ABSTRACT

SHAH, RADHIKA. Evaluation of Zebrafish Novel Immune-Type Receptor 9 (Nitr9). (Under the direction of Dr. Jeffrey A. Yoder.)

Natural killer (NK) cells are lymphocytes of the innate immune system that express several cell surface receptors including both activating and inhibitory forms. The natural killer cell receptors (NKRs) distinguish neoplastic or virally infected cells from normal host cells and regulate cytotoxic function. NKRs of different mammalian species differ dramatically and are the products of different multigene families. Due to the recent and rapid evolution of these receptors it has been difficult to identify orthologs of NK cell receptors (NKRs) in non-mammalian vertebrate species. In this regard, a multigene family of novel immune type receptors (NITRs) has been identified in bony fish species that are structurally similar to mammalian immunoglobulin (Ig)-type NKRs. In zebrafish, 14 NITR gene families have been identified, of which Nitr9 is the only activating receptor. In an effort to better understand the role of Nitr9 in zebrafish immunity, it was necessary to develop tools and methods to enable identification, purification, characterization and function of Nitr9 expressing cells.

In Chapter 2, the generation of two anti-Nitr9 monoclonal antibodies (mAbs) that were utilized to determine the Nitr9 protein expression profile *in-vivo* is described. Immunofluorescence and flow cytometric analysis of cells transiently

transfected with *nitr9* demonstrated that the two mAbs can detect Nitr9 expressing cells. However; due to low levels of expression it was difficult to identify Nitr9 expressing cells *in-vivo* and to subsequently purify the cells. Thus, the goal of the studies presented in Chapter 3 was to determine a method to boost Nitr9 expression *in-vivo* and facilitate the identification and purification of Nitr9 expressing cells with the two mAbs. Preliminary results suggest that *nitr9* gene expression is higher in the intestine of *rag1* deficient zebrafish, which lack a functional adaptive immune response, compared to *rag1* expressing zebrafish. This may provide a potential model to identify Nitr9 expressing cells.

Evaluation of Zebrafish Novel Immune-Type Receptor 9 (Nitr9)

by
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DEDICATION

"Jai Sacchai Ma"

To my late grandfather (nana), who not only passed on his love for knowledge and respect for education to me but also always inspired me to do my best. Through his own example, my grandfather instilled in me a sense of discipline and dedication towards my work.

To my mom (Rita Shah) and dad (Niyogi Shah)
for their support and encouragement
and my sister (Malavika Shah) for always being there.

BIOGRAPHY

Radhika Shah was born on January 22, 1980 in Mumbai, India. She lived in the vibrant city of Mumbai with her family. She completed her bachelor's with a double major in microbiology and biochemistry, and a master's degree in microbiology from St. Xavier's College, Mumbai. She joined the immunology graduate program at NC State University in August 2004. She began her PhD under the direction of Dr. Jeffrey A. Yoder in 2005. As part of her PhD research, her goal was to understand the role of Nitr9 in the innate immune system of zebrafish.

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CHAPTER 1: OVERVIEW OF MAMMALIAN NK CELL BIOLOGY AND ZEBRAFISH NITRS

1.1. INTRODUCTION

“Innate immunity has sometimes been viewed as ancient in origin, and therefore highly optimized by natural selection. But does this mean it is now set in evolutionary stone? Although the mechanisms of innate immunity predate those of adaptive immunity, it is not necessarily true that these mechanisms have stopped evolving. NK cells in humans and other species have many types of receptor, some conserved and others highly variable. These are not properties of a highly optimized system, but rather of one that has been successively pushed in many different directions by natural selection.” – Peter Parham, Nature, 2003 (1).

Parham appropriately states how the innate immune system is continuously evolving by the process of natural selection in which proteins, such as the natural killer cell receptors (NKRs), of the innate immune system are either conserved through evolution with respect to their function and signaling pathways or highly polymorphic to generate a wide range of binding specificities to their ligands thus increasing the likelihood that an infection will be terminated before the onset of disease. Given the degree of plasticity within receptors of the innate immune system only broad scale comparisons can be used to generate hypotheses regarding the evolution of this system. In addition, this variation cannot be predicted or understood by examining receptor structure and function in mammals alone (1, 2).

In the case of NKRs, major differences between species are apparent, however; these receptors are poorly understood below the phylogenetic levels of mammals, and even within mammals (3). To understand the evolution of the NKRs and their divergence between species it is necessary to study their structure and function in non-mammalian species. In this respect, zebrafish, a non-mammalian vertebrate, has gained prominence as a model system for studying gene structure-function relationships. Zebrafish share many orthologous genes with mouse and man and recent studies have identified several regions of the zebrafish and human genome that share conserved synteny. The resolution of the novel immune type receptor (NITR) genes in zebrafish, which are predicted functional orthologs of mammalian NKRs, likely provides an insight into the evolution of NKRs (4).

The focus of this chapter is to highlight important features about mammalian natural killer (NK) cell biology and provide current information about NITRs in zebrafish in an effort to understand how the physical environment in which these bony fish develop and reproduce has influenced evolution with respect to the NKRs of the innate immune system.

1.2. MAMMALIAN NK CELL BIOLOGY

- The Null Hypothesis:

A little more than 30 years ago, in 1975, Kiessling et al., and Herberman et al., were the first to define NK cells primarily on their ability to kill tumor cells rapidly, without any prior immunization (5-7). They noticed these cells had the morphology of small lymphocytes but could not be classified as T or B lymphocytes as the cells did not express any known surface markers. These cells were commonly referred to as "null" cells on the assumption that they lacked any cell surface markers. The authors found that the removal of T and B lymphocytes or of macrophages and monocytes in mice led to increased cytotoxicity by these killer cells. The killer cells were most abundant in the spleens as compared to the lymph nodes and bone marrow of mice and absent in the thymus. Spleen killer cells from genetically athymic mice were as active as those from normal mice of the same strain with peak killing activity around 1-3 months of age. Based on these results the authors concluded that these "null" killer cells represented a third group of lymphocytes, the natural killer (NK) cells (8, 9).

- ‘Markedly’ Different:

In 1983, Lanier et al. identified a subset of human peripheral blood lymphocytes that possessed natural cytotoxicity against the prototypic K562 tumor target cells. This subset of cells did not express surface immunoglobulin (Ig) or CD3 as reported for NK cells, but did express an Fc receptor, CD16, on its cell surface when analyzed by flow cytometry (10). For the first time it was evident that NK cells did express a cell surface marker but it was not the markers generally found on T and B cells (7, 10). It is now known that NK cells are a heterogenous population of lymphocytes that can be phenotypically characterized and isolated based on the expression of several cell surface markers. These include: activation markers (eg. CD69, CD25); adhesion molecules (eg. CD45RA, CD45RO, CD27); memory markers (eg. CD62L); inhibitory and activating receptors; and intracellular proteins such as granzymes and perforin that contribute to the lytic function of the cells (11). Different NK cell subsets exhibit differential expression of these molecules due to their distinct trafficking patterns and functions. In humans, two subsets of NK cells have been described based on their function, $CD56^{bright}/CD16^{-}$ expressing cells which are cytokine producers and $CD56^{dim}/CD16^{+}$ expressing cells which are the cytotoxic NK cells (12). In mice, the NK cells do not express CD56; instead the cytokine producers express $CD27^{hi}$ whereas the cytotoxic NK cells express $CD27^{lo}$ on their cell surface (13, 14). Thus it can be said that natural killer cells are a distinct subset of lymphocytes consisting of

cells that express several different cell surface markers and are not governed by a single receptor, as in the case of T and B lymphocytes.

