks were used per treatment, and the experiment was repeated three times. After 4 weeks, the vaccinated *rag1*$^{-/-}$ zebrafish were challenged by 1×10^4 CFU/fish IC injections of a virulent *E. ictaluri* strain (#93146). When challenged, naïve fish (n=20) were injected with virulent WT *E. ictaluri*. Fish were

observed and deaths recorded for 20 days. All moribund fish demonstrated signs of Enteric Septicemia of Catfish (ESC) infection in zebrafish (Petrie-Hanson, Romano et al. 2007) and *E. ictaluri* was recovered from randomly sampled fish.

5.2.3 Quantifying gene expression

Treatments were administered as described above except for the omission of Poly I:C due to the limitation in number of samples that could be processed within the sampling time frame. Fish were euthanized in buffered 0.02% MS222 and liver, kidney and spleen tissues from fish were surgically removed at 0h (non-injected fish, n=5) for basal expression), 1, 6, 12 and 24 hours post injection (hpi) (n=3) for each TLR ligand. Whole tissues were immediately transferred to 400µl Trizol reagent (Zymo Research, USA) and homogenized following standard procedures in our lab (Elibol-Fleming 2006). Total RNA was extracted from each liver, kidney and spleen sample using RNA extraction kits (Zymo Research, USA) according to the manufacturer's protocol. The quantity of extracted total RNA was determined by NanoDrop ND-1000 and ND-8000 8-Sample Spectrophotometer and stored at -80°C until used. 100ng cDNA was prepared from RNA by using Super script III VILO^{TM} cDNA Synthesis Kit (Invitrogen).

Arp, Ifnγ, t-bet, nitr9, nkla, nklb, nklc and *nkld* cDNA were measured using real time quantitative PCR. The *t-bet* primer and probe (Table 1) were designed by Beacon Design software (BioRad) and Primer3 plus (GraphPad) software, respectively. The source of the other primers and probes are included in Table 1. All primers and probes were purchased from Eurofins MWG, Operon, Huntsville, Alabama, USA. Amplification of the ubiquitously expressed acidic ribosomal phosphoprotein (*arp*) gene was used for the internal control (Ju, Xu et al. 1999). The amplification was performed in a 25µl

volume containing 10 µl target cDNA and 15 µl master mix containing: 8.8 µl Nuclease free water (GIBCO, Ultra Pure^{TM}), 1.5 µl $MgCl_2$ (5mM), 2.5 µl 10x buffer, 0.5 µl dNTPs, 0.2µl Taq Polymerase HS enzyme (Hot Start PCR Kit, TAKARA, Japan), 0.5 µl forward primer (20µM), 0.5 µl reverse primer (20µM) and 0.5 µl probe (10µM). Thermal cycler parameters for the PCR program were set as follows: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 61°C for 1 minute. All samples (biological reps) were run in triplicates i.e., 3 technical reps/sample.

Table 5.1 Oligonucleotide primers and probes used for qRT-PCR to quantify gene expression levels of *ifnγ, t-bet, nitr9, nkla, nklb, nklc* and *nkld*.

Gene	Oligonucleotide sequences (5'–3')	GenBank Accession No.
Arp	**Fwd:** CTGCAAAGATGCCCAGGGA **Rev:** TTGGAGCCGACATTGTCTGC **Probe:**[6~FAM]TTCTGAAAATCATCCAACTGCTGGATGACTACC [BHQ1a~ Q] (Vojtech, Sanders et al. 2009)	NM_131580
Ifnγ	**Fwd:** CTTTCCAGGCAAGAGTGCAGA **Rev:** TCAGCTCAAACAAAGCCTTTCG **Probe:**[6~FAM]AACGCTATGGGCGATCAAGGAAAACGAC[BHQ1a~ Q] (Vojtech, Sanders et al. 2009)	NM_212864
t-bet	**Fwd:**GATCAAGCTCTCTCTGTGATAG **Rev:**GCTAAAGTCACACAGGTCT **Probe:**[6~FAM]TTCTGAAGGTCACGGTCACA[BHQ1a~Q] *	NM_001170599.1
nitr9	**Fwd:** GTCAAAGGGACAAGGCTGATAGTT **Rev:**GTTCAAAACAGTGCATGTAAGACTCA **Probe:** [6~FAM]CAAGGTTTGGAAAAGCAC[BHQ1a~Q] (Shah, Rodriguez-Nunez et al. 2012)	AY570237.1
nkla	**Fwd:** TTTCTGGTCGGCTTGCTCAT **Rev:** TTCTCATTCACAGCCCGGTC **Probe:**[6~FAM]TCTGCAGCTCACTGGGAGGTTCGTGA[BHQ1a~Q]	NM_001311794
nklb	**Fwd:** TCCGCAACATCTTTCTGGTCA **Rev:** AGCCTGCTCATGAATGAAAATGA **Probe:**[6~FAM]CACGCCTGCAAATCTGAACCACCCA[BHQ1a~Q]	NM_001311792
nklc	**Fwd:** CTGCTTGTGCTGCTCACTTG **Rev:** AGCACACATGGAGATGAGAACA **Probe:** [6~FAM]GGGCTTGCAAGTGGGCCATGGGAA[BHQ1a~Q]	NM_001311793.1
nkld	**Fwd:** ACCCTGCTCATCTCCTCTGT **Rev:** CCCCAGCTAAAGCAAAACCC **Probe:**[6~FAM]TGCCTGGGATGTGCTGGGCTTGCAA[BHQ1a~Q]	NM_212741.1

Housekeeping gene *arp* (house-keeping gene) was used as a reference gene. *The *nkla, nklb, nklc, nkld* and *t-bet* primers and probes were designed by Primer3 plus (GraphPad) software.

5.2.4 Data analysis and statistical evaluation

Survival curves analysis were performed by the Kaplan-Meier survival plot using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The non-parametric statistic tests Gehan-Breslow-Wilcoxon test and Log ranked (Mantel-Cox) test were used to estimate the statistical significance between the survival curves of RE-33® only, TLR ligand groups only and RE-33® plus TLR ligand groups. The Gehan-Breslow-Wilcoxon method gives more weight to mortalities at early time points. The Log-rank (Mantel-Cox) test gives equal weight to all time points and is more preferred. Relative gene expression was determined using the Pfaffl method (Pfaffl 2004). Data obtained from qRT-PCR were expressed as fold change and were converted to log2 values. Data were analyzed by two-way analysis of variance using PROC MIXED (SAS for Windows 9.4, SAS Institute, Inc., Cary, NC). Separate models were used for each gene and tissue combination. The explanatory variables for all models were treatment, time post injection, and the treatment x time interaction. The effect of treatment was reported only if the treatment and treatment x time interaction was significant. An alpha level of 0.05 was used to determine the significance of all analyses.

5.3 Results

5.3.1 Survival Trials

RE-33® vaccinated *rag1*$^{-/-}$ zebrafish demonstrated significantly higher survival than non-vaccinated fish when injected with a field isolate of WT *E. ictaluri* 4 weeks after vaccination (Fig. 5.1 and Table 5.2). β glucan was the only TLR ligand that when administered by itself, provided significantly higher survival than RE-33® (Fig. 5.2). When β glucan was co-administered with RE-33®, survival was significantly higher than

RE-33® alone. Survival after the administration of β glucan+RE-33® was not significantly greater than survival after the administration of β glucan alone (Fig. 5.2 and Table 5.2).

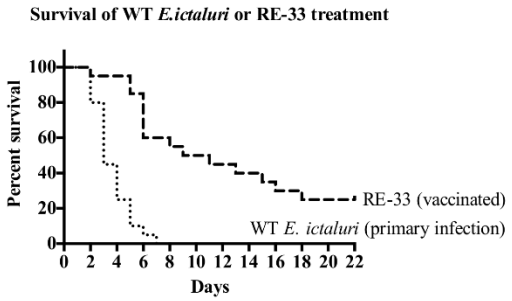

Figure 5.1 Comparison of survival of non-vaccinated *rag1*$^{-/-}$ mutant zebrafish and RE33® vaccinated fish after *E.ictaluri* challenge group.

There was significantly higher survival in the *rag1*$^{-/-}$ mutant zebrafish that was vaccinated compared to the group that was not. All control fish died by day 7. Treatment groups: WT *E. ictaluri* (dotted line) and RE-33® (dashed line)

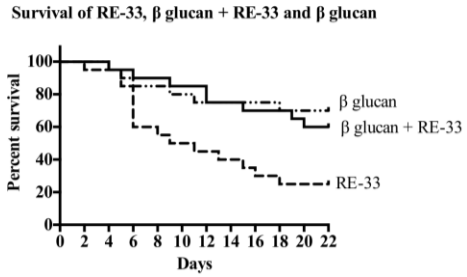

Figure 5.2 Comparison of survival of *rag1*$^{-/-}$ mutant zebrafish groups that were pre exposed to RE-33®, β glucan + RE-33® or β glucan and then challenged with WT *E. ictaluri* 4 weeks later.

The *rag1*$^{-/-}$ mutant zebrafish that received β glucan alone had significantly higher survival than the group that received RE-33® alone. β glucan + RE-33® demonstrated significantly higher survival than the group that received RE-33® alone. Survival in treatments that received β glucan and β glucan + RE-33® were not significantly different.

Treatment groups: RE-33® (dashed line), β glucan + RE-33® (solid line) and β glucan (dotted line).

Survival resulting from the administration of Poly I:C alone was not significantly greater than survival in fish that received RE-33® alone (Fig. 5.3 and Table 5.2). The co-administration of Poly I:C+RE-33® resulted in survival that was significantly higher than RE-33® for the first ten days of the study, but not for the last ten days. Survival after the administration of Poly I:C+RE-33® was not significantly greater than survival after the administration of Poly I:C alone.

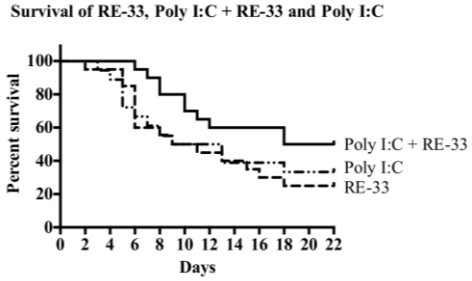

Figure 5.3 Comparison of survival of *rag1*$^{-/-}$ mutant zebrafish groups that were pre exposed to RE-33®, Poly I:C + RE-33® or Poly I:C and then challenged with WT *E. ictaluri* 4 weeks later.

The group that received the Poly I:C alone demonstrated no significant difference for survival from the vaccinated (RE-33®) group There was no difference in survival between the group that received Poly I:C + RE-33® and the group that was vaccinated (RE-33®) alone. Treatment groups: RE-33® (dashed line), Poly I:C + RE-33® (solid line) and Poly I:C (dotted line).

Survival resulting from the administration of R848 alone was not significantly higher than survival from survival in fish that RE-33® alone (Fig. 5.4 and Table 5.2). R848+RE-33® treated *rag1*$^{-/-}$ zebrafish demonstrated significantly higher survival than

RE-33® only treated fish after challenge. R848 alone provided the same protection as R848+RE-33® treated fish.

When the co-administered treatments were compared to each other, there were no significant difference between the survival for β glucan+RE-33® and R848+RE-33® treated fish. Both treatments demonstrated higher survival than fish that received Poly I:C+RE-33®.

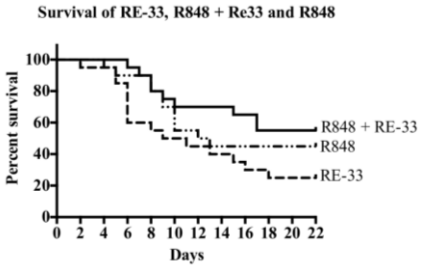

Figure 5.4 Comparison of survival of *rag1*$^{-/-}$ mutant zebrafish groups that were pre exposed to RE-33®, R848 + RE-33® and R848 and then challenged with WT *E. ictaluri* 4 weeks later.

The group that received R848 alone demonstrated no significant difference in survival from the RE-33® alone group. The group that received the R848 + RE-33® demonstrated significantly higher survival than the group that received RE-33® alone. Treatment groups: RE-33® (dashed line), R848 + RE-33® (solid line) and R848 (dotted line).

Table 5.2 Comparison of survival of fish administered TLR ligands or RE-33® alone, or combinations of TLR ligands and/or RE-33®. *Rag1*$^{-/-}$ mutant zebrafish were challenged with wild type *E. ictaluri* 4 weeks following TLR ligands and/or RE-33® administration.

Treatment	Breslow-Wilcoxon	Mantel-Cox
RE-33® v control	p<0.0001	p<0.0001
β glucan v RE-33®	p=0.0246	p=0.0135
β glucan+RE-33® v RE-33®	p=0.0096	p=0.0116
β glucan+RE-33® v β glucan	p=0.7392	p=0.6265
Poly I:C v RE-33®	p=0.3536	p=0.2511
Poly I:C+RE-33® v RE-33®	p=0.0329	p=0.0544
Poly I:C+RE-33® v Poly I:C	p=0.0843	p=0.1575
R848 v RE-33®	p=0.1740	p=0.1879
R848+RE-33® v RE-33®	p=0.0189	P=0.0285
R848+RE-33® v R848	p=0.4519	p=0.4728

5.3.2 Quantitative Gene Expression

WT *E. ictaluri* exposure (bacterial challenge)

In *rag1*$^{-/-}$ mutant zebrafish, the effects of WT *E. ictaluri* exposure were significantly up-regulated liver *nitr9* at 6 hpi, liver *ifnγ* at 12 hpi, liver *ifnγ* and liver *nkla* at 48 hpi, and kidney *nitr9* and *nklb* at 6 hpi, kidney *nitr9* and *nklb* at 12 hpi, kidney *ifnγ* at 24 hpi and *nitr9, nkla* and kidney *nklc* at 48 hpi (Table 5.3 Summary and Table A3). Liver *nkla* was significantly down-regulated at 48 hpi.

RE-33® exposure

RE-33® caused significantly up-regulated liver *ifnγ* and *nklb* at 48 hpi and significantly up-regulated kidney *nklc* at 12 hpi and *ifnγ* at 48 hpi. Following the WT *E. ictaluri* exposure 4 weeks after RE-33®, there was significantly increased liver *nitr9* at 6 hpi, *nkla* at 12 hpi and *ifnγ* and *nkla* at 48 hpi. Whereas in kidney tissue, there was significantly increased levels of *t-bet* at 6 hpi, *nkld* at 12 hpi and *nkla* at 48 hpi (Table 5.3 Summary and Table A3).

β glucan treatment

The primary exposure of β glucan caused significantly increased liver *t-bet* expression at 6 hpi and decreased *nkld* at 48 hpi. In kidney tissue, the primary β glucan exposure caused significantly increased *t-bet* at 6 hpi. Following challenge with WT *E. ictaluri*, there was significantly increased *ifnγ, nkla, nklb, nklc,* and *nkld at 6hpi* in the liver and significantly increased *nklb* at 6 hpi*, ifnγ* and *nkld* at 12 hpi*, ifnγ* at 24 hpi and *nkla* at 48 hpi in kidney tissue (Table 5.3 Summary and Table A3).

β glucan + RE-33® treatment

The effect of the primary exposure of β glucan + RE-33® in liver caused an increase in *t-bet* at 6 hpi, *nkla* at 12 hpi, *nitr9* at 24 hpi and 48 hpi. *Nkld* was down-regulated at 48 hpi. Following challenge with bacteria, expression was significantly up-regulated in *nkld* at 12 hpi, *ifnγ, nitr9, t-bet, nkla, nklb, nklc* and *nkld* at 24 hpi and *nkla* was significantly down-regulated at 48 hpi. The effect of primary exposure to β glucan + RE-3c® in kidney caused an increase in *t-bet* at 6 hpi, and *nkla* and *nklc* at 12 hpi. Following challenge with bacteria, there was an up-regulation in *nkld* at 12 hpi, *nklb* and *nkld* at 24 hpi and *ifnγ* at 48 hpi (Table 5.3 Summary and Table A3).

R848 treatment

The effect of primary R848 exposure in liver caused an increase of *ifnγ* at 24 hpi, and *nkla, nklb, nklc, nkld* at 48 hpi. *T-bet* was down-regulated at 48 hpi. Following challenge with bacteria, there was an increase in *nitr9* at 12 hpi. *T-bet* and *nkla* expressions were down-regulated at 48 hpi. R848 exposure in the kidney caused increases in *ifnγ, nkla, nklc* and *nkld* at 24 hpi, and *nklc* and *nkld* at 48 hpi. *T-bet* was down-regulated at 6 hpi. Following challenge with bacteria, there was an increase in *nkla*

at 48 hpi. *T-bet* and *nkld* were down-regulated at 6 hpi and *ifnγ* was down-regulated at 12 hpi (Table 5.3 Summary and Table A3).

R848 + RE-33® treatment

The effect of R848 + RE-33® in liver caused an increase in *nitr9* at 24 hpi and *nkla*, *nklb*, *nklc,* and *nkld* at 48 hpi. Following challenge with bacteria, there was in increase in *nitr9* at 24 hpi and *ifnγ* at 48 hpi. The primary exposure in kidney caused an upregulation of *ifnγ, nkla, nklc,* and *nkld* at 48 hpi. Following bacterial exposure in the kidney, there was an up-regulation of *nkld* at 24 hpi and *nkla* at 48 hpi (Table 5.3 Summary and Table A3).

5.4 Discussion

T and B cell deficient *rag1*$^{-/-}$ zebrafish develop protective immunity against *E. ictaluri* (Hohn and Petrie-Hanson 2012). In this study we demonstrated that exposure to TLR ligands provide long term protection similar to vaccination. We also used co-administration of TLR ligands and live attenuated bacteria to define the role of NK cells in protective responses of these fish and found enhanced protection in some cases. Our study demonstrates that β glucan provides protection when administered alone and increases protection when co-administered with an attenuated live vaccine in mutant zebrafish. Most studies that evaluated the effect of β glucan on immune responses in fish have not evaluated co-administration of β glucan with a vaccine using a parenteral route, and no other studies have investigated these administrations in T and B cell deficient fish. In a study on furunculosis using immune competent Atlantic salmon, β glucan co-administered with a vaccine improved vaccine efficacy against *Aeromonas salmonicida* (Rørstad, Aasjord et al. 1993). Dalmo and Jarl Bogwald suggested that efficacy of using

β glucan with vaccines could vary by the host species and the pathogen (Dalmo and Bøgwald 2008).

Similar findings were reported in a study involving *Candida albicans* and β glucan in murine monocytes, in which functional re-programming of monocytes via dectin-1 was associated with protection (Quintin, Saeed et al. 2012). Inspite of the several studies that have documented the enhanced effects of β glucan on fish immunity (reviewed in Vaclav Vetvicka 2013) (Petit and Wiegertjes 2016), there is limited knowledge of the mechanisms in which β glucan and other TLR ligands modulate fish immunity. The effect of β glucan on the modulation of gene expression leading to stimulation of fish immunity is not clear. Earlier studies using β glucan showed that β glucan application significantly stimulated the phagocytic system and also played a role in general defense against tumors and infections (Novak and Vetvicka 2008) (Novak and Vetvicka 2009).

In teleosts, the mechanism of action of R848 is mediated by the TLR7/8 signaling pathway, in which MyD88 and NK-κB are essential players (Zhou and Sun 2015). R848 is known to enhance humoral and cellular Th1 immune responses (Vasilakos, Smith et al. 2000, Quiniou, Boudinot et al. 2013). R848 has been successfully used as an anti-viral agent in humans (Miller, Meng et al. 2008) and Japanese flounder (Zhou and Sun 2015). The use of R848 demonstrated the induction of anti-bacterial activity in tongue sole (Li and Sun 2015). In our study, administration of R848 alone did not result in significantly higher survival compared to RE33®. However, co-administration of R848+RE-33® significantly increased survival compared to R848 alone (Fig. 3.4). Zhou and Sun reported several functions of R848 in Japanese flounder including stimulating

proliferation of peripheral blood leukocytes (PBL), reduced apoptosis of PBL, replication inhibition of megalocytivirus and enhanced mRNA expression of *tlr7, il-6, Myd88, ifnγ* and *irf3* and down-regulation *il-1β* (Zhou and Sun 2015). The effect of R848 on immune responses of T and B cell deficient animals has not been investigated.

When Poly I:C was administered alone or co-administered with RE-33® 1 month prior to *E. ictaluri* challenge, it improved survival in our *rag1*$^{-/-}$ mutant zebrafish, but not as much as β glucan and R848. This suggests that induction of protection to *E. ictaluri* in lymphocyte deficient fish requires the stimulation of TLRs other than TLR3. However, TLR3 is involved in recognizing bacteria and viruses (Schmidt, Leung et al. 2004). Poly I:C is known to strongly induce type 1 interferon and this can suppress viral replication and dissemination in the host. It also causes macrophages to direct type 1 response focusing on intracellular pathogens. This suppression enhances survival when live virus is administered, allowing the fish to survive the primary infection long enough to develop acquired immunity (Takami, Kwon et al. 2010, Oh, Takami et al. 2012). Used in this manner, it may not enhance acquired immunity, but protection can ultimately develop. In Chapter 2, we saw that *mx* was substantially up-regulated indicating that a strong induction of type 1 interferon had occurred. Alternatively, the benefit of using Poly I:C in vaccines may relate to enhance T and B lymphocyte based immune responses. Poly I:C has been shown to be beneficial when used in vaccine studies with immune competent aquaculture species (Strandskog, Skjæveland et al. 2008, Nishizawa, Takami et al. 2009, Takami, Kwon et al. 2010, Nishizawa, Takami et al. 2011, Oh, Takami et al. 2012). Poly I:C is successfully used as a cancer vaccine adjuvant in humans (Ammi, De Waele et al. 2015).

In the current study, β glucan and β glucan + RE-33® treated fish and R848 + RE-33® treated fish demonstrated higher survival than fish that received Poly I:C + RE-33®, Poly I:C, R848 or RE-33® alone when challenged with WT *E. ictaluri* four weeks after these treatments were administered (Figs. 3.1, 3.2 and 3.3). The molecular and cellular mechanisms mediating this enhanced survival have not been determined. Other studies have shown that macrophages and NK cells can undergo epigenetic changes, causing a phenomenon known as "trained immunity", following a primary stimulus (vaccination). In mammals, these cells are capable of mounting adaptive cellular mechanisms in the absence of T and B cells upon reinfection (Quintin, Saeed et al. 2012). The concept of trained immunity has not been explored in fish.

Table 5.3 Summary of expression changes of significantly up-regulated and down-regulated genes at different hours post injection (hpi) following primary or secondary immune stimulation in *rag1*$^{-/-}$ mutant zebrafish.

Treatment	Tissues	Up regulated <10 fold change		Up regulated 10-100 fold change		Up regulated >100 fold change		Down regulated genes	
		Pri	Sec	Pri	Sec	Pri	Sec	Pri	Sec
E. ictaluri	Liver			*Ifnγ* (12hpi) *Nklc* (48hpi)		*Ifnγ* (6hpi) *Ifnγ* (48hpi)		*Nkla* (48hpi)	
	Kidney	*Nitr9* (6hpi) *Nitr9* (12hpi) *Nitr9* 48hpi) *Nkla* (48hpi)				*Ifnγ* (24hpi)			
RE33	Liver		*Nkla* (48hpi)			*Ifnγ* (48hpi) *Nklb* (48hpi)	*Ifnγ* (48hpi) *Nitr9* (6hpi) *Nkla* (12 hpi)		

Table 5.3 (continued)

	Kidney	*Nkla* (48hpi)				*Ifnγ* (48hpi) *Nklc* (12hpi)	*Tbet* (6hpi) *Nkld* (12hpi)		
B glucan	Liver					*Tbet* (6hpi)	*Ifnγ* (6hpi) *Nkla* (6 hpi) *Nklb* (6hpi) *Nklc* (6hpi) *Nkld* (6hpi)	*Nkld* (48hpi)	*Nkla* (48hpi)
	Kidney		*Nkla* (48hpi)		*Ifnγ* (24hpi)	*Tbet* (6hpi)	*Ifnγ* (12hpi) *Nklb* (6hpi) *Nkld* (12 hpi)		
B glucan + RE33	Liver			*Nitr9* (48hpi)		*Tbet* (6hpi) *Nitr9* (24hpi) *Nkla* (12hpi)	*Ifnγ* (24hpi) *Tbet* (24hpi) *Nitr9* (24hpi) *Nkla* (24hpi) *Nklb* (24hpi) *Nklc* (24hpi) *Nkld* (12hpi) *Nkld* (24hpi)	*Nkld* (48hpi)	*Nkla* (48hpi)
	Kidney		*Nkla* (48hpi)			*Tbet* (6hpi) *Nkla* (12hpi) *Nklc* (12hpi)	*Ifnγ* (48hpi) *Nklb* (24hpi) *Nkld* (12hpi) *Nkld* (24hpi)		

Table 5.3 (continued)

R848	Liver					*Ifnγ* (24hpi) *Nkla* (48hpi) *Nklb* (48hpi) *Nklc* (48hpi) *Nkld* (48hpi)	*Nitr9* (12hpi)	*Tbet* (48hpi)	*Tbet* (48hpi) *Nkla* (48hpi)
	Kidney				*Nkla* (48hpi)	*Ifnγ* (24hpi) *Nkla* (24hpi) *Nklc* (24hpi) *Nklc* (48hpi) *Nkld* (24hpi) *Nkld* (48hpi)		*Tbet* (6hpi)	*Tbet* (6hpi) *Nkld* (6hpi) *Ifnγ* (12hpi)
R848 + RE33	Liver					*Nitr9* (24hpi) *Nkla* (48hpi) *Nklb* (48hpi) *Nklc* (48hpi) *Nkld* (48hpi)	*Ifnγ* (48hpi) *Nitr9* (24hpi)		*Ifnγ* (12hpi) *Nkla* (48hpi)
	Kidney		*Nkla* (48hpi)			*Ifnγ* (48hpi) *Nkla* (48hpi) *Nklc* (48hpi) *Nkld* (48hpi)	*Nkld* (24hpi)		

Rag1$^{-/-}$ mutant zebrafish were intra coelomically injected with RE33® or one of The TLR ligands β glucan, Poly I:C or R848. The TLR ligands were also co-administered with RE33®. Fish were challenged with WT *E. ictaluri* 4 weeks later and gene expressions determined in liver and kidney tissues. Table 3.3 is the summary of expression changes of significantly up-regulated and down-regulated genes after the

administration of these treatments. Zebrafish have NK cells and non-specific cytotoxic cells (NCCs) (Petrie-Hanson, Hohn et al. 2009). NCCs are small agranular lymphocytes. They have been isolated and described, and reported to be NK cell precursors (Moss, Monette et al. 2009). NCCs are unique to teleosts and are small and large agranular lymphocytes. In Chapter 5, we reported the presence of NK cells in zebrafish kidney, liver and spleen that ranged in sizes from 4um (small) to 9.5um (large) (Muire, Hanson et al. 2017). Other than these two reports, zebrafish NK cells and NCCs have not been explored. A zebrafish NK cell line is not available and this makes it challenging to study the cells' activation and dynamics during infection. It is a challenge to isolate a pure NK cell population based on a size and granularity on a forward/side scatter dot-plot due to the interference of NCCs (reported as mammalian NK cells equivalents) (Yoder 2004). The *rag1*$^{-/-}$ mutant zebrafish T and B cells (Petrie-Hanson, Hohn et al. 2009) and the advantage of using this model is to rule out the contribution of T and B cells in immune responses. The tissue locations of NK cells and NCCS are not clear at present. In Chapter 5, we show that a large granular nitr9^{+} NK cell population is within the myeloid population. A similar cell population was recently reported that corroborates this cell population (Pereiro, Varela et al. 2015).

Zebrafish have four Nk-lysin genes: *nkla, nklb, nklc* and *nkld*. A recent study of Nk-lysins in the NK cells and NCCs of wild type and *rag1*$^{-/-}$ mutant zebrafish reported that the kidney precursor cell population was the highest producer of the 4 Nk-lysins. Differential expression patterns of NK lysins suggests specialization of the cell types in the production of these proteins (Pereiro, Varela et al. 2015). Since *rag1*$^{-/-}$ fish lack T and B cells, the NK cells are the major producers of the Nk-lysins (Petrie-Hanson, Hohn et al.

2009, Pereiro, Varela et al. 2015). Zebrafish have the highest repertoire of Nk-lysin genes described (Pereiro, Varela et al. 2015). Such a diversity could indicate a specialization of the different proteins into different functions and as consequence the preferential expression of these genes by different cell types (Pereiro, Varela et al. 2015). The morphology of different NK cell populations in the liver, kidney and spleen of *rag1*$^{-/-}$ mutant zebrafish is discussed in Chapter 2.

Transcript of *Nklc* represents 71% of the total Nk-lysin expression in the *rag1*$^{-/-}$ mutant zebrafish kidney. Transcript of *Nkla*, *nklb* and *nkld* are 18%, 11% and <1% of the total, respectively (Pereiro 2015). In our study, when naïve fish were challenged with *E. ictaluri, nklc* was significantly up-regulated in the liver. *Ifnγ* was significantly up-regulated at 6, 12 and 48 hpi in liver. *Ifnγ* was up-regulated at 24 hpi in kidney. *Nitr9* was up-regulated in the kidney at 6hpi, 12hpi and 48hpi. Indicating that *nitr9* expression was up-regulated until 12hpi and decreased, but again up-regulated at 48hpi, the time when *E. ictaluri* pathogenesis sets in, suggesting an anti-bacterial role of *nitr9*. Following RE-33® injection (live attenuated vaccine) and after challenging the fish 4 weeks post vaccination at 48 hpi *ifnγ* was up regulated in liver and kidney (only *E. ictaluri* control). However, there was no over expression of *ifnγ* in the kidney of the challenged fish, but there was an expression of *t-bet* (NK cell transcriptional factor) in the kidney of vaccinated-challenged fish, indicating a proliferation of NK cells at 6hpi. *T-bet* was downregulated in primary and secondary infection following R848, which could be due to a feed-back mechanism during an active immune response. In the R848 treated non-challenged fish, *ifnγ* was up-regulated at 24 hpi in liver and kidney unlike β glucan, where *ifnγ* was not up-regulated during primary infection. *Ifnγ* was also up-regulated in

the liver and kidney of β glucan and β glucan + RE-33® administered challenged-fish, indicating the importance of *ifnγ* in killing the bacteria and mediating an immune response. In β glucan and β glucan + RE-33® administered fish, *t-bet* expression was mainly in liver and kidney at 6hpi. In the case of R848 and R848 + RE-33® administered fish, the level of *t-bet* expression was not statistically different from PBS but was downregulated at 48 hpi during primary and secondary stimuli.