- MHC I – A Negative Control?

It was believed that unlike T cell recognition, NK cell recognition was not restricted by MHC. In 1986 Karre observed that mouse NK cells preferentially kill certain tumors lacking MHC I. The fact that NK cells had the ability to efficiently kill cells lacking MHC confirmed the hypothesis that MHC expression was not required for NK cell function. However; Karre still believed that MHC I played a significant role in NK cell function and proposed the theory that NK cells were inhibited, not activated, by interacting with MHC I (7, 15). Based on Karre's theory Lanier et. al conducted studies on HLA recognition by human NK cells and discovered the inhibitory receptor, KIR3DL1, which recognizes HLA-Bw4, thereby providing evidence that NK cells are indeed inhibited by MHC I molecules (7, 16). There was rapid progress in understanding the inhibitory receptors with the discovery of the multi-gene families of KIRs in humans, and Ly49 receptors in mice (discussed below) (17). This was followed by the revelation that some receptors in the KIR and Ly49 family possessed homologous extracellular domains but different transmembrane and cytoplasmic domains as compared to the inhibitory receptors. Functionally, these receptors were reported to activate the NK cell to lyse target cells (18-20). The presence of both,

inhibitory and activating receptors led to the concept that NK cells were possibly regulated by the integration of positive and negative signals and regulation was not solely dependent on inhibition by MHC I (21).

- Negative Signaling:

Signaling through inhibitory receptors was well defined and established in the early 1990s. The inhibitory receptors possess one or more cytoplasmic immunoreceptor tyrosine- based inhibition motifs (ITIMs) that consist of the prototypic sequence Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val, where x denotes any amino acid. These ITIMs are well conserved and found in all inhibitory NK receptors (22, 23). Upon ligand binding by the extracellular domains of the inhibitory receptors the ITIMs in the cytoplasmic tails are phosphorylated, likely by Src family kinase, and phosphatases are recruited through their SH-2 domains. These phosphatases dampen or prevent NK cell effector function of cytokine production and cytotoxicity towards normal host cells (23-26).

- Positive Association:

The activating receptors possess a positively charged amino acid in their transmembrane domain. Results of studies published by Vivier et al. supported the concept that a small protein coimmunoprecipitated with certain KIRs (7, 20). Soon

after, in 1997, Lanier et al. discovered a signaling adaptor protein, DAP12, containing a negatively charged amino acid in its transmembrane region. In addition, the DAP12 sequence was also shown to possess an immunoreceptor tyrosine-based activation motif (ITAM) with the consensus sequence, Asp/Glu-x-x-Tyr-x-x-Leu/Ile-x_{6-8}-Tyr-x-x-Leu/Ile where x denotes any amino acid. It was then found that DAP12 forms an activating receptor complex with the activating KIR, CD94/NKG2C, and Ly49D receptors (27-29). Another adaptor protein, DAP10 was also identified and shown to partner with the CD94/NKG2 activating cell receptor complex (30). It was established that the activating receptors associate with adaptor proteins through their charged residues upon target cell recognition and ligand binding. The tyrosine in the ITAM of the adaptor protein is phosphorylated and tyrosine kinases Syk and ZAP70 are recruited via their SH2 domains to stimulate downstream events, causing a Ca^{2+} influx, degranulation, and transcription of cytokine and chemokine genes (23, 31, 32). Overall, it can be concluded that a single signaling pathway does not dominate the differentiation and effector functions mediated by NK cells but a balance of signals from both inhibitory and activating receptors are required (7).

- <u>"Balancing" Act</u>

Normal host cells ubiquitously express MHC-I on their cell surface. The inhibitory receptors typically recognize these MHC-I molecules, as a 'self' ligand, and inhibit

destruction of normal host cells. Transformed, infected or stressed cells can lose expression of self MHC class I molecules. The loss or reduction of MHC-I expression initiates NK cell activation and thereby target cell lysis (33, 34). This phenomenon is known as missing-self recognition. Virally infected cells down regulate MHC I as a means to evade cytolysis by CD8 T cells, and it is likely that the missing self recognition is a counter strategy by the innate immune system to lyse the infected cells (34). In some cases, even untransformed or uninfected cells that do not express a full complement of MHC I molecules are susceptible to NK cell lysis. This was first shown in studies of bone-marrow transplantation in which donor bone-marrow cells lacking an MHC class I allele of the recipient were often rejected by NK cells (34, 35). Missing self-recognition does not depend solely on MHC I down regulation but requires the expression of stimulatory ligands to activate the lytic pathway (23, 34, 36, 37). It is also possible that NK cells are activated due to induced self recognition where the stimulatory ligands expressed on target cells may overcome the constitutive inhibition delivered by inhibitory receptors and activate the cytolytic pathway. Missing-self and induced-self recognition may operate simultaneously to provide NK cells with the maximal ability to discriminate between normal cells and transformed or infected target cells (23, 34).

- Mammalian natural killer cell receptor gene families:

Consistent with their functions as innate sentinels, NK cells lack the ability to generate antigen-specific receptors by somatic recombination, even though they possess rearranging genes (38-40). Instead, they express a repertoire of cell surface receptors that control their activation, proliferation and effector functions, as described above (23). 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), whereas gene families that encode the type I TM receptors with Ig-like domains cluster in the leucocyte receptor complex (LRC) (41-43). 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 comparison to only the single Ly49 pseudogene identified in humans. Conversely, in humans the KIRs of the LRC functionally replace the rodent Ly49 genes and encode an entire family of Ig-like NK cell receptors whereas, only a single KIR is encoded by the rat LRC and two KIR genes are found in mice that are not linked to the LRC (39, 44). Despite the differences in these gene sets, the structure of the receptor proteins encoded by the gene sets and their species specific

expansions in rodents and humans, the function, expression and diversity of human KIRs and mouse Ly49 family members are extra-ordinarily similar (45).

Both, the KIR and Ly49 genes: a) belong to small gene families of 10-15 closely linked genes; b) demonstrate genetic polymorphism; c) encode inhibitory receptors with ITIMs in their cytoplasmic domains that inhibit NK cell function via phosphorylation of the ITIM, which in turn recruit and activate phosphatases, SHP-1, SHP-2 and SHIP; d) encode activating receptors that possess a positively charged amino acid in their TM region, which associates with immuno-receptor tyrosine based activation motif (ITAM) containing adaptor proteins that in turn recruit and activate protein tyrosine kinases such as Syk/ZAP70 or PI3K that leads to target cell lysis; e) are expressed primarily on NK cells; and f) interact with MHC-I molecules (23, 38, 43, 46-48).

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 (48-50). 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 below, may be involved in mediating NK cell receptor function.

- Why Are NK Cells Important?

NK cells are cytotoxic lymphocytes of the innate immune system and are widespread throughout the lymphoid and nonlymphoid tissues (40). In most tissues, NK cells represent a minor fraction of total lymphocytes (from 2% in mouse spleen to 10% in mouse lung and from 2-18% in human peripheral blood). Human NK cell turnover in blood is around 2 weeks, consistent with the data in the mouse (51-54). They act as a first line of defense against aberrant cells in the body that have been infected, transformed or stressed (55). NK cells play a vital role in resistance to viral infections. They eliminate target cells by release of cytokines and the engagement of activating cell surface receptors to ligands on the target cells (39, 40, 56, 57). Infection by many viruses, such as herpes simplex virus-I, influenza virus and ectromelia poxvirus can be controlled by NK cells in mice. An increased susceptibility or resistance to the herpes virus MCMV after NK cell depletion or NK cell adoptive transfer respectively, has also been reported. NK cells also mediate antiviral effects to several human viruses including HCMV, varicella zoster virus, human papilloma virus, and Epstein Barr virus (39, 40).