A set of four Nk-lysin paralogs were described in zebrafish and their transcriptional response to viral infection was characterized (Pereiro, Varela et al. 2015). Although all Nk-lysin genes were expressed at mRNA level in all the tissues, proportionally, nkla is the most expressed gene in liver, kidney, tail, head and muscle; *nkla* and *nklb* genes were expressed equally in gills; nklb was predominantly expressed in intestine; *nklc* was expressed in spleen and *nkld* was highly prevalent in gills, kidney and spleen (Pereiro, Varela et al. 2015). Pereiro et al 2015 observed that there was a higher absolute expression of the Nk-lysins in the *rag1*$^{-/-}$ when compared to the WT zebrafish (Pereiro, Varela et al. 2015). This could support the theory that in the absence of the T and B cells, the NK cells tend to fill in the vacuum and hence could lead to higher expression of NK cell specific Nk-lysins. Nkla was expressed in the *rag1*$^{-/-}$ mutants but to a lesser extent when compared to the WT zebrafish, whereas, *nklb, nklc* and *nkld* were highly expressed in *rag1*$^{-/-}$ mutant zebrafish. Therefore, the results from Pereiro et al suggest that CTLs are the major producers of *nkla* and the NK cells mainly express *nklb*, *nklc* and *nkld*. All the 4 Nk-lysins were expressed in the *rag1*$^{-/-}$ mutants and the WT zebrafish. Therefore, the Nk-lysins serve as good candidate genes for tracking down NK cells in zebrafish. Zang et al found that NK-lysins exhibit tissue specific expression and,

when overexpressed in fish, regulate immune gene expression (Zhang, Long et al. 2013). In our study, the expression of the NK-lysin paralogs were differentially expressed in the liver and kidney at different time points. Nk-lysins serve as molecular markers for NK cells identification/detection in zebrafish systems.

In both vaccination (RE33®) and WT challenge *nkla* was up-regulated only in the kidney but at a very low expression level at 48hpi, but *nkla* was downregulated in the liver at 48hpi post challenge in naïve (non-vaccinated) fish. We report that following challenge (secondary infection) with *E. ictaluri* in all treatment groups, *nkla* was the most expressed gene at 48 hpi. *E. ictaluri* pathogenesis sets in at 48h post infection and the ESC symptoms are evident in the fish. We have shown in our survival curves that all control fish that were injected with WT *E. ictaluri* died at day 7 in the trial. Interestingly, downregulation of liver *nkla* (secondary infection) at 48hpi was noted in both TLR ligand administered groups only after bacterial challenge. But the mRNA levels of *nkla* were reported to be up-regulated at earlier time points (6hpi and 24hpi) only in β glucan and β glucan +RE33® groups. In the β glucan and β glucan + RE-33® groups downregulation of liver *nkld* (primary infection) was noted. In flounder, constitutive expression of Nk-lysin was detected mainly in gills, heart, head kidney, intestines, peripheral blood leukocytes, and spleen, and no expression was detected in brain, skin and stomach (Hirono, Kondo et al. 2007). In channel catfish, high levels of Nk-lysin expression were detected in gill, head kidney, intestine, and spleen, and no expression was detected in muscle (Wang, Wang et al. 2006). *E. ictaluri* challenge in channel catfish, induced NK-lysin type1 and type 2 expression in head kidney (Wang, Wang et al. 2006, Pridgeon, Mu

et al. 2012) which correlate with our observation with *nkla, nklb, nklc* and *nkld* expression in kidney influenced by TLR ligand exposures.

Moore et al 2016, analysed the expression level of Nk-lysin 4 in *rag2* mutant zebrafish and reported a 10-fold increase (i.e.,6%) in expansion of nkl.4^{+} cells in the WKM. This cell population corresponds to a unique cytotoxic lymphocyte like cell population. Moreover, these nkl.4^{+} populations expressed the lck gene, suggesting that they are likely related to cytotoxic T cells, NK cells, or NKT cells. Additionally, an expansion of the myeloid cell population was reported, suggesting that an influx of leukocytes is a compensation mechanism in the impaired T and B cell zebrafish to protect it from infection (Moore, Garcia et al. 2016) and these cells could represent trained macrophages (reviewed in (Petit and Wiegertjes 2016) and the large granular lymphocytes (NK cells), respectively (Muire, Hanson et al. 2017). In another study, when adult WT zebrafish were intraperitoneally injected with SVCV, they observed that the transcript levels of zebrafish NK-lysins sometimes decreased after viral infection. There was a significant expression of *nkla* and *nkld* observed in kidney at 3, 6 and 24hp challenge. *Nklb* and *nklc* did not have any effect. Whereas *nkla* and *nkld* in kidney displayed the greatest increase after infection with SVCV (Pereiro, Varela et al. 2015), these NK-lysins displayed differing responses to *S. iniae* infection (Murji 2015). This may reflect NK-lysin specialization for different pathogens. The observed variations in post-infection transcript levels may also be explained by the number of bacteria injected into the zebrafish. Basal level mRNA expression of Japanese flounder Nk-lysin was reported in gills, heart, head kidney, intestine, peripheral blood leukocytes (PBLs), spleen and trunk kidney at higher levels and in liver, muscle and ovary at lower levels. However,

null expression of Nk-lysin was noted in the brain, skin and stomach of SPF Japanese flounders (Hirono, Kondo et al. 2007).

Nkla gene expression reached the highest expression at 24h (FC=4.85) with respect to the control challenge (Pereiro, Varela et al. 2015). In the case of *nkld* expression, there was a significantly high expression at 6h (FC=4.05) and returned to its basal levels after 24h (Pereiro, Varela et al. 2015). In our study, β glucan treated fish, following secondary infection, liver *nkla* was up-regulated at 6hpi (>100fold) and then gradually downregulated at 48 hpi in liver but was still up-regulated in kidney at 48 hpi (<10 fold) although at a very small fold change. Similar observations were noted for liver *nkla* expression in fish treated with β glucan + RE-33® where the *nkla* mRNA was up-regulated at 24 hpi (>100 fold) and then down regulated at 48 hpi in liver. Although the *nkla* expression was up-regulated in kidney at 48 hpi (<10 fold) but a lower level. This observation suggests that administration of β glucan alone or in co-administration with RE-33® does not involve *nkla* and *nkld* in the bacterial lysis activity. In the case of R848 exposure (primary stimulation), liver *nkla* was up-regulated at 48 hpi (>100hpi) and kidney *nkla* was up-regulated at 24 hpi (>100 fold). Following secondary stimulation, liver *nkla* was downregulated at 48 hpi and kidney *nkla* was up-regulated at 48 hpi (>100 fold). There was no up/down-regulation of *nklb* in the control fish treated with *E. ictaluri*. *Nklb* was up-regulated in liver at 48 hpi in RE-33® vaccinated fish. *Nklb* was up regulated at 6hpi in liver and 24hpi in liver and kidney post challenge in β glucan and β glucan + RE-33® treated fish, respectively. Liver *Nklb* was up-regulated in liver at 48 hpi in R848 and in R848 + RE-33® treated fish. Interestingly, *nkla* was downregulated in liver in both TLR ligand administered groups only after bacterial challenge. Septic shock

causes an influx of cytokines that can mediate a negative effect on the host. During this time, the host adapts to this "cytokine storm" by becoming immunocompromised and are more susceptible to infections that often leads to fatal outcomes (Słotwiński, Sarnecka et al. 2015) (Wiersinga, Leopold et al. 2014). This could be the reason for downregulation of the Nk-lysins. Another reason for downregulation could be due to a feed-back mechanism, occurring during NK cell stimulation and pathogenesis. Other studies in fish treated with R848 have reported up-regulated expression of genes associated with TLR signaling, notably IFN I, IFN-γ, IL-1β, IL-8, TNF-α, and interferon regulatory factor 3 (IRF3) (Palti 2011) (Purcell, Kurath et al. 2004) (Svingerud, Solstad et al. 2012). In our study, up-regulation of the NK-lysins was observed in the liver and kidney of β glucan and β glucan + RE-33® treated fish groups during the secondary stimulus (*E.ictaluri* challenge). In contrast, in R848 and R848 + RE-33® treated fish groups, Nk-lysins were up-regulated during the primary stimulus (vaccination). These results indicate that R848 induces a greater immune response following primary stimulus comparable to that observed in secondary stimulus (*E. ictaluri* challenge), which suggests that R848 has a lasting protective effect but not as robust as β glucan, β glucan + RE-33 or R848 + RE-33®. This observation infers that R848 provides only a primary protection but β glucan provided a longer secondary immune memory protection.

According to Hong et al. 2006, the kinetics leading to increases and decreases of chicken Nk-lysin transcript levels coincide with parasite oocyst production, thus suggesting that transcription levels increase when more pathogens are present (Hong, Lillehoj et al. 2006). Once *E. ictaluri* was injected intramuscularly in zebrafish, the pathogen may have travelled to surrounding tissues and induced transcription of the Nk-

lysin genes. This could have led to variations in NK-lysin response, as some tissues may have been exposed to the pathogen at different times. In chickens, the activity of the peptide was dependent on intact disulfides, and both the bactericidal effect and the cytolytic effect of the peptide were inhibited when Nk-lysin was downregulated (Andersson, Gunne et al. 1996). In channel catfish, all Nk-lysin genes are expressed, but exhibit distinct expression profiles in various tissues (Wang, Wang et al. 2006). In channel catfish, it is possible that the appearance of mature CTLs affects the number of NK cells and/ or the transcription of certain genes and therefore, these changes in the cell populations could be altering the expression level of Nk-lysin genes. Overall, we observe that T and B cell deficient zebrafish, *ifnγ*, Nk-lysins and *nitr9* (in co-administered TLR ligand and vaccine group) were up-regulated mostly following challenge with *E. ictaluri*, indicating the response of macrophages and NK cells during the onset of infection. This could suggest that β glucan promotes memory in the naïve cells which is why we observe increased expression of the Nk-lysins mRNA following pathogenic challenge.

5.5 Conclusion:

Our findings reveal pathways that are important for the way fish that have limited lymphocyte based immunity can respond to primary infections or vaccination. T and B cell deficient *rag1*$^{-/-}$ mutant zebrafish demonstrate specific protection (Hohn and Petrie-Hanson 2012), and our current study was performed to better characterize the cell population(s) involved in that response by interpreting transcript level changes in tissues. Gene expression profiles and survival (following bacteria challenge) results gave us an idea about the measure of immune function. Differential expression of immune response genes suggests how tissue environments induce changes in innate immune cells that can

result in these cells having the capacity to mediate protective immunity at subsequent pathogen exposure.

Significant increases in *ifnγ* expression in Poly I:C treatment did not occur (Muire, Hanson et al. 2017) and when Poly I:C was co-administered with vaccination, survival was not increased. Administration of β glucan, β glucan + RE-33® and R848 + RE-33® 4 weeks prior to bacterial challenge resulted in the greatest survival. Intracoelomic injection of β glucan and R848 caused >100 fold increase in *ifnγ* expression in liver and kidney tissues (Muire, Hanson et al. 2017). *Rag1*$^{-/-}$ mutant zebrafish can develop protective immunity, and TLR ligands (β glucan and R848) stimulate this protective response when administered alone or co-administered with RE-33®.

5.6 References

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CHAPTER VI

THE DEVELOPMENT OF *LCK:mCherry RAG1*$^{-/-}$ MUTANT ZEBRAFISH AND *RAF1A*$^{-/-}$ MUTANT ZEBRAFISH MODELS TO INVESTIGATE THE ROLE OF NK CELLS IN TELEOST IMMUNITY

Abstract

Zebrafish are a powerful model to study the vertebrate immune system. The lack of availability of monoclonal antibodies for zebrafish immune cell markers has made it challenging to use zebrafish as a model for several studies. As an alternative, several labs are working to develop transgenic zebrafish and knock out models to study particular cells. Transgenic fish that express mCherry protein is driven from the LCK promoter. Lymphocyte specific tyrosine kinase (*lck*) is expressed by T cells and NK cells. We developed the wild type:*lck*:*mCherry* as well as *rag1*$^{-/-}$:*lck:mCherry* zebrafish models, using the Tol2 kit method, to elucidate the functions of *lck* expressing Natural Killer (NK) cells in the absence of T cells. At 4 days post fertilization (dpf) lck^{+} cells were seen in the head kidney and blood vessels. At 7 dpf *lck*$^{+}$ cells were seen in thymus of wild type*:lck:mCherry* zebrafish. Lck^{+} cells were seen only in the head kidney and the blood vessels in *rag1*$^{-/-}$*:lck:mCherry* zebrafish. No lck^{+} cells were seen infiltrating the thymus in *rag1*$^{-/-}$*:lck:mCherry* zebrafish. Our observations suggest the lck^{+} population at 4 dpf in the *rag1*$^{-/-}$ mutant zebrafish comprises the NK cells. To further elucidate the function of NK cells without the interference of trained macrophages in innate immune protection, we

developed a *raf1*$^{-/-}$ mutant zebrafish model using CRISPR-Cas9 gene knock out technique. *Raf1a* is a serine/threonine kinase and is activated via the MEK-ERK1/2 pathway and activates monocytes to become trained macrophages.

6.1 Introduction

Zebrafish have proved to be highly advantageous in research due to their small size, fast generation time, transparent embryos and ease to carry out gene mutations and knock down studies. The tol2kit is a multisite gate way based construction kit for tol2 transposon transgenesis constructs (Kwan, Fujimoto et al. 2007). The importance of using transgenic zebrafish in research is that it allows one to study the function of a particular gene either by inactivating the gene function or by fusing a reporter gene to the promoter of the gene of interest and tracking its function in vivo. In this study we worked to differentiate natural killer (NK) cells from macrophages. The CRISPR-cas9 gene knock out (KO) technology system has been used as a successful tool to target important genes in many cell lines and organisms, including human (Mali, Yang et al. 2013, Louwen, Staals et al. 2014), bacteria (Louwen, Staals et al. 2014), zebrafish (Hwang, Fu et al. 2013), pigs (Hai, Teng et al. 2014), goats (Ni, Qiao et al. 2014), plants (Miao, Guo et al. 2013), *C. elegans* (Waaijers, Portegijs et al. 2013), yeast (Niu, Shen et al. 2014), *Drosophila* (Gratz, Ukken et al. 2014), monkeys (Niu, Shen et al. 2014), rabbits, rats and mice (Mashiko, Young et al. 2014).

Lymphocyte specific tyrosine light chain kinase gene (*lck*) is a T cell specific tyrosine kinase which is expressed by immature and mature T cells as well as in NK cells. The mCherry expression is driven by the lck promoter activation. The *lck:mCherry* zebrafish model was used in previous studies to elucidate the functions of T cells

(Langenau, Ferrando et al. 2004, Trede, Langenau et al. 2004, Brugman, Witte et al. 2014). A recent study used the *lck:mCherry* zebrafish to study lymphocyte like cells (LLCs) (Carmona, Teichmann et al. 2017).

Raf1a is a proto oncogene, a serine/threonine kinase and is activated via the MEK-ERK1/2 pathway. *Raf1a* functions downstream of p21 Ras (Zhang, X. F. et al 1993 and Ghosh, S. et al 1994) and serves as an upstream regulator of the Ras-Raf1a-MEK-MAP kinase signal transduction cascade that is activated in response to a wide variety of signals, including growth factors, differentiation hormones, tumor promoters, inflammatory cytokines, calcium mobilization, DNA-damaging agents and oxygen radicals.

T-bet (also known as Tbox-21) belongs to the T-box family of genes, consisting of over 20 members characterized in mammals. They contain a conserved sequence, around 200 amino acids in length, called the 'T-box', which, in *t-bet*, is centrally located, whereas, in other members, it is located at the amino terminus. T-bet is the transcription factor required for the differentiation of the naïve T cell to Th1 cell and of innate lymphoid group 1 cells, which consists of Natural Killer (NK) cells and the innate lymphoid cells (ILC) in humans and mice. NK cells are prototypical type I (ILC). Additionally, the T-box transcription factors, eomesodermin *(Eomes)* (E4BP4) and *t-bet* direct the fate and function of cytotoxic cell lineages including NK cells and $CD8^+$ T cells. Runx3, GATA-3, *t-bet* and Eomes mediate regulation of NK cells response. The expression of *t-bet* suppresses Eomes and results in *Eomes*-/- NK cells. This means that the repression of *t-bet* is essential for the expression of the *Eomes* +/+ NK cells (Daussy, Faure et al. 2014). Thus, *t-bet* controls the developmental stability of immature NK cells,

while Eomes regulates NK maturation. The zebrafish *t-bet*/ *tbx21I* cDNA (EMBL accession no. AM942761) consists of a 36 bp 5'-untranslated region (UTR), a 419 bp 3'-UTR and a single open reading frame of 1830 bp (Mitra, Alnabulsi et al. 2010). The gene organizations of zebrafish *t-bet* were also determined and found to be very similar to their human homologues. Both the human and zebrafish *t-bet* genes contain the same number of exons and introns i.e., six exons and five introns.