In vitro studies using tumor cells from humans and several other mammalian species implicate a role of NK cell receptors in tumor surveillance and elimination. *In vivo* rejection of several transplanted tumors in mice has been shown to be dependent on the presence or absence of NK cell receptor ligands expressed by the tumor cells. The lack of MHC-I (e.g. the classical RMA/S, MHC-I deficient mouse lymphoma cells), the upregulation of NKG2D ligands or CD70, CD27 ligands has been shown to render tumor cells susceptible to NK cell-mediated lysis (58). NK cells have been shown to control the growth of B cell lymphomas that arise spontaneously in mice which lack perforin and β_2-microglobulin. In malignancies such as acute myeloid leukemia, allogeneic stem cell transplantation in tumor patients lacking donor KIR ligands can lead to improved engraftment and post-transplant survival in the absence of graft-versus-host disease. In addition, NK cells have been reported, to play an important role in tumor surveillance in models of spontaneous and induced tumors (40).

Uterine NK cells that represent a distinct subset of NK cells have been shown to participate in remodeling of the feeding arterial systems that support maternal endometrial tissue at sites of implantation and support placental development (40, 59).

Recently, in a model of contact hypersensitivity NK cells were reported to mediate hapten-specific recall responses, independent of T and B cells (60). NK cells have

also been shown to home to inflamed skin in vernal keratoconjunctivitis, atopic dermatitis, and psoriasis (61-63). NK cells have been implicated in organ transplantation, autoimmunity, asthma and in control of parasitic infections such as toxoplasmosis, trypanosomiasis, leishmaniasis, and malaria (40, 64).

The lack of NK cell selective deficiencies in humans has hindered the characterization of NK cell function *in vivo* in general and in antitumor surveillance in particular (40, 65). Thus, thirty years after the discovery of NK cells, several aspects of NK cell function *in vivo* remain to be elucidated.

1.3 ZEBRAFISH AND NOVEL IMMUNE TYPE RECEPTORS:

- Zebrafish as a model for immunology:

The small size, easy maintenance, relatively rapid life cycle with simple breeding protocols, genetic amenability and low costs makes the zebrafish a suitable laboratory animal. Other unique advantages of this model include their ex-utero development and fertilization, high fecundity and translucent embryos (66). These features complemented by the fact that zebrafish have a fully developed adaptive and innate immune system that closely parallels that of humans and other mammals renders it an attractive new vertebrate model for immunological research. Evolutionarily, the zebrafish are one of the earliest species in the vertebrate lineage with a functional adaptive immune response comprised of T lymphocytes with a highly diversified TCR, B lymphocytes that encode Ig gene families and MHC-I, II and III genes (67). Functionally, antigen presenting cells present epitopes to zebrafish T cells as seen in mammals, and B cells share IgM and IgD antibodies with mammals, also B cells secrete antibodies upon immunization. In addition, zebrafish possess chemokines, cytokines and a well developed complement system. Unlike higher vertebrates, the adaptive immune systems of these fish lack lymph nodes, germinal centers, antibody class switching and affinity maturation (67-69). These inefficiencies of the zebrafish adaptive immune system are compensated by a robust innate immune response with monocytes, macrophages, granulocytes, mast

cells and toll like receptor homologs (67-71). However, NK cells and their role in cytotoxic function have not been defined in zebrafish (70). In this regard, providing evidence that NK cell receptor function has been conserved by means of the NITRs in zebrafish will warrant the zebrafish as an alternative model to study NK receptor function.

Zebrafish are also becoming a powerful model to study host-pathogen interactions and cancer biology (67, 72). Many models that use natural fish pathogens closely related to those that infect mammals have been developed. Viral pathogens for zebrafish include infectious hematopoietic necrosis virus (HNV), infectious pancreatic necrosis virus (IPNV), spring viremia of carp virus (SVCV), snakehead rhabdo virus (SHRV), viral hemorrhagic septicemia virus (VHSV) and nervous necrosis virus (NNV) (40, 67, 73). In addition, tumors can be induced in zebrafish, in most of the tissues, using mutagens such as N-ethyl-N-nitrosourea (3). Recently, transplantable tumor lines have been generated in zebrafish (74). These infection and cancer models can eventually be applied to study NK cell and NITR function *in vivo* in zebrafish.

- Novel Immune Type Receptors:

The first NITR cDNA was cloned more than 12 years ago by Rast, Haire and Litman from the Southern pufferfish (75, 76). NITRs have now been identified in at least 13 species of bony fish, such as zebrafish, rainbow trout, channel catfish, Japanese flounder, medaka, Fugu and *Tetraodon nigroviridis* and likely exist in all bony fish (4, 75, 77-83). Structurally, the NITRs belong to the immunoglobulin superfamily (IgSF) and a typical NITR gene encodes a type I TM glycoprotein with an extracellular variable (V) and intermediate (I) domain, a TM region and a cytoplasmic tail. Both ectodomains may contain joining (J) like sequences, even though there is no evidence of somatic recombination (4, 75). The NITR V domains possess highly conserved residues that are also conserved in T cell receptor (TCR) and Immunoglobulin (Ig) V domains. The V domains include three hypervariable regions which correspond to the complementarity determining regions in TCRs and Igs (4, 84, 85). Recent crystal structures of V domains demonstrate that they are structurally similar to the V domains of antigen receptors (75, 86). The NITR I domain contains six conserved cysteines and many of the same conserved residues observed in the V domains of NITRs, TCRs and Igs (87). It is likely that the NITRs represent either modern forms of progenitor genes or derived products of TCR and Ig loci, which would function in an innate capacity.

Similar to the mammalian NKRs, NITRs can be classified into two primary functional groups: 1) inhibitory, based on the presence of a cytoplasmic ITIM or itim (ITIM like) sequence and 2) activating, based on the presence of a positively charged residue within the transmembrane domain (4, 75). As with mammalian NKRs the numbers of inhibitory forms of NITRs predominate over the activating forms. For example, 1 of 39 NITRs in zebrafish and 3 of 44 NITRs in medaka are predicted to have activating potential (4, 81). Although it is unclear how many NITRs are present on a single cell, the higher number of inhibitory receptors may contribute to a functional redundancy ensuring that activating pathways are normally suppressed (75). Although the ligands for NITRs have not been identified yet, it is likely that NITRs will recognize a wide range of ligands given the high level of diversity within each NITR family. It has been postulated that similar to mammalian NKRs, inhibitory NITRs may recognize MHC-I molecules and activating NITRs may recognize viral, stress related proteins and/or MHC molecules. It has recently been shown that the activating catfish IpNITR11, expressed by a clonal cytotoxic NK-like cell line binds to a ligand on the surface of a non-self allogeneic target B cell line (86).