Here, we present results from our attempt to generate a *t-bet* transgenic construct; the preliminary results from *rag1*$^{-/-}$*:lck:mCherry* zebrafish; and the methods and results from CRISPR-Cas9 mediated *raf1a* KO zebrafish. We developed these models with an intent to elucidate the role of NK cells in zebrafish immunity, due to the scarcity of molecular tools or antibodies to target and study zebrafish NK cells. I would like to acknowledge the American Association of Immunologist (AAI) for selecting Dr. Petrie-Hanson's laboratory as the recipient of a Travel for Techniques Award to learn the CRISPR/cas9 system for generating knockout zebrafish. This award funded my travel to Dr. Jeffrey Yoder's laboratory at North Carolina State University to learn the CRISPR-Cas9 technique, and I thank Dr. Jeffrey Yoder and Dustin Wcisel for teaching me the CRISPR-Cas9 technique. I also thank Dr. Matthew Jenny from University of Alabama, Tuscaloosa for helping with the *lck:mCherry* transgenic fish project.

6.2 Material and methods

6.2.1 Zebrafish care

Zebrafish were mated, raised and maintained at 28°C in the specific pathogen free hatchery in the College of Veterinary Medicine, Mississippi State University (MSU) following standard lab protocols. Experimental protocols were approved by the MSU

Institutional Animal Care and Use Committee (IACUC). A homozygous breeding colony of *rag1*$^{-/-}$ mutant zebrafish was previously established (Petrie-Hanson, Hohn et al. 2009) and bred at the CVM-SPF hatchery and the experimental fish are progeny from that colony.

6.2.2 Amplification of desired sequence

This method uses the three-insert multisite gateways system. The three inserts are the entry vectors: 5' entry clone (p5E-XX), middle clone (pME-XX) and the 3' entry clone (p3E-XX). The 5' entry clone contains the promoter element of the gene of interest, with attL4 and attR1 sites flanking the insert. The 5' entry clone has the *t-bet* (prepared in house) (Fig. 6.1) or *lck* (purchased from: https://www.addgene.org/58890/) gene promoter elements as inserts. In the middle clone the insert is a reporter: *mCherry* with attL1 and attL2 sites flanking the insert. The 3' entry clone contains a polyA signal or a 3' tag (*myc:*EGFP fusions) with attR2 and attL3 sites flanking the insert. The 3' entry clone has a polyA signal.

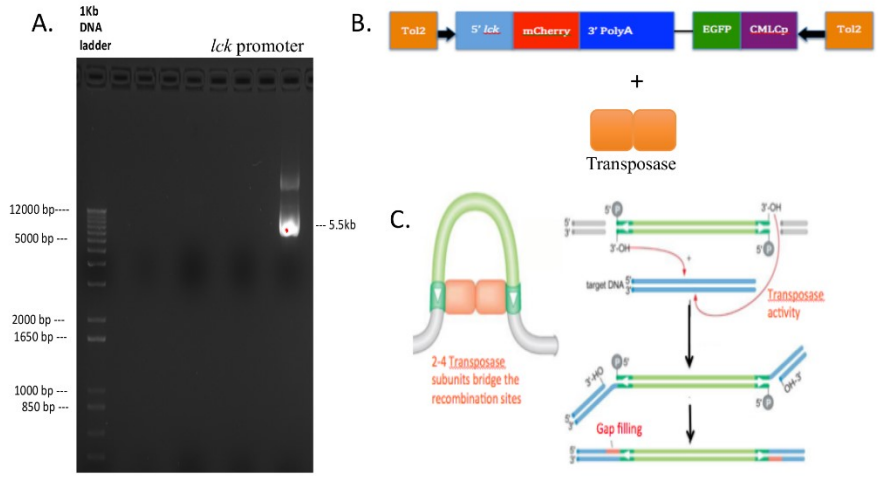

Figure 6.1 *lck:mCherry* 5' entry construct

(A) Overview of *lck:mCherry* entry construct; (B)5.5Kb lck promoter; (C)Site specific recombination and transposase mediated cut and paste mechanism of transposition (animation adapted from (Watson and Watson 2008).

6.2.3 Generation of *t-bet* 5' entry clone

Bacterial artificial chromosome (BAC) was extracted using BAC extraction kit (zymoresearch) from [BAC] CH211-194K22 transformed *E.coli* cells (http://zfin.org/action/marker/view/ZDB-BAC-050218-1713). In order to amplify the *t-bet* gene promoter sequence upstream of exon 1, we PCR amplified the target sequence by using appropriate primer sets designed for ~6.5kb upstream of the first exon and few base pairs downstream of the start site. Since the start site for *t-bet* is in the 1st exon. There is no need to go downstream of the first exon. There is a conserved element ~1kb upstream of the first exon which could be the non-coding exon. The primers designed for *t-bet* amplification were: forward - aaaatcgatTATGTAATATAAGCACTTAGCT and reverse – aaaggatccCTTCTTACCGTCCTTGTTTCGT. The amplified region of *t-bet*

was cloned in the vector 228 (from Dr. Matthew Jenny, University of Alabama, Tuscaloosa, AL), to generate the "*t-bet* 5' entry clone".

6.2.4 Designing target oligo(s) and primers *for Raf-1a* KO zebrafish mutants using CRISPR-Cas9 technique

The target for the CRISPR-cas9 mutation (Fig. 6.2) selected was designed using http://useast.ensembl.org/index.html and http://zifit.partners.org/ZiFiT/Disclaimer.aspx. Target sequence was: 5'-taatacgactcactataGGAACTTGTAACCACATCTTgttttagagctagaaatagc-3', this region was within exon 5 and within the protein kinase domain of RAF1a. The ultramer oligo for the guide RNA synthesis was 5'AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'

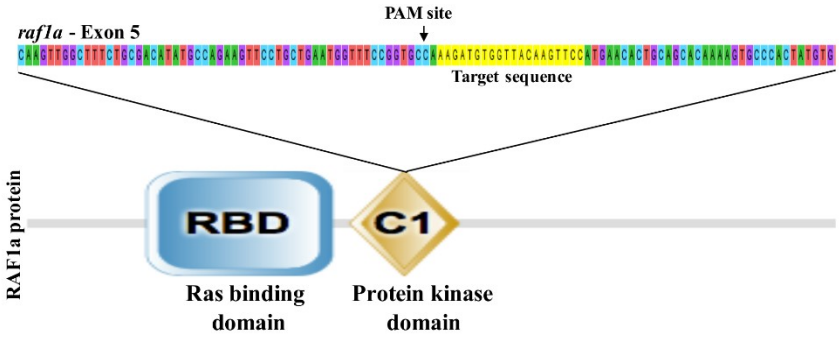

Figure 6.2 Target site for CRISPR-Cas9 mutations and protein coding region in *raf1a.* RAF1A protein domains. RBD: Ras binding domain; C1: protein kinase domain. (Animation drawn on www.smart.embl-heidelberg.de).

6.2.5 GuideRNA (gRNA) synthesis

To align target oligos and guideDNA: dNTPs, target oligo, guideDNA (default) and taq polymerase were combined. The following thermal cycles were applied: denaturation at 98C for 2min, annealing at 50C for 10min and extension at 72C for 10min. This was followed by in-vitro RNA transcription using manufacturers protocol (Ambion Invitrogen - mMESSAGE mMACHINE® T7 Transcription Kit (Catalog number: AM1344). gRNA sample was DNAse treated followed by a RNA clean up procedure (RNA Clean & Concentrator™-5 (Catalog number R1015). gRNA was stored at -80C until use.

6.2.6 Spawning and microinjections

For the *lck:mCherry* injections *we used rag1*$^{-/-}$ mutant and WT zebrafish. For the *raf1*$^{-/-}$ KO CRISPR-Cas9 mutations, we used NHGRI-1 WT zebrafish strain. Fish were set up to spawn (males and females mixed at a ratio of 2:1) per holding tank with spawning nets to get at least 200 healthy eggs per group. Spawning tanks were provided with air stones and covered all night. After the injection set up was ready, spawning tanks were brought out to a will lit area to drop eggs. Eggs were microinjected at the 1 to 2 celled stage (i.e. approx. within 1h post fertilization) at both times. Eggs in the tol2kit experiment were *lck:mCherry* construct and transposase enzyme. Eggs in the CRISPR-Cas9 experiment were injected with gRNA and Cas9 enzyme.

6.2.7 Confirming microinjections

Forty-eight hours post microinjections, fry were screened with an OLYMPUS 1X50 stereomicroscope with OLYMPUS U-RFL-T burner and positive fry with the

lck:mCherry transgene were raised to adult. About 8 of the *raf1*a gRNA injected embryos or adults were genotyped for mosaic mutation. Genomic DNA was extracted from individual embryo or fin clips from adult fish and PCR (Promega GoTaq - GoTaq® DNA Polymerase) was performed with forward: 5'-CAGTCGGGCGTCATCACTGTCTTGACCATCTCTTTTCA-3' and reverse: 5'-AGTCCAGTCTACACACATAGTGG -3' primers designed upstream and downstream of the target site. The thermal conditions were as follows: initial denaturing: 95C for 2 minutes, denaturing: 95C for 30 seconds, annealing 54C for 30 seconds, extension: 72C for 30 seconds, final extension: 72C for 7 min, hold: 4C. Each sample was diluted as 1:50, 1:20 or 1:10 (based on the thickness of the band on the gel) and subjected to fragment analysis. Data collected from fragment analysis was analysed using Peak Scanner software by Thermo Fisher-Applied Biosystems.

6.2.8 Genotyping adult zebrafish and mating patterns

The F0 adult zebrafish with a mosaic mutation was crossed with a wild type zebrafish. The progeny (F1) were raised to adults and were fin clipped and genotyped as above. Heterozygous mutations were confirmed by TA cloning the PCR product in to a pCR4 vector (Invitrogen) and sequencing in forward (T3 primer) and reverse (T7 primer) directions. Sequencing data was analyzed by MEGA M7, Sequencher 5.4.6 and Lasergene softwares. F1 zebrafish with heterozygous mutation in one of the *raf1a* alleles were in-crossed. $1/4^{th}$ of the F2 progeny are expected to have a homozygous mutation for *raf1a* (Fig 6.3).

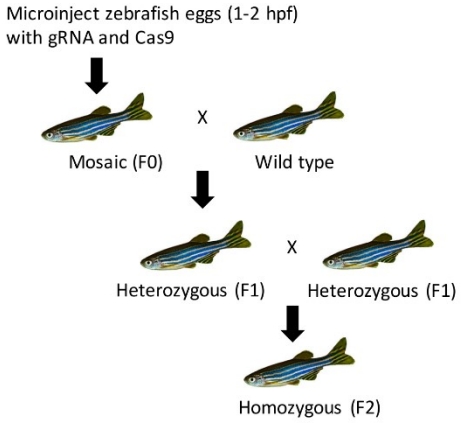

Figure 6.3 Schematic of mating patterns.

6.2.9 Microscopic detection and imaging

Images of 4 days post fertilization (dpf) and 7 dpf *lck*$^{+}$*:mCherry* fry were acquired with a Cytation 5 image reader at 10X and 60X magnification under bright field or texas red (detects mCherry) or FITC (detects green fluorescence protein) filters. GFP fluorescence was excited at 488 nm detected between 415 and 570 nm. *mCherry* fluorescence was excited at 561 nm and detected between 570 and 655 nm. Fry were anesthetized in 0.4% MS-222 (Tricaine) solution and immobilized for a short time in 3% methyl cellulose during the image capture period and were revived minutes after placing them back in fresh water.

6.3 Results

6.3.1 *T-bet* amplification of 6.5 kb upstream of exon 1

We were able to amplify a ~3.9 kb, instead of a 6.5 kb fragment, upstream element of the *t-bet* gene (Fig 6.4).

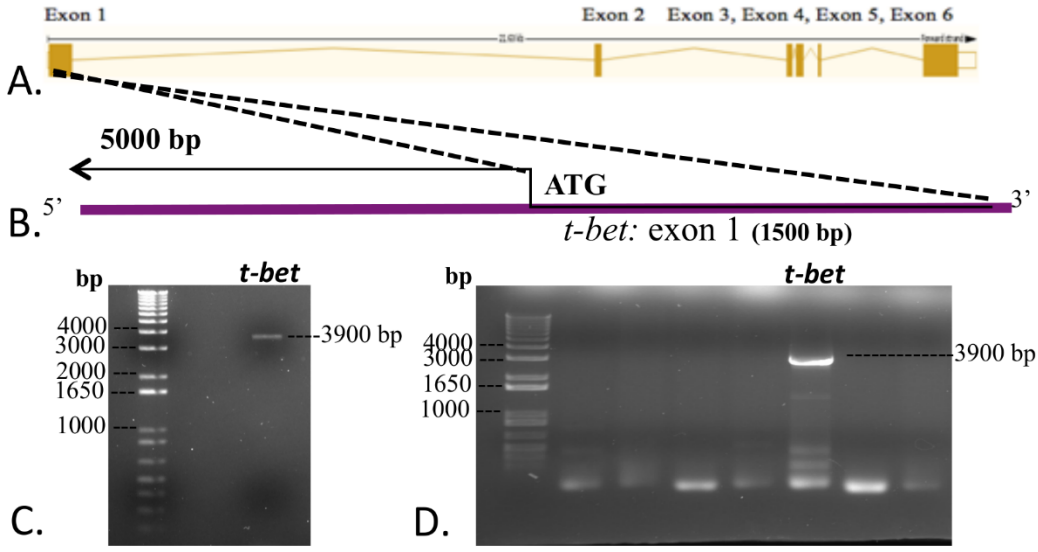

Figure 6.4 Construction of *t-bet* entry clone.

(A)*T-bet* gene with exons 1- 6; (B)Schematics of the amplifying region of *t-bet* exon 1 and putative promoter; (C)3.9Kb amplified product from BAC; (D)3.9Kb cloned product from colony PCR.

6.3.2 NK cells distribution in wild type and *rag1*$^{-/-}$*lck:mCherry* zebrafish

At 24 hpf (hours post fertilization), expression of the myocardial gene (positive control for transgenic insert) was noted in embryos. At 2 days post fertilization (dpf), expression of *lck:mCherry*$^{+}$ cells were noted in the embryos, mainly in the circulatory system in wild type embryos. At 4 days *lck:mCherry*$^{+}$ cells were noted in the circulatory system in wild type and *rag1*$^{-/-}$ mutant zebrafish embryos (Fig 6.5). At 7 dpf *lck:mCherry*$^{+}$ T lymphocytes were homing in the thymus in the wild type zebrafish. Interestingly, at 7 dpf, there was no T lymphocyte homing in the *rag1*$^{-/-}$ mutant zebrafish. (Fig 6.6) This suggests that the *lck:mCherry* $^{+}$ cells in the *rag1*$^{-/-}$ mutant zebrafish are NK cells. However, the F0 generation always has mosaic expression that is not as strong and consistent as an F1. Not all blood cells expressed *mCherry*. Only a small fraction of

individual circulating cells expressed *mCherry* indicating the occurrence of NK cells at 4 dpf.