The NITR genes have been best characterized in zebrafish. Twelve different NITR gene families (*nitr1* – *nitr12*) that encode approximately 36 different zebrafish NITR genes are located within the NITR gene cluster on chromosome 7, which may be derived from the ancient chromosome that gave rise to the human chromosome 19q, that encodes the LRC region (4). Two additional families, *nitr13* and *nitr14*, located

on chromosome 14 also have been described (82). Like the polygenic and polymorphic nature of the KIR loci, the NITR genes show high interindividual heterozygosity and are expressed only in the lymphoid cell population in zebrafish, excluding *nitr14*, which is expressed in lymphoid and myeloid cells (J.A. Yoder personal communications).

The predicted protein structures of the different zebrafish NITR families are shown in Figure 1. Of the several inhibitory NITRs, the *nitr3* gene family is interesting since it is expressed during embryogenesis and in mature lymphocytes (unpublished data by Yoder, J.A., et al.). 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. This inhibition is regulated at the level of the ITIM, indicating that inhibitory NITRs may utilize similar intracellular signaling pathways as mammalian inhibitory NKRs to inhibit target cell lysis in zebrafish (80).

Only a single zebrafish gene, *nitr9*, encodes an activating receptor that contains the positively charged amino acid, arginine, in its TM 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 TM 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 (3, 5). Zebrafish Nitr9 shares a similar downstream signaling pathway as activating human KIRs (3). RT-PCR analysis indicated that both zebrafish Nitr9 and the adaptor protein, Dap12 are expressed in lymphocytes. Upon antibody cross-linking Nitr9 associates with Dap12 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. (4, 77, 80, 84, 88, 89).

Collectively, these observations and results of the NITR gene families have led to a model suggesting that NITRs are functionally linked to human KIRs (shown in Table 1). NITRs and KIRs are encoded by rapidly evolving multi-gene families, that include both inhibitory and activating forms. 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 (75). Nevertheless, NITRs and KIRs possess similar signaling domains so that the receptors can couple with appropriate signaling adaptors, which are ancient and much more conserved (45).

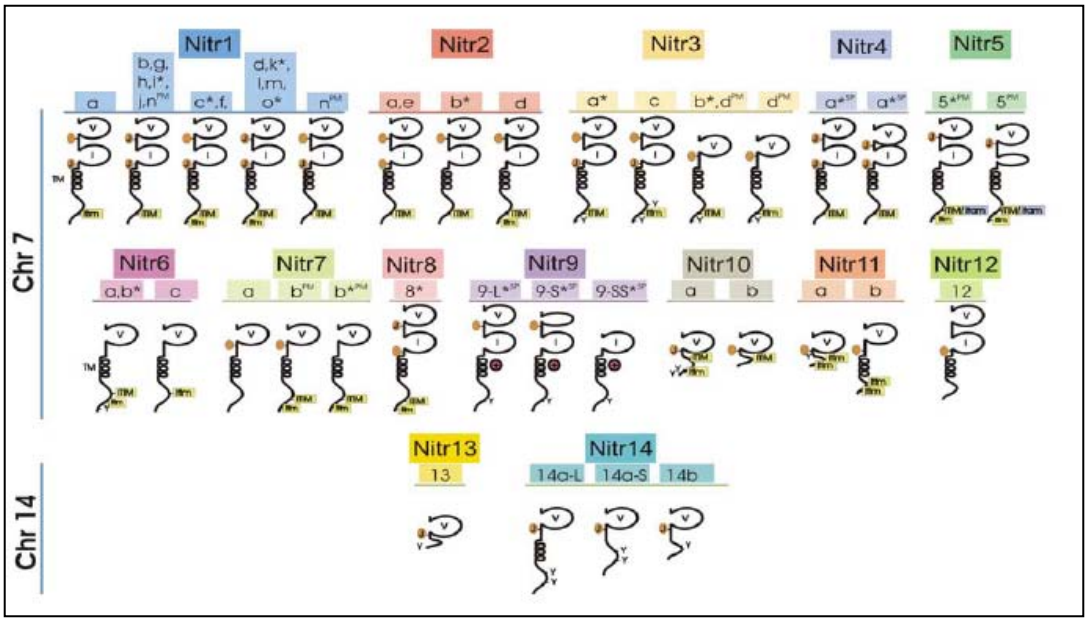

Figure 1: Schematic Representation of Diverse NITR Proteins in Zebrafish (5, 6). Most NITRs are located on chromosome 7 and two are found on chromosome 14. Protein domains include: a variable Ig domain (V); an intermediate Ig domain (I); a TM region (coiled structure) and a cytoplasmic tail. A prototypic joining-like region (J); a J-like variant (orange oval); polymorphic (PM), RNA splice variants (SP) and full length transcripts (*) have been identified. Inhibitory forms, with a conventional immunoreceptor tyrosine-based inhibition motif (ITIM) or a variant of an ITIM (itim) are predicted. The single activating gene, *nitr9* that consists of a positive charged residue midmembrane (⊕) and includes three predicted protein structures encoded by three mRNA splice variants of *nitr9* are indicated as Nitr9-long (Nitr9-L), Nitr9-short (Nitr9-S) and Nitr9-supershort (Nitr9-SS). Figure courtesy of J. A. Yoder.

Table 1: Characteristics of multigene families present in humans, mice and zebrafish.

As shown below, NITRs are similar to mammalian NK cell receptors in structure and share similar cytoplasmic signaling pathways. Based on this information it is proposed that the NITRs are similar to mammalian NK cell receptors and regulate cytotoxic function.

	Human KIRs	**Mouse Ly49 receptors**	**Zebrafish NITRs**	**References**
Gene family:	LRC	NKC	NITR	(4), (41-43)
Structure: Extracellular domain	Ig-type	C-type lectin	Ig-type	(4), (50)
Transmembrane region	Activating receptors possess a positively charged amino acid	Activating receptors possess a positively charged amino acid	Activating receptors possess a positively charged amino acid	(84), (23)
Cytoplasmic tail	Inhibitory receptors possess 1 or 2 ITIMs	Inhibitory receptors possess 1 or 2 ITIMs	Inhibitory receptors possess 1 or 2 ITIMs or ITIM-like sequences	(4), (23)
Signal Transduction: Activating receptors	Associate with ITAM containing adaptor proteins e.g. DAP12 to initiate downstream signaling	Associate with ITAM containing adaptor proteins e.g. DAP12 to initiate downstream signaling	Associates with ITAM containing adaptor protein, zebrafish Dap12 to initiate downstream signaling	(84), (23)

Table 1 continued.

Inhibitory receptor	Phosphorylation of tyrosine in ITIM leads to activation of phosphatases that dephosphorylate effector molecules (e.g. MAPK) in the activation pathway	Phosphorylation of tyrosine in ITIM leads to activation of phosphatases that dephosphorylate effector molecules (e.g. MAPK) in the activation pathway	ITIM dependent inhibition of MAPK activation	(48), (80)
Ligands: Activating Receptors	Viral epitopes, stress induced ligands, MHC I	Viral epitopes, stress induced ligands, MHC I	?	(55)
Inhibitory receptor	MHC-I molecules	MHC-I molecules	?	(50)
Function: Activating receptor	Cytotoxicity and cytokine release	Cytotoxicity and cytokine release	?	(33)
Inhibitory receptor	Inhibit lysis of normal host cells	Inhibit lysis of normal host cells	?	(33)

1.4 CONCLUSION:

Despite considerable progress in identifying genes of the immune system, developing the zebrafish as a new immunological model system is an extensive undertaking. Perhaps the most commonly used reagents for identification, purification and characterization of different blood cell lineages in humans and mice has been the production of monoclonal antibodies. However; generation of monoclonal antibodies against the blood cells of zebrafish has been difficult, possibly due to the different glycosylation patterns that are recognized as foreign by the rodent immune system (90). This has led to a paucity of antibodies to study blood cell lineages of zebrafish (91). Thus to identify NK cells and elucidate their role in fish immune responses it is first necessary to identify the cell surface markers expressed on teleost NK cells and then generate antibodies against these markers to purify and characterize the cells. Based on current knowledge about the zebrafish NITRs, the studies that follow focus on the single activating receptor Nitr9 with the goal of determining its protein expression profile, developing a means to identify and purify Nitr9 expressing cells and develop a method to increase Nitr9 expression *in vivo*.