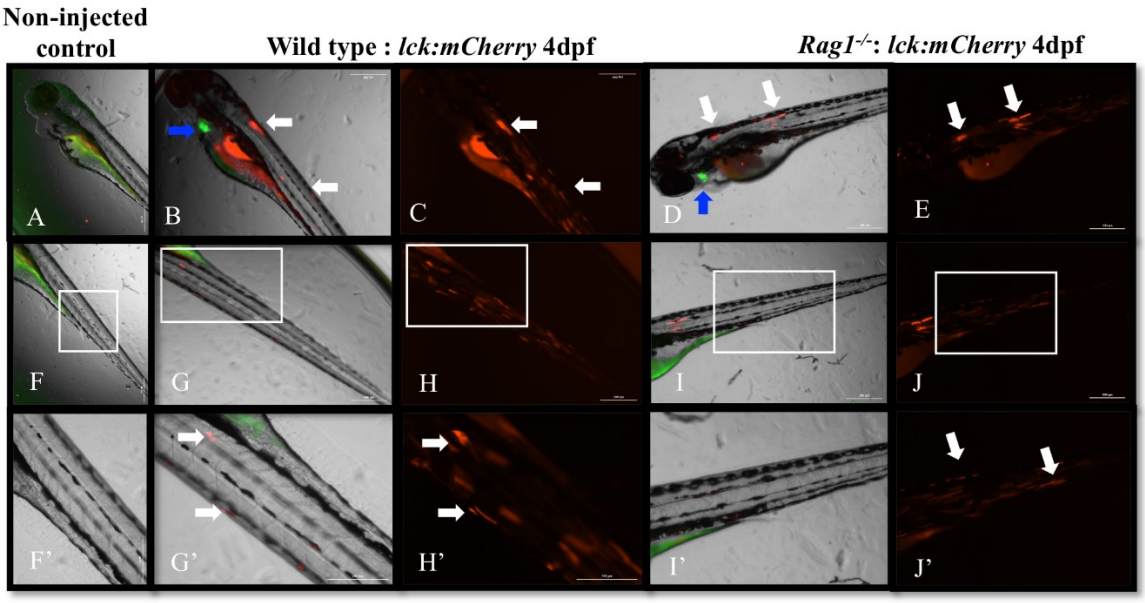

Figure 6.5 *Lck:mCherry* expression in Wild type and *rag1*$^{-/-}$ mutant zebrafish at 4 days post fertilization (dpf). Blue arrows indicate the myocardial gene expression (positive control for transgene insert). White arrows indicate lck+ cells via mCherry expression.

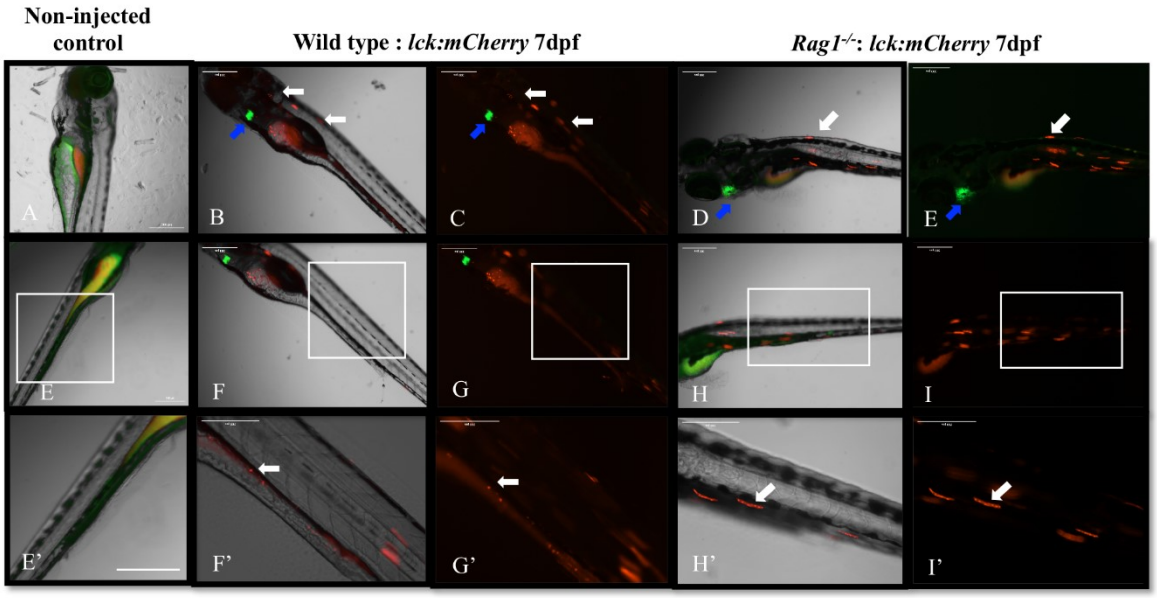

Figure 6.6 *Lck:mCherry* expression in Wild type and *rag1*$^{-/-}$ mutant zebrafish at 7 days post fertilization (dpf). Blue arrows indicate the myocardial gene expression (positive control for transgene insert). White arrows indicate lck+ cells via mCherry expression.

6.3.3 Genotyping F0 for *raf1a* mosaic mutations

Results from fragment analysis suggested a 25% mutation rate of *raf1a* (Fig 6.7). Following screening by fragment analysis, the samples with multiple short peaks were considered positive for mosaic mutation and were subjected to sequencing from further analysis (Fig 6.8).

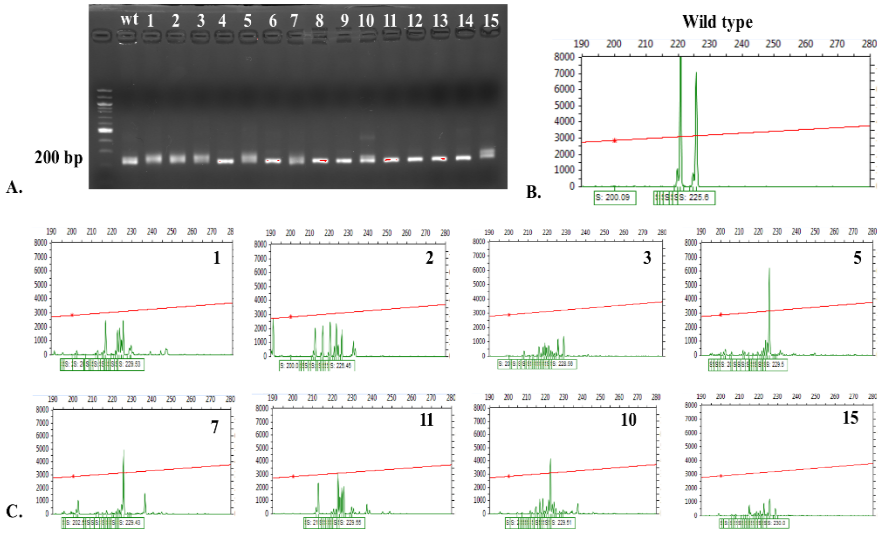

Figure 6.7 Fragment analysis to screen for *raf1a* mosaic mutation in F0 generation.

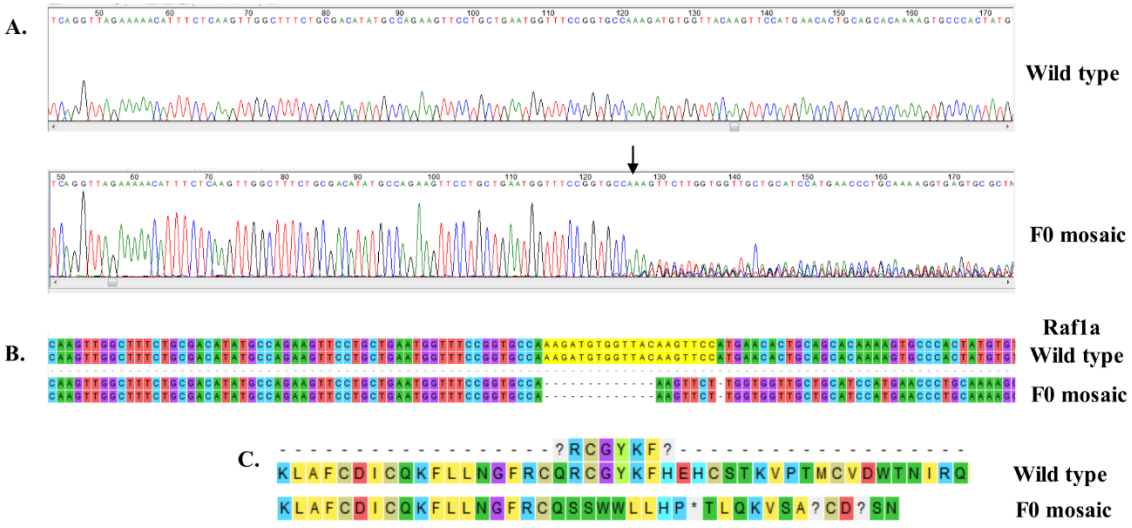

Figure 6.8 Screening F0 generation for *raf1a* mosaic mutation

(A)Chromatograms showing the difference between wild type alleles and heterozygous mutant alleles; (B)Wild type and mosaic allele DNA and protein sequences

6.3.4 Genotyping F1 for *raf1a* heterozygous mutations

Sequencing data from F1 generation showed WT (Fig 6.9 A) and three types mutations within the target sequence at the PAM (TGG) site. Mutation 1 is a 3 bp deletion (Fig 6.9 B). The translation of this mutation did not result in a premature stop codon but resulted in a frame shift mutation. Mutation 2 is a CTCT insertion (Fig 6.9 C). The translation of this mutation resulted in a premature stop codon and addition of serine residues right after the PAM site. Mutation 3 is an 11bp deletion (Fig 6.9 D). The translation of this mutation resulted in a premature stop codon.

Figure 6.9 Genotyping results of F1 generation for *raf1*$^{+/-}$ heterozygous mutation

(A)Wild type allele; (B)Mutation with 3 bp deletion. No premature stop codon but resulted in a frame shift mutation; (C)Mutation with CTCT insertion and a premature

stop codon and addition of serine residues right after the PAM site; (D)Mutation with 11bp deletion and a premature stop codon.

6.4 Discussion:

Tol2Kit method uses att site specific recombination based cloning (multisite gate way technology). The use of the Tol2 transposon backbone takes advantage of the systems transgenesis efficiency (Kawakami 2004). Expression from tol2 constructs seems sufficiently non-mosaic that it may be possible to carry out many experiments in injected embryos (i.e. transient transgenics) rather than using stable lines. The tol2kit method includes a method to visualize transgenes expressing non-target proteins like a *cmlc2:EGFP* transgenesis marker included in the vector backbone to drive EGFP expression. The general requirements for cloning a target gene into a vector are to ideally, take the 1st exon, the 1st intron and 5Kb upstream of the 1st exon; the start site should be in the first or second exon. If the 1st intron is too long (10-20kb), it is difficult to clone it into a vector. Here in the case of *t-bet* the exon 1 is 1500 bp.

T-box transcription factor TBX21/*t-bet* is a protein in humans (Zhang and Yang 2000), mouse (Jenner, Townsend et al. 2009) and zebrafish (Mitra, Alnabulsi et al. 2010). The TBX21 and other related phylogenetically conserved genes share a common DNA-binding domain called T-box. T-box genes encode transcription factors involved in the regulation of developmental processes. *T-bet* has shown to activate T helper 1 (Th1) cells as well as NK cells in human and mice (Szabo, Kim et al. 2000, Jenner, Townsend et al. 2009). This gene has been cloned and sequenced in zebrafish (Mitra, Alnabulsi et al. 2010). Similar to its role in mice and humans, T-bet stimulates the activation of Th1 cells. In a study in molecular cloning and expression analysis of *t-bet* in ginbuna crucian carp,

kidney cells were sorted by FACS and subjected to RT-PCR analysis, which revealed that *t-bet* was highly expressed in surface IgM negative lymphocytes and absent on surface IgM positive lymphocytes, suggesting the importance of the involvement of *t-bet* in the immune system and can serve as an important tool to analyze teleost cell mediated immunity (Takizawa, Araki et al. 2008). In the case of T cells, the homozygous mutant mice for *t-bet* did not differentiate CD4 T cells and showed a marked decrease in *IFNγ* synthesis during a primary stimulation. In the case of heterozygous mice for *t-bet* showed a slight expression of CD4 T cells and NK cells and a milder defect in *IFNγ* compared to the homozygous mutants (Szabo, Sullivan et al. 2002). In the absence of *T-bet*, CD4 T cells fail to differentiate into the Th1 lineage and differentiate to the default Th2 state and NK cells showed markedly reduced cytolytic activity. Hence the role of *t-bet* is also known to mediate NK cell cytotoxicity.

We attempted to develop a *rag1*$^{-/-}$*:t-bet:EGFP* transgenic zebrafish to study the ontogeny of NK cells. The *rag1*$^{-/-}$ mutant fish do not have the ability to undergo recombination activation of the T and B cell receptors, thus rendering the T and B lymphocytes in the inactive state. This makes it feasible to study NK cells activation via *t-bet:EGFP* promoter expression. In our study, we aimed to amplify 6.5kb upstream of exon 1. However, we were not able to amplify 3.9 kb of the fragment even after several attempts. The reason for the failed DNA amplification could be due to the presence of microsatellites in the region upstream of the *t-bet* exon 1. Microsatellites are short (1-6bp), tandem repeats (10-5- copies) in DNA. They are randomly distributed and comprise of mono to tetra nucleotides $(AT)_{n.}$ Therefore, the polymerase might keep slipping off the DNA stand hence resulting in failed amplification of PCR product.

Since we were unable to amplify the *t-bet* promoter, we chose another NK cell specific marker, lymphocyte protein tyrosine kinase (*lck*) to study the zebrafish NK cells. Fry were screened for *cmlc2:EGFP* expression 1 dpf in the heart to confirm the insertion of the *lck:mCherry* insert in the genome. The zebrafish thymus is completely developed by 60 hpf and by 68 hpf is colonized by T lymphocyte progenitors, which give rise to mature T cells at 72 hpf (Langenau, Ferrando et al. 2004). A study by Langenau et al 2004, reported *lck-EGFP* expression in the thymus at 3 or 4 dpf, indicating T cell homing to the thymus. Post 4 dpf, the mature T cells migrate in the blood stream which is indicated by *lck-EGFP* expression in the tail portion (Langenau, Ferrando et al. 2004). At 7 dpf the T cells in the thymus doubled in number giving a brighter signal. Similar to our observation, the study by Langenau et al also reported auto-fluorescence in the yolk sac. In another study, Sylvia Brugman et al used the Tg (lck:mCherry) zebrafish model to study the influence of T cell mechanisms on chemokine (CxCl8) regulation in zebrafish intestines (Brugman, Witte et al. 2014).

In our study, we observed an expression of *lck:mCherry* in the wild type zebrafish as well as in the *rag1*$^{-/-}$ mutant zebrafish at 4 dpf and 7 dpf. At 7 dpf, T cell homing in the thymus was noted in the wild type:*lck:mCherry* zebrafish. T cell homing in the thymus was not observed in the *rag1*$^{-/-}$:*lck:mCherry* zebrafish. However, there was *lck:mCherry* expression observed in the head kidney and blood circulatory vessels in wild type and *rag1*$^{-/-}$ mutant zebrafish. As a negative control, non-transgenic fish were used. The order of development of lymphoid organs is as follows: the thymus is the first lymphoid organ to develop and become lymphoid; this is followed by the kidney and lastly the spleen (reviewed by (Bajoghli, Guo et al. 2011). Kidney is the main site for granulopoiesis in

zebrafish. Immunocompetence in zebrafish, as measured by humoral response to T-dependent and -independent antigens, is not reached until 4–6 wpf (Lam, Chua et al. 2004). Expression of TCRα was observed throughout the thymus at 6 wpf and *rag1* expression was localized to the peripheral cortex (Danilova, Hohman et al. 2004, Bajoghli, Guo et al. 2011).

The *raf1a* gene is the homologue of the viral raf (V-raf) gene. Raf1 is known to regulate the MAP kinase pathway. MAP kinase kinase kinase (MAP3K) functions downstream of the Ras family of membrane associated GTPases to which it directly binds and activates itself. This is followed by binding to RAF1 protein. This binding is associated with phosphorylation to activate dual specificity protein kinase MEK1 and MEK2, which in turn phosphorylate to activate the serine/threonine specific kinase, ERK1 and ERK2. Activated ERKs are pleotropic effectors of cell physiology and play an important role in the control of gene expression involved in cell division, apoptosis, cell differentiation and cell migration.

In this study, we used the CRISPR-Cas9 technique to knock down a *raf1a* gene in zebrafish. The CRISPR-cas9 technology is a recent advance in the field of reverse genetics and knockout (KO) studies and has proven to be a better method for KO zebrafish when compared to the zinc finger nucleases (ZFN) and transcription activator like nucleases (TALENS) methods (Auer and Del Bene 2014).

In 2013, Hwang *et al* were the first group to successfully demonstrate the use of the CRISPR/Cas9 gene KO system to mediate site-specific mutagenesis in zebrafish (Hwang, Fu et al. 2013, Hwang, Fu et al. 2013). In this study, a custom designed sgRNA had to be created by replacing the 20bp responsible for target recognition with the

sequence of interest, so that, the sgRNA has atleast 20bp homology with the target DNA. Following the in-vitro transcription of the customized sgRNA, the Cas9 protein along with the sgRNA are co-injected into single cell stage zebrafish embryos (Kim, Kim et al. 2014, Sung, Kim et al. 2014). Following injections, the N terminus of the 20bp sequence in the sgRNA hybridizes to its complementary locus (target DNA) in the genome by Watson and Crick base pairing fashion. This is followed by the immediate recruitment of Cas9 nuclease to the target site. Cas9 nuclease introduces double stranded breaks in the DNA at the target site just upstream of the PAM (TGG) sequence. The PAM is a standard 3 bp codon which includes any one nucleobase with GG (guanine). The PAM sequence directs the gRNA and cas9 to the target site in the genome to create random double stranded breaks (DSB). DSBs are usually repaired by homology-directed repair (HDR) or the NHEJ pathway. HRD pathway is mediated when a single stranded nick is created, which reduces the occurrence of indel mutations and allows for precise replacement mutations to be made (Overballe-Petersen, Harms et al. 2013). Whereas, the NHEJ pathway results in the incorporation of indels at the site of repair. Formation of indels within the open reading frame causes frame shifts leading to premature stop codon and results in the loss-of-function alleles. Thus, creating a KO zebrafish. Recent advances using the CRISPR/Cas9 approach successfully generated DSBs in multiple targets by co-injecting several custom sgRNAs with Cas9 protein into zebrafish embryos (Le 2013). By using CRISPR/Cas9 we can attain loss-of-function of alleles either by disruption of the open reading frame by indel mutation or by deletion of whole loci from the genome.

Depending on the duration and magnitude of activation, *raf1a* kinase plays a crucial role in several normal and pathological cellular processes including proliferation,

differentiation, development, senescence, programmed cell death, cell cycle progression, immune responses and carcinogenesis (Wood, Sarnecki et al. 1992) (Morrison 1990, Marshall 1995) (Heidecker, Kölch et al. 1992) (Hu, Stites et al. 2013). *Raf1a* is also responsible in maintaining normal neurogenesis and a neuroprotective ability (Rössler, Giehl et al. 2004).

Previous studies in mammals have reported some disorders associated with defects in *raf1* expression. Defects include congestive heart failure and arrhythmia leading to premature heart failure; leopard syndrome 2 (LPRD2) characterized by pulmonic senosis, electrocardiograph conduction abnormalities, retardation of growth, abnormalities of growth and sensorineural deafness; Noonan syndrome 5 characterized by short stature, facial dysmorphic features such as hypertelorism, congenital heart defects and hypertrophic cardiomyopathy. Similarly, a mutation in the ATP binding pocket of *Raf* (RafK375M) acts as a dominant negative in *Xenopus* embryos, resulted in embryos with shortened body axes and defects in the processes by which mesoderm is formed and specified. Likewise, mice lacking *A-Raf*, *B-Raf*, or *c-Raf-1* exhibit several defects in development and organogenesis. *Drosophila D-Raf* acts downstream of a number of receptor tyrosine kinases that are required for normal development, including the EGF, FGF, torso, and *sevenless* receptor.

In humans, the region between 398 and 401bp is responsible for the activity of the *raf1a* gene activity. Activation of the *Raf1a* serine/threonine protein kinases is tightly regulated by multiple phosphorylation events. To examine whether the RafAAA mutation would affect normal development, synthetic RNAs encoding wildtype human *Raf1a* were injected into one to two cell zebrafish embryos. The mutation replaces a critical lysine

residue at the ATP binding pocket of *raf1a* resulting in an inactive protein. Injection of wildtype *raf1a* had no effect on normal zebrafish development, whereas about 30% of embryos injected with mutated *raf1a* (replaced lysine residue at ATP binding site) at 24 hpf produced a phenotype in embryos with no tail or severely shortened, and the over- all length of the anterior-posterior axis is shortened. Examination of injected zebrafish embryos at 12 hpf frequently revealed that the notochord was bent or shortened. Thus, injection of *Raf1a* with a mutation only in the phosphatidate (ATP) binding site is sufficient to block normal signaling events that are required for the expression of *ntl* in the newly formed mesendodermal layer, resulting in developmental defects. To avoid this lethal mutation, we designed the target site in the protein kinase domain coding region on exon 5 between 552bp and 571bp. The Ras binding domain (RBD) is between 49 – 124 aa. The protein kinase domain (132 – 177aa) codes for intracellular signal transduction and hence will not affect the development of zebrafish.

From previous studies, a mutation in the phosphatidate binding site II of *raf1a* results in a compromised development program of zebrafish and this leads to embryos with bent tail trunk structures and a shortened anterior – posterior axis. Such phenotypic changes were not observed in our study.

Sonja et al 2007 have demonstrated a role for *raf1a* in controlling adaptive immune response through the activation of dendritic cells. The binding of microbial pathogens to C-type lectin type DC-SIGN expressed by dendritic cells activates *raf1a*-acetylation-dependent signaling pathway to modulate toll like receptor (TLR) signaling pathways and this *raf-1* mediated pathway is involved in regulation of adaptive immune cells mainly T helper cells. Activation of *raf1a* leads to the acetylation of p65, one of the

key activating subunits of NF-κB transcription factor (Gringhuis, den Dunnen et al. 2007).

Activated β glucan receptor dectin I mediates a non-canonical *raf1a* pathway to activate functional reprograming of monocytes to transform into trained macrophages, leading to enhanced cytokine production in vivo and in vitro (Quintin, Saeed et al. 2012) (Saeed, Quintin et al. 2014). This reprograming included changes in histone trimethylation at H3K4, which suggests the involvement of epigenetic mechanisms in this phenomenon in macrophages and NK cells (Netea, Latz et al. 2015) (Quintin, Cheng et al. 2014) (Hoving, Wilson et al. 2014) (Netea, Joosten et al. 2015) (van der Meer, Joosten et al. 2015) (Dambuza and Brown 2015). The *raf1a*$^{-/-}$ mutant zebrafish will help up further determine the concept of trained immunity and may be employed to design improved vaccine strategies.

The occurrence of *lck:mCherry* expression in *rag1*$^{-/-}$ mutant zebrafish suggests that *lck* is also expressed in zebrafish NK cells and these cells first appear at 4 dpf. We noticed an equal expression of *lck:mCherry* in *rag1*$^{-/-}$ and wild type fish, which could be because this this the F0 generation and the integration of the construct in the genome could result in mosaic expression. A clear idea of the expression of *lck* gene could be made in the F1 generation (F0 x WT). The *rag1*$^{-/-}$*:lck:mCherry* model could prove beneficial to eliminate the challenges associated with the lack of NK cell specific monoclonal antibodies and help researches to sort out the lck+ NK cell population to further elucidate its role in immune protection.

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APPENDIX A

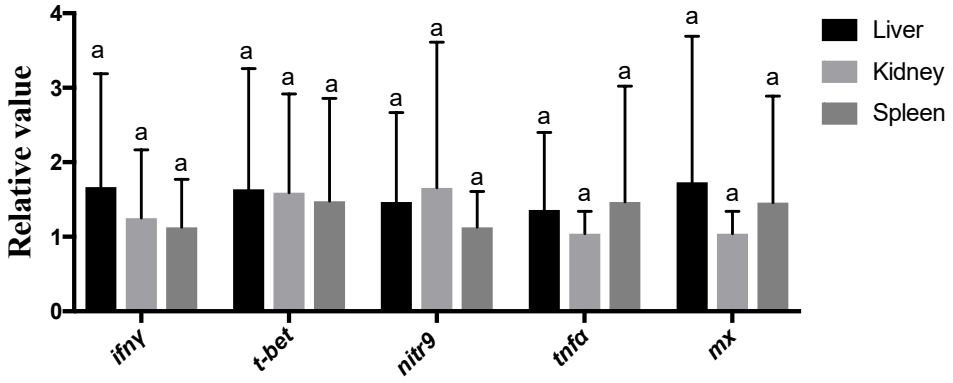

Figure A.1 Expression analyses of *mx, tnfα, ifnγ, t-bet* and *nitr9* in liver, kidney and spleen of non-injected (control) adult *rag1*$^{-/-}$ mutant zebrafish (n=6) were analyzed by RT-qPCR.

Gene expression levels of *mx, tnfα*, *ifnγ*, *t-bet* and *nitr9* were normalized with housekeeping gene, *arp,* expression levels. No significant differences were observed in the gene expressions between tissues prior to injecting *rag1*$^{-/-}$ mutant zebrafish with TLR ligands. Data are presented as mean fold change ± standard deviation.

Table A.2 Differential gene expression following stimulation of toll like receptors ligands in *rag1*$^{-/-}$ mutant Zebrafish.

Treatment	Gene	Tissue	Time	p value	Mean ± SD (Treatment)	Mean ± SD (PBS)
β glucan	***mx***	Liver	All time points	0.7871		
		Kidney	All time points	0.1386		
		Spleen	All time points	0.2652		
	tnfα	Liver	1 hpi	0.0122	9.97±3.09	1.14±0.78
			6 hpi	0.0802	4.77±1.33	0.91±0.08
			12 hpi	0.0415	13.55±4.41	3.55±1.03
			24 hpi	0.9427	4.71±3.03	6.21±1.77
		Kidney	1 hpi	0.0006	4.35±1.15	0.25±0.14
			6 hpi	0.0002	8.34±0.53	0.21±0.04
			12 hpi	0.0463	1.99±1.38	0.29±0.07
			24 hpi	0.7631	3.74±1.36	0.61±0.92
		Spleen	All time points	0.2386		
	ifnγ	Liver	1 hpi	<0.0001	1502.27±300.76	167.53±122.24
			6 hpi	<0.0001	1226.20±518.96	115.95±11.93
			12 hpi	<0.0001	1741.50±904.84	207.35±103.17
			24 hpi	<0.0001	2232.38±966.22	578.55±225.71
		Kidney	1 hpi	0.0004	68.61±27.67	7.05±3.89
			6 hpi	<0.0001	193.93±81.42	9.63 ±4.98
			12 hpi	0.0072	62.57±20.94	12.63±4.59
			24 hpi	0.4280	96.18±28.98	59.48±49.36
		Spleen	1 hpi	<0.0001	70.24±30.89	13.40±8.90
			6 hpi	<0.0001	110.87±18.95	8.38±3.71
			12 hpi	<0.0001	94.85±30.77	31.10±26.71
			24 hpi	<0.0001	116.27±36.22	44.98±12.16
	t-bet	Liver	All time points	0.4319		
		Kidney	1 hpi	0.5820	0.24±0.07	0.35±0.17
			6 hpi	0.0332	0.59±0.19	0.28±0.09
			12 hpi	0.9899	0.28±0.07	0.31±0.05
			24 hpi	0.7258	0.35±0.07	0.47±0.16

Table A2 (continued)

		Spleen	All time points	0.1656		
	nitr9	Liver	All time points	0.3400		
		Kidney	1 hpi	0.9361	1.31±1.24	1.48±0.40
			6 hpi	0.8726	1.30±0.05	1.10±0.36
			12 hpi	0.0062	4.17±0.86	5.12±0.84
			24 hpi	<0.0001	1.89±1.46	7.1±1.03
		Spleen	All time points	0.1060		
Poly I:C	***mx***	Liver	1 hpi	0.0145	3.80±0.98	2.12±1.06
			6 hpi	0.0014	17.01±7.64	2.33±1.26
			12 hp	<0.0001	23.27±9.71	2.78±1.48
			24 hpi	0.0002	14.50±7.93	3.11±1.50
		Kidney	1 hpi	0.9973	3.19±1.52	3.07±2.35
			6 hpi	0.0182	19.59±15.54	1.86±1.53
			12 hpi	0.0003	40.86±24.64	1.09±0.44
			24 hpi	0.0052	13.04±5.56	1.08±0.48
		Spleen	1 hpi	0.9999	3.15±1.22	2.60±0.36
			6 hpi	0.0028	8.03±5.67	0.13±0.18
			12 hpi	0.0107	19.76±9.02	0.82±0.44
			24 hpi	0.3500	5.95±3.46	1.42±1.07
	tnfα	Liver	All time points	0.1843		
		Kidney	1 hpi	0.9997	0.97±0.26	1.12±0.36
			6 hpi	0.9783	3.51±2.87	1.00±0.75
			12 hpi	0.4310	1.77±0.42	0.61±0.37
			24 hpi	0.0194	0.17±0.02	0.67±0.24
		Spleen	1 hpi	0.9829	4.01±0.69	3.19±0.19
			6 hpi	0.9894	1.59±1.00	1.09±0.48
			12 hpi	0.4594	0.59±0.18	2.38±0.98
			24 hpi	0.0041	0.17±0.03	2.13±0.55
	ifnγ	Liver	All time points	0.3992		
		Kidney	1 hpi	0.9090	4.22±1.20	2.81±0.13
			6 hpi	0.6046	8.38±4.31	4.59±2.68
			12 hpi	0.0025	6.31±2.14	0.99±0.95

Table A2 (continued)

			24 hpi	0.9973	2.76±2.38	2.57±0.64
		Spleen	All time points	0.0521		
	t-bet	Liver	All time points	0.1279		
		Kidney	1 hpi	0.2044	0.58±0.20	0.35±0.17
			6 hpi	0.0004	1.21±0.30	0.28±0.09
			12 hpi	0.0913	0.64±0.26	0.31±0.05
			24 hpi	0.9978	0.44±0.13	0.47±0.16
		Spleen	All time points	0.1470		
	nitr9	Liver	All time points	0.5372		
		Kidney	1 hpi	0.0274	18.77±7.86	1.13±0.52
			6 hpi	0.7303	2.09±1.62	0.58±0.19
			12 hpi	0.7253	1.23±1.13	2.69±1.49
			24 hpi	0.2842	1.09±1.00	3.77±0.54
		Spleen	All time points	0.1954		
R848	***mx***	Liver	1 hpi	0.0003	11.79±3.23	2.12±1.06
			6 hpi	0.0002	30.46±12.59	2.33±1.26
			12 hpi	<0.0001	26.04±10.29	2.78±1.48
			24 hpi	<0.0001	58.25±20.03	3.11±1.50
		Kidney	1 hpi	0.0009	11.41±9.57	3.07±2.35
			6 hpi	0.0008	32.60±10.69	1.86±1.53
			12 hpi	0.0052	13.74±9.12	1.09±0.44
			24 hpi	0.0014	19.03±9.75	1.08±0.48
		Spleen	1 hpi	0.6934	5.67±1.05	2.60±0.36
			6 hpi	<0.0001	4.37±1.20	0.06±0.03
			12 hpi	0.0137	7.73±2.56	0.82±0.44
			24 hpi	0.2371	4.70± 2.43	1.42 ±1.07
	tnfα	Liver	All time points	0.1815		
		Kidney	All time points	0.4432		
		Spleen	All time points	0.1167		
	ifnγ	Liver	1 hpi	0.9897	16.27±5.22	29.08±24.58
			6 hpi	0.0310	100.37±40.04	8.85±2.37
			12 hpi	0.0040	65.41±32.45	2.49±2.24