- Hypothesis:

1. Nitr9 is an activating transmembrane receptor:

   RT-PCR analysis indicates that Nitr9 is expressed in adult tissues including the ovary, liver, kidney, intestine and spleen (unpublished data – Jeffrey A. Yoder). The Nitr9 gene family comprises of three splice variants that encode three different receptors: Nitr9-L, Nitr9-S and Nitr9-SS. All three receptors encode an identical transmembrane domain that includes a positively charged amino acid, as observed in activating mammalian NKRs. Thus based on its predicted protein structure, it can be hypothesized that Nitr9 is an activating transmembrane receptor.

2. Nitr9 is expressed on NK cells or cytotoxic T cells:

   RT-PCR analysis indicates that Nitr9 is expressed exclusively in the lymphoid population of adult zebrafish kidney cells and is absent in the myeloid population (unpublished data – Jeffrey A. Yoder). In addition *in-vitro* studies indicate that Nitr9 associates with zebrafish Dap12 adaptor protein and activates the ERK signaling pathway as seen with mammalian activating NKRs (84). These observations lead to the hypothesis that Nitr9 is expressed on NK cells or cytotoxic T cells.

3. Nitr9 provides cytotoxic function:

   From the above hypotheses, Nitr9 is an activating receptor present on zebrafish cytotoxic cells including NK cells or cytotoxic T cells that activates 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.

- <u>Objectives:</u>

1. Develop a tool to identify Nitr9 protein in zebrafish:
   a) Anti-Nitr9 mAb development
2. Characterize Nitr9 expression by:
   a) Western Blot Analyses – to determine the protein expression profile in adult tissues
   b) Immuno-fluorescence – to determine the type of cells that express Nitr9
   c) Fluorescent antibody cell sorting (FACS) – to isolate and purify the Nitr9 expressing cells
3. Determine a change in transcriptional response of Nitr9
   a) In Rag1-/- zebrafish, by real-time PCR
   b) After viral infection, by real-time P

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CHAPTER 2: IDENTIFICATION OF ZEBRAFISH NITR9 WITH ANTI-NITR9 MONOCLONAL ANTIBODIES

Submitted to *Zebrafish*

Contribution by our Collaborators:

- Robert Haire bacterially expressed and purified the Nitr9 Intermediate domain that was used as an antigen to immunize mice for monoclonal antibody production and Donna Eason screened the hybridoma supernatants by immunofluorescence in Dr. Gary Litman's laboratory at University of South Florida.

2.1 ABSTRACT

Novel immune-type receptors (NITRs) are encoded by multigene families of inhibitory and activating receptors that are predicted to function in innate immunity. NITRs may be restricted to bony fish species but share structural and functional characteristics with mammalian natural killer cell receptors (NKRs). Within the zebrafish NITR family, *nitr9* is the only gene predicted to encode an activating receptor. Alternative RNA splicing generates three distinct *nitr9* transcripts, each of which encodes a different Nitr9 isoform. One isoform of Nitr9 has been shown to partner with and signal via Dap12 paralleling the signaling cascade initiated by mammalian activating NKRs. In an effort to better understand the role of Nitr9 in zebrafish immunity, anti-Nitr9 monoclonal antibodies were developed and used to characterize Nitr9 protein expression in transfected cells and in zebrafish tissues.

2.2 INTRODUCTION

Mammalian natural killer (NK) cells are large, granular lymphocytes of the innate immune system and express several cell surface receptors that regulate cytotoxic function through a complex network of signaling pathways. NK cell receptors include both activating and inhibitory forms that are proficient in distinguishing neoplastic or virally infected cells from normal host cells.[1,2] The regulation of natural killer cell cytotoxicity is dependent on the integration of signals from these activating and inhibitory receptors.[3] Although, it is postulated that NK cell receptors arose early in the vertebrate phylogeny, functional data primarily are based on studies of mammalian NK cell receptors. In order to better appreciate the origins and evolution of NK cell receptors and their function it is critical to define equivalent receptor forms in non-mammalian species. The bony fish represent one of the earliest vertebrate lineages with a functional innate and adaptive immune response that closely parallels that of humans and other mammals.[4] A large multigene family of recently and rapidly evolving inhibitory and activating novel immune-type receptors (NITRs) has been identified in multiple fish species including zebrafish, pufferfish, medaka, catfish and trout[5]. Complete analyses of the NITR gene clusters sequences have been performed only from the zebrafish and medaka genomes.[6,7,8,9] Within the zebrafish genome, *nitr9* is the only NITR gene predicted to encode an activating receptor.[8] Three alternatively spliced transcripts of *nitr9* have been characterized

that encode three distinctly different receptors: Nitr9-long (Nitr9L), Nitr9-short (Nitr9S) and Nitr9-supershort (Nitr9SS), which differ in their extracellular domains. Nitr9L utilizes a positively charged residue within its transmembrane domain to partner with the adaptor protein Dap12. Cross-linking the Nitr9L-Dap12 complex can stimulate the same intracellular signaling pathways as mammalian activating NK cell receptors, consistent with a possible role in cytotoxic function.[10] Herein we describe the derivation of two anti-Nitr9 monoclonal antibodies (mAbs) and use them to characterize the three isoforms of Nitr9 as well as to detect their expression on transfected cells.

2.3. MATERIALS AND METHODS

- Nitr9 expression constructs

Epitope (FLAG)-tagged Nitr9 expression cassettes were constructed with the *pLF* plasmid which incorporates an amino-terminal leader sequence and FLAG tag.[10] Endogenous Nitr9 expression cassettes were constructed with *pcDNA3* (Invitrogen; Carlsbad, CA). The coding sequences of zebrafish *nitr9L, nitr9S and nitr9SS* were amplified by PCR and cloned into *pLF or pcDNA3.* FLAG-Nitr9 and endogenous Nitr9 cassettes were then shuttled into *pIRES2-EGFP* (Clontech; Mountain View, CA) generating: *pFLAG-Nitr9L/EGFP*, *pFLAG-Nitr9S/EGFP, pFLAG-Nitr9SS/EGFP,*

pNitr9L/EGFP, *pNitr9S/EGFP* and *pNitr9SS/EGFP* plasmids (Figure 1). Primer sequences used in all cloning steps are included in Table 1.

- Antibody development and purification

All three isoforms of Nitr9 share a common extracellular immunoglobulin domain of the intermediate or "I" type (Figure 1). The coding sequence of the Nitr9 I domain (residues 131-239 of ZFIN:ZDB-GENE-041001-6) was amplified by PCR and cloned into pETBlue-1 (Novagen; Gibbstown, NJ) and used to transform *E. coli* Tuner™ cells (Novagen). Cells were induced and the Nitr9 I domain was recovered in inclusion bodies.