Table A2 (continued)

			24 hpi	0.0007	41.83±21.16	1.10±0.90
		Kidney	1 hpi	<0.0001	12.61±4.14	2.81±0.13
			6 hpi	<0.0001	153.23±90.34	4.59±2.68
			12 hpi	0.0030	31.45±25.55	0.99±0.95
			24 hpi	<0.0001	24.34±15.29	2.57±0.64
		Spleen	1 hpi	0.7600	2.13±1.24	3.46±0.47
			6 hpi	0.0229	6.37±2.12	0.90±0.19
			12 hpi	0.0179	8.35±3.22	0.70±0.30
			24 hpi	0.0055	5.54±1.78	0.87±0.66
	t-bet	Liver	1 hpi	<0.0001	106.64±77.90	1.29±0.68
			6 hpi	0.0006	11.69±5.48	1.41±0.28
			12 hpi	0.9699	2.16±0.88	2.71±1.07
			24 hpi	<0.0001	179.29±84.28	3.85±1.56
		Kidney	1 hpi	0.0006	3.80±2.80	0.35±0.17
			6 hpi	0.0006	7.15±1.99	0.28±0.09
			12 hpi	0.9913	0.68±0.80	0.31±0.05
			24 hpi	0.0789	2.47±1.17	0.47±0.16
		Spleen	All time points	0.0950		
	nitr9	Liver	1 hpi	<0.0001	49.13±27.15	0.51±0.31
			6 hpi	0.0002	6.22±2.76	0.42±0.35
			12 hpi	0.6616	0.97±0.12	0.56±0.07
			24 hpi	0.9573	3.11±1.64	2.53±1.74
		Kidney	1 hpi	0.2292	0.90±0.56	0.77±0.73
			6 hpi	0.0010	10.18±2.59	0.58±0.19
			12 hpi	0.0811	0.51±0.15	2.69±1.49
			24 hpi	0.1154	0.91±0.30	3.77±0.54
		Spleen	All time points	0.4584		

The numbers highlighted grey denote not significant.

Table A.3 Differential gene expressions of up-regulated and down-regulated genes at different hours post injection (hpi) following primary or secondary immune stimulation in *rag1* mutant zebrafish.

Treatment	Gene	Tissue	Time	p value	Mean ± SD (Vaccination)	Mean ± SD (Challenge)	Mean ± SD (PBS)
E. ictaluri	*Ifnγ*	Liver	6 hpi			101.15±10.91 (0.0172)	34.16± 21.18
			12hpi			91.87±91.2 (0.0056)	1531.35±2009.44
			24 hpi			36.82±17.08	2.05±0.75
			48 hpi			1961.90±303.72 (0.0065)	543.08±502.89
		Kidney	6 hpi			29.43±13.27	29.13±11.36
			12 hpi			28.05±1.75	118.05±49.41
			24 hpi			992.04±142.99 (0.0001)	14.72±7.22
			48 hpi			15.85±11.27	5.00±2.07
	t-bet	Liver	6 hpi			344.85±94.31	329.56±252.22
			12 hpi			72.70±16.52	38.98±50.43
			24 hpi			76.32±44.75	7.74±10.01
			48 hpi			147.56±179.56	3479.65±1030.15
		Kidney	6 hpi			49065.40±23728.97	113274.49±75465.53
			12 hpi			23551.85±13505.67	5510.68±2292.01
			24 hpi			42372.51±24723.35	49539.49±46201.41
			48 hpi			22819.74±4531.83	37631.19±65706.75
	Nitr9	Liver	6 hpi			290.43±158.53	33.37±31.31
			12 hpi			72.78±63.63	28.77±1.85
			24 hpi			36.64±7.68	2.14±1.85
			48 hpi			82.12±48.10	948.39±284.44

Table A3 (continued)

		Kidney	6 hpi			9.51±7.45 (0.0134)	1.41±0.94
			12 hpi			12.07±8.30 (0.0001)	0.08±0.03
			24 hpi			0.57±0.73	1.12±1.56
			48 hpi			0.35±0.33 (0.0005)	0.14±0.14
	Nkla	Liver	6 hpi			13.03±6.80	10.75±5.98
			12 hpi			3.23±1.52	0.37±0.40
			24 hpi			40.74±51.84	6.19±4.15
			48 hpi			11.38±7.00	298.480±322.20 (0.0019)
		Kidney	6 hpi			13.59±1.53	13.14±9.95
			12 hpi			7.41±2.80	0.01±0.02
			24 hpi			12.28±1.13	2.14±1.69
			48 hpi			5.99±3.81 (0.0001)	3.07±2.12
	nklb	Liver	6 hpi			67.32±19.22	17.11±13.14
			12 hpi			0.20±0.17	0.52±0.90
			24 hpi			22.23±11.09	22.51±24.92
			48 hpi			27.45±5.29	227.03±200.91
		Kidney	6 hpi			20.36±4.91	21.97±10.06
			12 hpi			2.82±1.23	1.38±1.41
			24 hpi			1463.84±441.32	2335.70±3036.07
			48 hpi			761.72±714.97	234.37±211.06
	nklc	Liver	6 hpi			200.98±64.57	168.99±183.16
			12 hpi			36.08±8.60	1.94±2.18
			24 hp			84.63±74.10	5.57±3.58
			48 hpi			71.03±31.95 (0.0006)	9.95±4.84
		Kidney	6 hpi			179.12±73.53	283.95±161.04

Table A3 (continued)

			12 hpi			58.21±24.66	0.02±0.02
			24 hpi			78.23±37.45	40.85±6.03
			48 hpi			49.23±26.49	104.72±29.51
	nkld	Liver	6 hpi			52.83±24.31	32.28±10.54
			12 hpi			11.26±8.83	1.22±0.88
			24 hpi			20.48±14.36	6.37±4.04
			48 hpi			13.22±6.74	612.19±176.53
		Kidney	6 hpi			116.80±40.62	155.22±80.33
			12 hpi			35.37±21.81	0.85±0.31
			24 hpi			49.75±9.56	6.90±1.90
			48 hpi			31.72±20.90	52.76±20.32
RE33	*Ifnγ*	Liver	6 hpi		5.59±1.97	160.27±153.07	34.16± 21.18
			12hpi		334.80±78.58	579.04±413.99	1531.35±2009.44
			24 hpi		424.72±241.17	246.11±95.84	2.05±0.75
			48 hpi		2786.47±3229.87 (0.0479)	2132.95±774.88 (0.0019)	543.08±502.89
		Kidney	6 hpi		6.52±2.79	104.10±39.13	29.13±11.36
			12 hpi		188.68±206.82	50.22±34.05	118.05±49.41
			24 hpi		119.74±4.53	25.52±9.37	14.72±7.22
			48 hpi		1678.42±244.57 (0.0001)	328.19±161.69	5.00±2.07
	t-bet	Liver	6 hpi		5.52±2.34	1649.27±1507.74	329.56±252.22
			12 hpi		368.05±233.92	21.40±17.95	38.98±50.43

Table A3 (continued)

			24 hpi		1399.45±482.59	218.60±29.45	7.74±10.01
			48 hpi		1396.93±664.59	446.78±179.79	3479.65±1030.15
		Kidney	6 hpi		119752.56±459300.59	280549.85±275127.30 (0.0141)	113274.49±75465.53
			12 hpi		114340.63±51519.94	4937.97±2163.96	5510.68±2292.01
			24 hpi		41419.45±13373.78	35912.47±10420.89	49539.49±46201.41
			48 hpi		114575.387±4531.83	39919.68±4531.83	37631.19±65706.75
	Nitr9	Liver	6 hpi		14.71±8.13	284.22±198.34 (0.0210)	33.37±31.31
			12 hpi		151.03±123.42	174.05±137.81	28.77±1.85
			24 hpi		214.63±36.68	78.88±56.57	2.14±1.85
			48 hpi		460.90±421.22	96.87±4.23	948.39±284.44
		Kidney	6 hpi		0.13±0.11	1.32±0.54	1.41±0.94
			12 hpi		0.39±0.36	0.19±0.18	0.08±0.03
			24 hpi		0.15±0.04	0.54±0.43	1.12±1.56
			48 hpi		1.38±0.85	0.21±0.21	0.14±0.14
	Nkla	Liver	6 hpi		2.08±0.77	59.39±45.37	10.75±5.98
			12 hpi		207.90±190.35	234.09±197.54 (0.0153)	0.37±0.40
			24 hpi		165.43±32.86	74.23±22.38	6.19±4.15
			48 hpi		310.64±355.32	173.17±138.55	298.480±322.20
		Kidney	6 hpi		5.34±2.51	46.71±37.14	13.14±9.95
			12 hpi		58.36±54.30	98.68±58.01	0.01±0.02
			24 hpi		5.35±1.69	2.00±1.71	2.14±1.69
			48 hpi		36.47±35.25	4.31±1.68 (0.0001)	3.07±2.12
	nklb	Liver	6 hpi		4.58±1.47	75.62±69.43	17.11±13.14

Table A3 (continued)

			12 hpi		235.43±148.21	297.35±230.11	0.52±0.90
			24 hpi		1.49±0.91	34.41±6.09	22.51±24.92
			48 hpi		962.57±779.90 (0.0002)	304.65±279.99	227.03±200.91
		Kidney	6 hpi		559.15±154.85	1585.37±1052.55	21.97±10.06
			12 hpi		7389.26±4622.03	2296.84±897.65	1.38±1.41
			24 hpi		17.17±2.03	347.99±12.87	2335.70±3036.07
			48 hpi		11885.26±7915.68	1641.89±1423.73	234.37±211.06
	nklc	Liver	6 hpi		26.27±13.27	303.95±212.73	168.99±183.16
			12 hpi		151.32±46.71	155.90±104.01	1.94±2.18
			24 hp		179.95±22.19	71.37±13.17	5.57±3.58
			48 hpi		667.80±334.72	173.15±103.17	9.95±4.84
		Kidney	6 hpi		97.40±55.68	221.14±103.08	283.95±161.04
			12 hpi		594.25±418.49 (0.0301)	114.87±80.51	0.02±0.02
			24 hpi		53.12±6.03	30.26±3.09	40.85±6.03
			48 hpi		91.01±49.35	34.59±33.05	104.72±29.51
	nkld	Liver	6 hpi		8.84±3.81	60.62±33.67	32.28±10.54
			12 hpi		144.33±50.78	117.22±41.32 (0.0107)	1.22±0.88
			24 hpi		50.79±1.17	21.89±6.99	6.37±4.04
			48 hpi		321.13±201.98	82.95±118.05	612.19±176.53
		Kidney	6 hpi		53.51±24.34	125.16±63.21	155.22±80.33
			12 hpi		66.43±29.87	49.21±37.11	0.85±0.31
			24 hpi		28.07±3.11	17.45±9.77	6.90±1.90
			48 hpi		74.52±5.49	26.43±16.51	52.76±20.32
B glucan	*Ifnγ*	Liver	6 hpi		523.57±399.49	1369.23±985.77 (0.0116)	34.16± 21.18

Table A3 (continued)

			12hpi		47.16±42.80	580±833.21	1531.35±2009.44
			24 hpi		62.91±16.02	208.38±183.73	2.05±0.75
			48 hpi		139.16±127.82	238.60±135.71	543.08±502.89
		Kidney	6 hpi		14.47±10.66	100.94±80.85	29.13±11.36
			12 hpi		15.87±13.99	575.15±299.31 (0.0276)	118.05±49.41
			24 hpi		5.92±2.51	92.66±48.02 (0.0001)	14.72±7.22
			48 hpi		27.42±5.98	39.86±28.72	5.00±2.07
	t-bet	Liver	6 hpi		7290.57±3349.88 (0.0001)	1954.90±710.20	329.56±252.22
			12 hpi		233.90±143.78	298.29±199.05	38.98±50.43
			24 hpi		1914.98±443.83	66.85±57.53	7.74±10.01
			48 hpi		1372.79±664.59	179.49±179.79	3479.65±1030.15
		Kidney	6 hpi		1064737.21±607786.55 (0.0011)	200692.20±94460.04	113274.49±75465.53
			12 hpi		26190.54±8181.75	117300.99±41162.87	5510.68±2292.01
			24 hpi		77970.24±69076.64	20607.28±15163.86	49539.49±46201.41
			48 hpi		120924±169	65882.08±4531.83	37631.19±65706.75
	Nitr9	Liver	6 hpi		216.42±125.46	591.12±239.39	33.37±31.31
			12 hpi		59.59±44.26	63.00±39.99	28.77±1.85
			24 hpi		203.50±67.25	50.33±44.41	2.14±1.85
			48 hpi		287.70±172.20	62.15±60.98	948.39±284.44
		Kidney	6 hpi		0.75±0.26	1.80±0.79	1.41±0.94
			12 hpi		0.20±0.07	1.80±1.41	0.08±0.03
			24 hpi		1.69±1.11	1.62±1.18	1.12±1.56

Table A3 (continued)

			48 hpi		0.49±0.31	0.56±0.26	0.14±0.14
	Nkla	Liver	6 hpi		37.21±17.18	249.51±208.10 (0.0127)	10.75±5.98
			12 hpi		10.05±10.29	24.01±16.93	0.37±0.40
			24 hpi		35.18±31.92	8.23±7.50	6.19±4.15
			48 hpi		56.92±31.32	5.25±2.85	298.480±322.20 (0.0015)
		Kidney	6 hpi		22.32±14.95	23.92±7.16	13.14±9.95
			12 hpi		11.08±3.23	31.73±11.21	0.01±0.02
			24 hpi		1.77±1.50	7.47±2.08	2.14±1.69
			48 hpi		15.54±9.63	2.19±1.50 (0.0001)	3.07±2.12
	nklb	Liver	6 hpi		52.44±21.51	734.20±430.87 (0.0244)	17.11±13.14
			12 hpi		12.63±4.83	17.52±7.59	0.52±0.90
			24 hpi		45.41±24.46	58.00±50.72	22.51±24.92
			48 hpi		103.18±123.01	62.06±47.68	227.03±200.91
		Kidney	6 hpi		760.30202.16	5536.22±2044.37 (0.0244)	21.97±10.06
			12 hpi		720.01±272.52	9866.47±11062.12	1.38±1.41
			24 hpi		113.26±30.46	719.59±97.27	2335.70±3036.07
			48 hpi		2068.87±1012.37	836.02±513.59	234.37±211.06
	nklc	Liver	6 hpi		449.66±346.55	2134.91±896.55 (0.0001)	168.99±183.16
			12 hpi		171.78±84.55	40.47±24.94	1.94±2.18
			24 hp		386.37±154.04	44.21±33.94	5.57±3.58
			48 hpi		940.16±641.58	60.59±26.64	9.95±4.84
		Kidney	6 hpi		404.05±154.75	279.94±59.11	283.95±161.04
			12 hpi		180.33±134.85	311.62±108.45	0.02±0.02

Table A3 (continued)

			24 hpi		572.19±446.40	50.06±35.28	40.85±6.03
			48 hpi		619.41±157.78	49.49±39.27	104.72±29.51
	Nkld	Liver	6 hpi		103.42±64.37	588.73±81.86 (0.0001)	32.28±10.54
			12 hpi		29.50±27.83	14.74±13.28	1.22±0.88
			24 hpi		94.02±36.81	5.17±3.92	6.37±4.04
			48 hpi		123.14±73.52	18.97±10.26	612.19±176.53 (0.0107)
		Kidney	6 hpi		289.61±189.40	194.29±64.14	155.22±80.33
			12 hpi		89.28±20.36	147.11±44.21 (0.0008)	0.85±0.31
			24 hpi		373.31±20.34	34.58±15.46	6.90±1.90
			48 hpi		193.93±35.16	34.21±25.26	52.76±20.32
B glucan +RE33	*Ifnγ*	Liver	6 hpi		20.50±7.35	237.16±64.05	34.16± 21.18
			12hpi		348.17±52.59	1539.20±273.06	1531.35±2009.44
			24 hpi		172.93±102.33	1400.53±214.21 (0.0075)	2.05±0.75
			48 hpi		293.04±176.36	322.96±88.11	543.08±502.89
		Kidney	6 hpi		13.88±6	62.54±32.10	29.13±11.36
			12 hpi		300.38±104.17	357.19±174.53	118.05±49.41
			24 hpi		36.09±15.07	46.25±19.48	14.72±7.22
			48 hpi		53.33±35.06	954.75±859.15 (0.0001)	5.00±2.07
	t-bet	Liver	6 hpi		4503.40±810.75 (0.0010)	505.14±130.43	329.56±252.22
			12 hpi		2587.18±3131.19	948.25±455.48	38.98±50.43
			24 hpi		1.87±0.44	3103.95±2467.42 (0.0001)	7.74±10.01
			48 hpi		97.99±34.40	80.60±179.79	3479.65±1030.15

Table A3 (continued)

		Kidney	6 hpi		790148.22±775649.65 (0.0011)	29171.11±20790.32	113274.49±75465.53
			12 hpi		312618.13±171006.95	112173.38±87112.28	5510.68±2292.01
			24 hpi		237970.06±59619.65	60893.41±6130.78	49539.49±46201.41
			48 hpi		37455.34±4531.83	51462.46±4531.83	37631.19±65706.75
	Nitr9	Liver	6 hpi		232.07±171.01	142.49±18.28	33.37±31.31
			12 hpi		99.72±31.97	150.94±18.58	28.77±1.85
			24 hpi		2312.75±839.75 (0.0001)	576.44±200.12 (0.0001)	2.14±1.85
			48 hpi		67.49±20.62 (0.0058)	45.93±19.60	948.39±284.44
		Kidney	6 hpi		0.25±0.12	0.43±0.32	1.41±0.94
			12 hpi		0.79±0.69	4.11±3.25	0.08±0.03
			24 hpi		1.16±0.77	1.89±0.16	1.12±1.56
			48 hpi		3.45±5.07	0.97±0.68	0.14±0.14
	Nkla	Liver	6 hpi		22.28±14.04	17.05±2.89	10.75±5.98
			12 hpi		328.45±58.15 (0.0435)	83.47±34.86	0.37±0.40
			24 hpi		19.35±4.00	332.07±142.16 (0.0004)	6.19±4.15
			48 hpi		61.12±19.45	51.54±46.31	298.480±322.20 (0.0015)
		Kidney	6 hpi		5.81±2.74	2.76±2.39	13.14±9.95
			12 hpi		105.22±67.28 (0.0089)	53.01±38.13	0.01±0.02
			24 hpi		34.95±30.08	6.42±1.93	2.14±1.69
			48 hpi		15.75±7.86	2.16±0.96 (0.0001)	3.07±2.12
	nklb	Liver	6 hpi		23.96±15.82	88.29±36.06	17.11±13.14
			12 hpi		188.91±46.78	80.67±8.05	0.52±0.90
			24 hpi		12.95±2.69	597.75±537.69 (0.0002)	22.51±24.92

Table A3 (continued)

			48 hpi		39.26±12.50	115.47±51.41	227.03±200.91
		Kidney	6 hpi		429.17±632.42	950.50±442.02	21.97±10.06
			12 hpi		11304.88±3781.58	41.46±17.56	1.38±1.41
			24 hpi		675.10±212.04	236.61±179.50	2335.70±3036.07
			48 hpi		2568.97±1566.36	2086.55±474.26	234.37±211.06
	nklc	Liver	6 hpi		167.99±63.12	249.35±134.88	168.99±183.16
			12 hpi		266.18±133.14	320.63±41.23	1.94±2.18
			24 hp		73.38±43.38	809.95±551.60 (0.0002)	5.57±3.58
			48 hpi		42.18±26.20	89.78±44.71	9.95±4.84
		Kidney	6 hpi		233.05±110.76	64.06±37.23	283.95±161.04
			12 hpi		1148.01±389.95 (0.0001)	88.86±35.78	0.02±0.02
			24 hpi		145.84±37.56	122.05±93.18	40.85±6.03
			48 hpi		94.88±49.96	209.49±131.08	104.72±29.51
	nkld	Liver	6 hpi		46.47±13.88	41.28±4.17	32.28±10.54
			12 hpi		183.83±136.32	108.03±1.63 (0.0216)	1.22±0.88
			24 hpi		16.78±1.26	246.20±119.84 (0.0001)	6.37±4.04
			48 hpi		19.06±4.82	26.60±15.38	612.19±176.53 (0.0015)
		Kidney	6 hpi		84.84±24.48	79.17±35.66	155.22±80.33
			12 hpi		289.29±171.12	165.04±106.38 (0.0002)	0.85±0.31
			24 hpi		44.58±13.07	109.00±80.96 (0.0299)	6.90±1.90
			48 hpi		14.95±1.72	73.31±26.75	52.76±20.32
R848	*Ifnγ*	Liver	6 hpi		220.90±71.93	38.89±17.43	34.16± 21.18
			12hpi		435.81±145.21	0.32±0.14	1531.35±2009.4
			24 hpi		3435.63±2069.55 (0.0001)	138.14±85.33	2.05±0.75

Table A3 (continued)

			48 hpi		500.66±435.05	269.98±148.81	543.08±502.89
		Kidney	6 hpi		339.69±184.66	7.22±3.83	29.13±11.36
			12 hpi		266.05±149.78	2.16±0.73	118.05±49.41 (0.0029)
			24 hpi		2168.24±756.85 (0.0001)	39.20±10.92	14.72±7.22
			48 hpi		255.35±120.63	291.81±96.43	5.00±2.07
	t-bet	Liver	6 hpi		25.01±26.20	314.34±75.17	329.56±252.22
			12 hpi		472.25±501.76	687.51±261.57	38.98±50.43
			24 hpi		586.32±458.59	133.21±79.68	7.74±10.01
			48 hpi		727.52±664.59	233.83±179.79	3479.65±1030.15
		Kidney	6 hpi		46060.72±21863.54	43092.01±22030.56	113274.49±75465.53
			12 hpi		38450.67±22670.11	74564.99±50454.61	5510.68±2292.01
			24 hpi		138659.09±22502.72	24256.95±3904.60	49539.49±46201.41
			48 hpi		122789.99±4531.83	14272.79±4531.83	37631.19±65706.75
	Nitr9	Liver	6 hpi		4.50±1.20	70.61±12.38	33.37±31.31
			12 hpi		94.16±41.06	359.10±114.05 (0.0013)	28.77±1.85
			24 hpi		200.16±74.26	49.46±28.35	2.14±1.85
			48 hpi		383.37±288.43	63.51±26.45	948.39±284.44
		Kidney	6 hpi		0.03±0.01	0.08±0.04	1.41±0.94
			12 hpi		0.67±0.51	2.38±1.29	0.08±0.03
			24 hpi		4.81±6.61	1.51±0.23	1.12±1.56
			48 hpi		1.17±0.85	0.61±0.22	0.14±0.14
	Nkla	Liver	6 hpi		2.72±0.72	23.86±22.14	10.75±5.98
			12 hpi		9.66±3.19	26.77±8.38	0.37±0.40
			24 hpi		123.41±27.15	19.86±27.15	6.19±4.15

Table A3 (continued)

			48 hpi		980.18±422.02 (0.0001)	82.13±33.52	298.480±322.20 (0.0284)
		Kidney	6 hpi		7.15±4.54	1.45±0.78	13.14±9.95
			12 hpi		1.29±1.09	2.92±1.07	0.01±0.02
			24 hpi		165.94±133.89 (0.0001)	2.24±1.08	2.14±1.69
			48 hpi		180.11±76.35	13.45±0.29 (0.0001)	3.07±2.12
	nklb	Liver	6 hpi		4.78±0.45	93.52±85.30	17.11±13.14
			12 hpi		30.20±40.28	34.05±15.78	0.52±0.90
			24 hpi		110.03±17.89	38.94±26.06	22.51±24.92
			48 hpi		1079.57±465.37 (0.0001)	47.85±21.11	227.03±200.91
		Kidney	6 hpi		446.26±165.13	2051.52±1329.96	21.97±10.06
			12 hpi		849.78±803.26	211.93±62.01	1.38±1.41
			24 hpi		4637.38±4055.53	475.24±289.39	2335.70±3036.07
			48 hpi		22518.52	666.44±379.35	234.37±211.06
	nklc	Liver	6 hpi		10.18±2.07	126.60±29.15	168.99±183.16
			12 hpi		27.53±11.84	257.44±186.13	1.94±2.18
			24 hp		1315.66±763.59	89.55±36.92	5.57±3.58
			48 hpi		12345.62±8618.66 (0.0001)	15.59±6.29	9.95±4.84
		Kidney	6 hpi		68.71±44.21	81.78±49.60	283.95±161.04
			12 hpi		74.01±80.52	79.12±35.94	0.02±0.02
			24 hpi		878.15±486.15 (0.0012)	43.19±21.08	40.85±6.03
			48 hpi		3455.31±813.90 (0.0001)	14.88±7.77	104.72±29.51
	nkld	Liver	6 hpi		3.35±1.27	21.44±7.60	32.28±10.54
			12 hpi		20.65±16.82	79.45±34.81	1.22±0.88

Table A3 (continued)

			24 hpi		224.47±200.59	25.62±15.75	6.37±4.04
			48 hpi		1710.82±799.97 (0.0001)	44.29±27.75	612.19±176.53
		Kidney	6 hpi		23.56±11.43	37.50±33.06	155.22±80.33 (0.0091)
			12 hpi		19.51±15.22	17.53±3.19	0.85±0.31
			24 hpi		415.25±239.42 (0.0054)	17.09±12.57	6.90±1.90
			48 hpi		1611.86±440.36 (0.0001)	5.71±1.15	52.76±20.32
R848 + RE33	*Ifnγ*	Liver	6 hpi		272.60±101.16	46.13±2.22	34.16± 21.18
			12hpi		413.55±299.22	18.51±4.42	1531.35±2009.44 (0.0033)
			24 hpi		409.95±107.55	106.40±111.26	2.05±0.75
			48 hpi		2572.19±2607.77	1126.93±445.83 (0.0001)	543.08±502.89
		Kidney	6 hpi		10.15±7.58	62.72±26.77	29.13±11.36
			12 hpi		419.26±247.54	2.24±0.79	118.05±49.41
			24 hpi		287.95±230.77	0.21±0.04	14.72±7.22
			48 hpi		1104.27±718.25 (0.0001)	1210.85±336.08	5.00±2.07
	t-bet	Liver	6 hpi		384.01±83.86	499.89±241.82	329.56±252.22
			12 hpi		156.91±28.68	65.78±15.98	38.98±50.43
			24 hpi		432.37±96.84	141.87±104.05	7.74±10.01
			48 hpi		4883.43±664.59	139.96±179.79	3479.65±1030.15
		Kidney	6 hpi		169154.27±8310.30	41416.10±13932.68	113274.49±75465.53
			12 hpi		220147.09±135282.08	24955.69±5560.68	5510.68±2292.01
			24 hpi		50343.08±8449.56	80945.91±26099.41	49539.49±46201.41
			48 hpi		92057.63±4531.83	35149.17	37631.19±65706.75

Table A3 (continued)

	Nitr9	Liver	6 hpi		284.50±96.64	80.36±24.50	33.37±31.31
			12 hpi		355.48±257.49	89.20±43.84	28.77±1.85
			24 hpi		1162.78±719.85 (0.0002)	225.29±277.85 (0.0489)	2.14±1.85
			48 hpi		888.51±800.75	129.12±44.74	948.39±284.44
		Kidney	6 hpi		2.10±1.36	0.19±0.11	1.41±0.94
			12 hpi		0.62±0.30	0.14±0.00	0.08±0.03
			24 hpi		0.19±0.13	0.25±0.05	1.12±1.56
			48 hpi		4.70±6.65	0.54±0.23	0.14±0.14
	Nkla	Liver	6 hpi		0.03±0.02	7.90±2.59	10.75±5.98
			12 hpi		28.38±21.79	44.41±23.96	0.37±0.40
			24 hpi		52.89±16.49	3.32±3.24	6.19±4.15
			48 hpi		1010.81±307.61 (0.0001)	50.83±23.92	298.480±322.20 (0.0091)
		Kidney	6 hpi		0.96±0.17	3.22±0.58	13.14±9.95
			12 hpi		8.20±3.33	5.53±5.06	0.01±0.02
			24 hpi		27.88±25.00	4.27±0.61	2.14±1.69
			48 hpi		141.66±52.85 (0.0004)	5.66±3.85 (0.0001)	3.07±2.12
	nklb	Liver	6 hpi		474.57±120.52	8.73±12.12	17.11±13.14
			12 hpi		39.57±5.96	44.42±3.75	0.52±0.90
			24 hpi		109.75±41.30	9.15±9.85	22.51±24.92
			48 hpi		573.40±152.77 (0.0002)	116.00±76.89	227.03±200.91
		Kidney	6 hpi		2729.82±743.27	8.76±7.19	21.97±10.06
			12 hpi		8963.79±11231.59	596.48±368.12	1.38±1.41
			24 hpi		4459.86±3310.35	9.39±7.05	2335.70±3036.07
			48 hpi		10226.27±4209.44	2470.30±2460.76	234.37±211.06
	nklc	Liver	6 hpi		767.01±594.25	224.65±135.28	168.99±183.16

Table A3 (continued)

			12 hpi		136.54±67.23	102.50±135.46	1.94±2.18
			24 hp		301.93±117.23	135.46±112.59	5.57±3.58
			48 hpi		6991.83±3146.41 (0.0002)	251.17±145.51	9.95±4.84
		Kidney	6 hpi		82.32±39.54	138.97±7.19	283.95±161.04
			12 hpi		138.68±63.30	46.25±120.68	0.02±0.02
			24 hpi		187.93±173.23	120.68±29.08	40.85±6.03
			48 hpi		775.17±161.97 (0.0117)	109.90±73.97	104.72±29.51
	nkld	Liver	6 hpi		16.72±4.20	36.45±16.05	32.28±10.54
			12 hpi		36.26±22.92	64.50±19.59	1.22±0.88
			24 hpi		113.51±76.29	12.72±9.43	6.37±4.04
			48 hpi		1347.18±219.53 (0.0001)	118.62±100.25	612.19±176.53
		Kidney	6 hpi		7.05±2.74	75.87±50.28	155.22±80.33
			12 hpi		107.27±30.07	48.84±19.30	0.85±0.31
			24 hpi		39.36±9.81	106.92±32.30 (0.0347)	6.90±1.90
			48 hpi		461.40±273.30 (0.0028)	117.40±80.89	52.76±20.32

Treatments included intra-coelomically injected WT *E. ictaluri*, RE-33®, β glucan or R848 and co-administration of β glucan or R848 with RE-33®. Control treatment was PBS. Level of expression is relative to time zero samples in *rag1*$^{-/-}$ mutant Zebrafish. The numbers highlighted grey denote statistical significance.