Swiss Webster mice were immunized with the bacterially expressed Nitr9 I domain, splenocytes were fused with P3X63Ag8.653 cells (CRL-1580: ATCC; Manassas, VA) and approximately 3,000 individual hybridoma supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) against the denatured recombinant Nitr9 I domain (Immunology Core Facility, University of North Carolina, Chapel Hill). The most strongly reactive ~100 supernatants were screened subsequently by parallel Western blot analyses and indirect immunofluorescence. Two single clones of interest were identified, 90.10.5 and 19.1.1 and their antibodies were isotyped (IsoStrips: Roche; Indianapolis, IN) as IgG2b, κ light chain and IgG2a, κ light chain,

respectively, and purified via protein A agarose columns (Upstate Cell Signaling Solutions; Lake Placid, NY).

- Cell culture and zebrafish

Human 293T cells were transfected using Fugene 6 (Roche) according to the manufacturer's instructions and harvested 48 hr after transfection. Adult zebrafish (Ekkwill; Ruskin, FL) were maintained and euthanized as described.[11]

- Western Analyses

Transfected 293T cells were washed with 1x phosphate buffered saline (PBS) and lysed with Mammalian Protein Extraction Reagent (M-PER®: Pierce; Rockford, IL). Tissues were removed from euthanized adult zebrafish and collected directly into Tissue Protein Extraction Reagent (T-PER®: Pierce) supplemented with protease inhibitors (Pierce) and homogenized. Lysates were centrifuged to remove nuclei and cell debris and protein concentrations were determined (BCA Protein Assay: Pierce). Proteins were resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes for Western analyses. Membranes were washed in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated in blocking buffer (100 mM boric acid, 25 mM Na-Borate, 75 mM NaCl, 5% goat

serum and 5% dry milk powder) for 1 hr. Membranes were incubated overnight with primary antibodies in blocking buffer at 4 °C. Primary antibodies include an anti-FLAG (M2) mouse mAb (Sigma-Aldrich; St. Louis, MO), an anti-GFP mouse mAb (Roche) and an anti-histone H2A rabbit polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA). Membranes were washed in TBST, followed by incubation with blocking buffer and either horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Roche) or horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology) and final washes with TBST. The Lumi-Light^{PLUS} Western Blotting substrate and detection system (Roche) was used to visualize reactivity.

- Endoglycosidase treatment

Cleared lysates (20 μg) from transfected cells were incubated with N-Glycosidase F (PNGase F: New England Biolabs; Ipswich, MA) for 1 hr at 37 °C. Cleared lysates (50 μg) from zebrafish tissues were precipitated with OrgoSOL™ buffer (G-Biosciences; Maryland Heights, MO), and resuspended in PNGase buffer for treatment with PNGase F.

- Immunohistochemistry

293T cells were transfected in 4 well chamber slides (Thermo Fisher Scientific; Rochester, NY). Transfected cells were washed (1x PBS), fixed with 3% paraformaldehyde for 20 min and treated with 50mM NH_4Cl, 1x PBS for 5 minutes. Cells were then permeabilized with 1.0% Triton-X-100, 1x PBS for 5 min, rinsed and blocked with 1% BSA, 1x PBS for 5 min. Permeabilized cells were incubated with the 90.10.5, 19.1.1 or anti-FLAG antibody for 1 hr, rinsed with 1x PBS, incubated with a phycoerythrin (PE) anti-mouse IgG antibody and DAPI (1:1000) for 1hr and washed with 1x PBS. Chambers were removed from the slides and coverslips were mounted using Immunomount (ThermoShandon; Pittsburgh, PA). Cells were photographed at 40x magnification using a Leica DM5000 microscope.

- Flow cytometry

Transfected 293T cells were incubated with the 90.10.5, 19.1.1 or anti-FLAG mAbs for 1 hr, washed (1x PBS) and incubated for 30 min with an allophycocyanin (APC) conjugated anti-mouse IgG secondary antibody. Labeled cells were washed and then fixed with 3% paraformaldehyde and subjected to flow cytometric analysis (BD FACSCalibur™: BD Biosciences; San Jose, CA).

2.4 RESULTS AND DISCUSSION

- Anti-Nitr9 antibody 90.10.5 binds all three isoforms of Nitr9 in Western analyses

In order to determine if mAbs 90.10.5 and 19.1.1 could detect all three isoforms of Nitr9 in Western analyses, 293T cells were transfected with plasmids encoding FLAG-tagged and endogenous isoforms of Nitr9 (Fig. 1) and cell lysates were subjected to Western blot analyses using the purified anti-Nitr9 mAbs. The 90.10.5 mAb recognized all three isoforms of the endogenous Nitr9 as well as the FLAG-tagged Nitr9L and Nitr9S proteins with a binding pattern equivalent to the anti-FLAG mAb positive control (Fig. 2A). However, the 90.10.5 mAb failed to recognize the FLAG-tagged isoform of Nitr9SS, possibly because the FLAG-tag hindered access to the antibody binding site of the Nitr9SS I domain. Two proteins bands were detected by mAb 90.10.5 for both FLAG-tagged and endogenous Nitr9L and Nitr9S transfections: these same bands were detected by the anti-FLAG mAb from the FLAG-tagged Nitr9L and Nitr9S transfections. Both observed Nitr9L proteins were larger than the predicted size of Nitr9L (34 kD) and one of the observed Nitr9S proteins was larger than the predicted size of Nitr9S (30 kD). The increase of observed M_r for Nitr9L and Nitr9S, as compared to their predicted M_r, is a result of differential gylcosylation (see below). Based on the chemiluminescence exposure times required for detecting the different isoforms of Nitr9, mAb 90.10.5 appears to

display a higher affinity for Nitr9L as compared to Nitr9S and Nitr9SS. mAb 19.1.1 did not bind endogenous Nitr9S and Nitr9SS proteins (data not shown) and was not characterized further for Western analyses.

- Nitr9 glycosylation in transfected cells

Nitr9L, Nitr9S and Nitr9SS possess three (NMSC, NDSR and NGSK), two (NMSC and NGSK), and one (NGSK) candidate N-linked gylcosylation sites, respectively. Two protein forms of Nitr9L, which are larger than its predicted M_r, are observed in transfected 293T cells. Two forms of Nitr9S also are observed in transfected cells, with one being larger than its predicted M_r. Treatment of lysates from transfected cells with endoglycosidase (PNGase F) result in the detection of a single protein of the expected size for both Nitr9L and Nitr9S (Fig. 2B). Both sets of results are consistent with *in vivo* glycosylation. The observed size of Nitr9SS in transfected cells does not appear to be altered by endoglycosidase treatment suggesting that it may not be glycosylated.

- Nitr9 proteins are differentially expressed in different tissues of zebrafish

Western blot analyses of endoglycosidase treated lysates, from adult zebrafish tissues confirmed translation of Nitr9 *in vivo*. Nitr9L was detected in the intestine,

spleen and kidney. Nitr9SS was detected only in the intestine (Fig. 2C). An artifactual band that corresponds to the predicted size of Nitr9S (30 kD) is detected with large total protein loads and long exposure times (e.g. 20-50 μg lysate and 10 min exposure) precludes any conclusions regarding Nitr9S in tissues. We previously demonstrated that transcripts of *nitr9* are detected from zebrafish lymphocytes but not from myeloid cells.[10] Interestingly, the amount of Nitr9L protein (per total protein) is highest in the intestine, which is a major secondary lymphoid organ and contains mature lymphocytes. Kidney marrow possesses fewer mature lymphocytes, which could account for the lesser amount of Nitr9L. Nitr9 isoforms also could mediate different tissue-specific functions and thereby explain the pattern of differential expression.

- Detection of Nitr9 isoforms in transfected cells by immunohistochemistry

In order to determine if mAbs 90.10.5 and 19.1.1 could detect all three isoforms of Nitr9 by immunohistochemistry (IHC), 293T cells were transfected with plasmids encoding both a FLAG-tagged or endogenous isoform of Nitr9 and EGFP: in this way, any cell expressing EGFP also expresses Nitr9 (Fig. 1). To ensure that the recombinant Nitr9 proteins could be detected by IHC, an anti-FLAG antibody was used to detect all three FLAG-tagged isoforms of Nitr9 in transfected cells (Fig. 3A). It was then shown that both 90.10.5 and 19.1.1 mAbs recognize FLAG-Nitr9L and

FLAG-Nitr9S by IHC, but both mAbs either fail to bind (90.10.5) or bind less effectively (19.1.1) to FLAG-Nitr9SS (Figs. 3B, 3C). In contrast, both 90.10.5 and 19.1.1 mAbs effectively recognize all three isoforms of endogenous Nitr9 when expressed in transfected cells albeit with an apparent higher background labeling of cells with 19.1.1 (Fig. 4). It is possible that the FLAG-tag disrupts folding of Nitr9SS or sterically interferes with antibody recognition of the I domain of FLAG-Nitr9SS; this also was observed with Western analyses (Fig. 2A).

Overall, 90.10.5 bound efficiently and specifically to Nitr9L and Nitr9S expressing cells and should be useful for detecting Nitr9 expressing cells *in vivo*. Attempts to employ 90.10.5 to label Nitr9 expressing cells from zebrafish tissues have not been successful (data not shown) likely due to the low levels of Nitr9 expression (see Fig. 2C).

- Detection of Nitr9 isoforms in transfected cells by flow cytometry

In order to determine if the 90.10.5 and 19.1.1 mAbs could detect all three isoforms of Nitr9 by flow cytometry, 293T cells were transfected with plasmids encoding a FLAG-tagged or endogenous isoform of Nitr9 and EGFP (Fig. 1). Flow cytometric analysis was performed using the anti-FLAG, 90.10.5 and 19.1.1 mAbs to label the Nitr9 expressing cells (Fig. 5). The percentage of double positive FLAG-Nitr9L

expressing cells was similar (55 – 63% of $EGFP^+$ cells) when either an anti-FLAG or anti-Nitr9 antibody was employed (Fig. 5A). mAbs 90.10.5 and 19.1.1 recognize transfected cells expressing the endogenous isoform of Nitr9L with a similar efficiency (61-73% of $EGFP^+$ cells) (Fig. 5B).

The anti-FLAG mAb failed to effectively recognize FLAG-Nitr9S (8% of $EGFP^+$ cells) and the 19.1.1 mAb failed to effectively label Nitr9S or FLAG-Nitr9S expressing cells (2-9% of $EGFP^+$ cells). Although the 90.10.5 mAb detected FLAG-Nitr9S weakly (31% of $EGFP^+$ cells), it did not bind Nitr9S on transfected cells (5% of $EGFP^+$ cells) (Figs. 5C, 5D). Although Nitr9S is produced by transfected cells (Fig 2) it may not be expressed on the cell surface. In order to determine if cell surface expression of Nitr9S requires co-expression of the signaling adaptor protein Dap12, cells were co-transfected with Nitr9S and Dap12 but cell surface labeling by the anti-Nitr9 mAbs did not increase (data not shown).

The anti-FLAG antibody effectively bound to FLAG-Nitr9SS (57% of the $EGFP^+$ cells); however, mAb 90.10.5 failed to bind FLAG-Nitr9SS (0% of $EGFP^+$ cells) but efficiently recognized Nitr9SS on transfected cells (66% of $EGFP^+$ cells). Similarly, the 19.1.1 mAb was more effective at recognizing Nitr9SS (75% of $EGFP^+$ cells) than the FLAG-Nitr9SS (31% $EGFP^+$ cells) (Figs. 5E, 5F). It is possible that the FLAG-tag hinders antibody recognition of FLAG-NITR9SS as seen in the Western analyses and immunofluorescence assays above.

Attempts to use 90.10.5 for detecting Nitr9 expressing cells from dissected tissues via flow cytometry have not been successful, most likely due to the low levels of Nitr9 expression (data not shown).

- Applications

The utility of the 90.10.5 mAb for detecting Nitr9 from zebrafish tissues was demonstrated by Western analyses. Both anti-Nitr9 mAbs can be used in IHC and flow cytometry. The limitations of each antibody for recognizing the three different Nitr9 isoforms have been established. Efforts are underway to increase Nitr9 expression within the zebrafish to facilitate the detection and characterization of Nitr9 expressing cells *in vivo*. In a small scale study, it was reported that, on average, *Listonella anguillarum* infection induced *nitr9* mRNA expression ~2-fold in whole zebrafish;[12] it is possible that other pathogens may produce a more robust increase in *nitr9* expression facilitating the use of these mAbs. Furthermore, these antibodies may prove useful for activating (cross-linking) or blocking Nitr9 function in both cell culture and *ex vivo* functional assays.

Acknowledgements

We are grateful to Bradley Bone and Karen Marcus for assistance with generating the hybridomas and performing ELISAs, Janet Dow for assistance with flow cytometry and Susan Tonkonogy for helpful discussions. This research was supported by grants awarded by the National Science Foundation MCB-0505585 (JAY) and the National Institutes of Health R01 AI057559 (GWL).

FIGURE 1. Schematic representation of the recombinant Nitr9 expression constructs.

All constructs include an internal ribosomal entry sequence (IRES2) permitting the expression of two proteins from a single transcript. (**A**) A single mRNA (left) is transcribed from the FLAG-Nitr9 constructs, which encode a FLAG-tagged Nitr9 isoform (employing a Nitr3 leader sequence) and EGFP. Translational start and stop sites are indicated by green arrows and red circles, respectively. Three plasmids, *pFLAG-Nitr9L/EGFP, pFLAG-Nitr9S/EGFP* and *pFLAG-Nitr9SS/EGFP,* were constructed encoding each isoform of Nitr9 (right).

(**B**) A single mRNA (left) is transcribed from the endogenous Nitr9 constructs, which encode an Nitr9 isoform and EGFP. Three plasmids, *pNitr9L/EGFP, pNitr9S/EGFP* and *pNitr9SS/EGFP,* were constructed encoding each isoform of Nitr9 (right). FLAG-tag (red) and Nitr3 leader peptide (blue) sequences are indicated. Transmembrane (TM) and immunoglobulin domains [of the variable (V) and intermediate (I) types] of Nitr9 are indicated. The I domain of Nitr9, which was used as the antigen for antibody production, is shown in orange. The positive charge within the TM domain is represented by a plus sign.

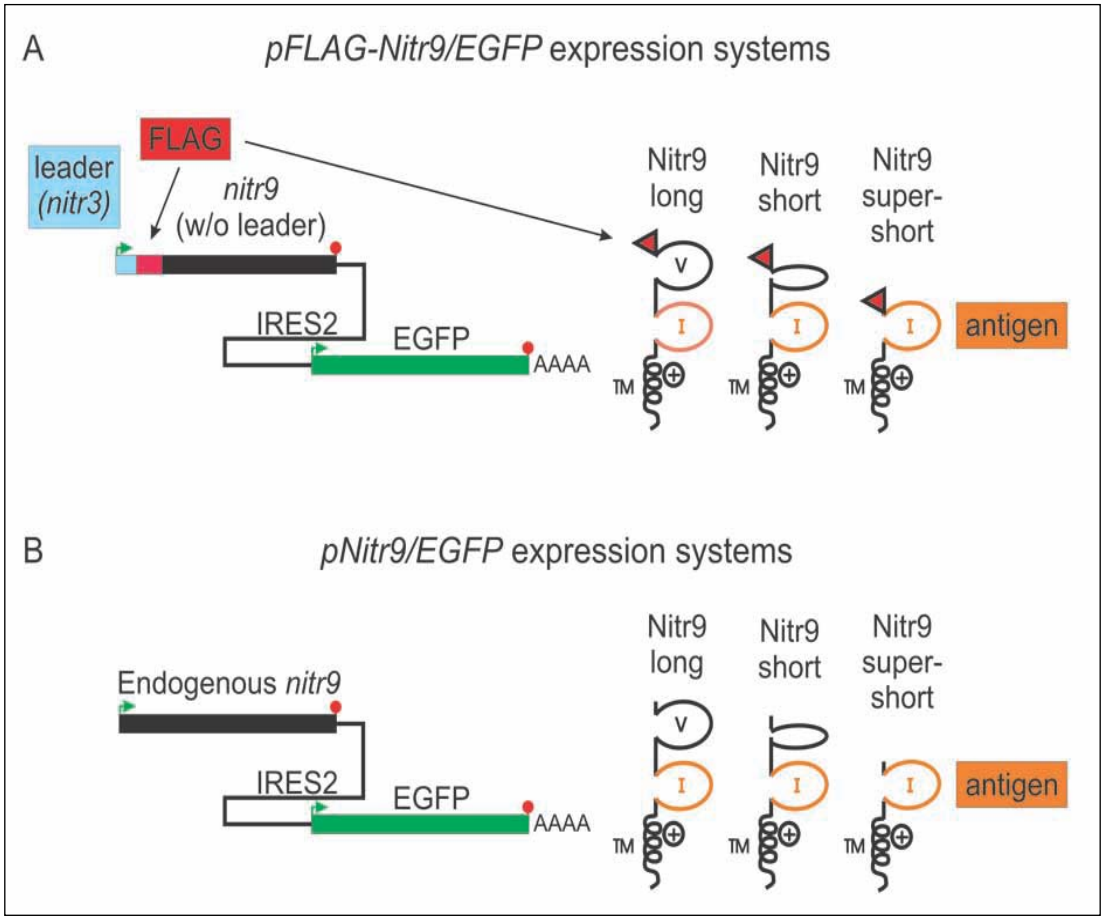
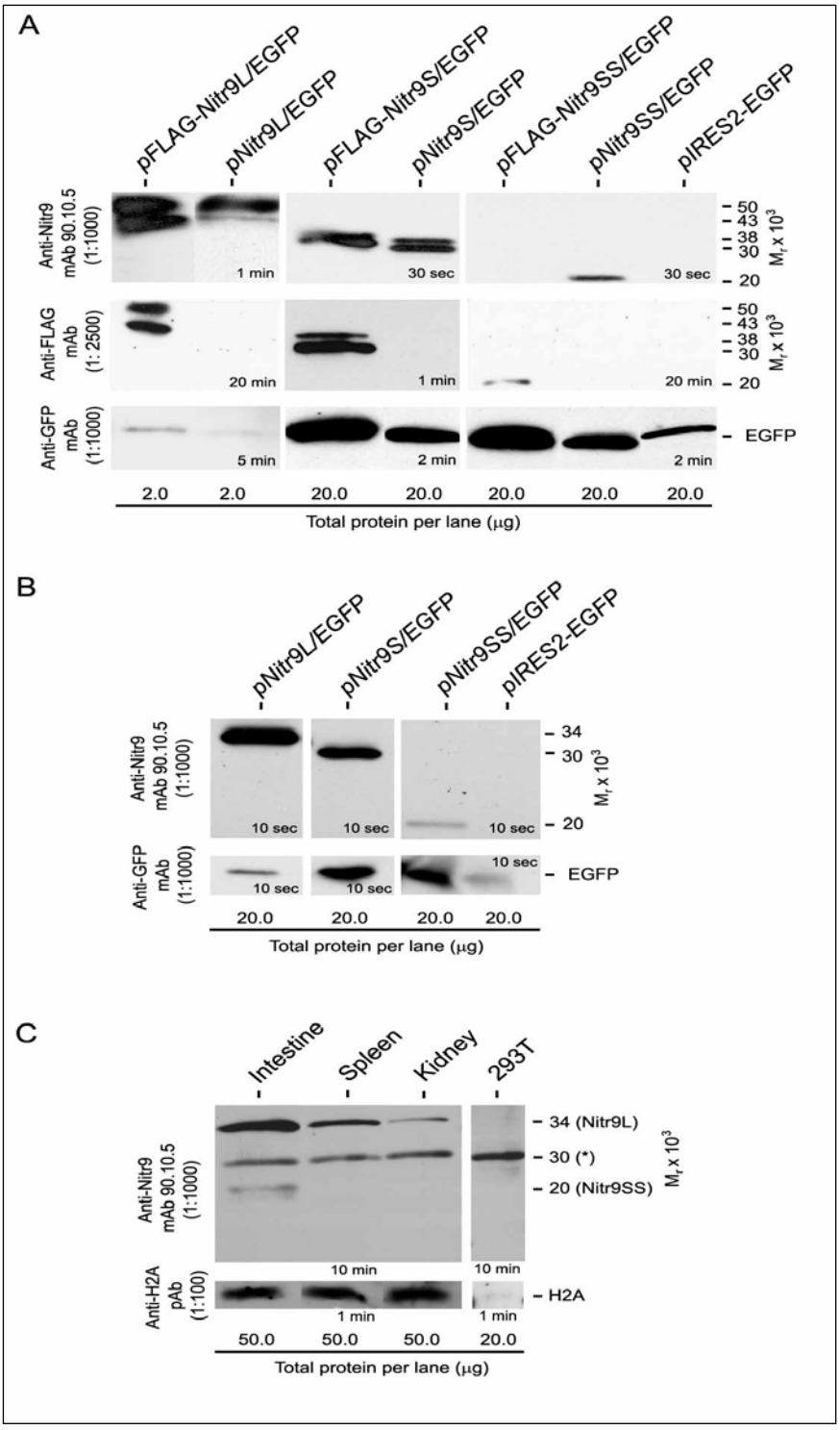

A
pFLAG-Nitr9/EGFP expression systems
FLAG
leader
(nitr3)
nitr9
(w/o leader)
IRES2
EGFP
AAAA
Nitr9
long
Nitr9
short
Nitr9
super-
short
V
I
TM
antigen
B
pNitr9/EGFP expression systems
Endogenous nitr9
IRES2
EGFP
AAAA
Nitr9
long
Nitr9
short
Nitr9
super-
short
V
I
TM
antigen

FIGURE 2. Detection of Nitr9 protein by Western analyses.

(**A**) Western blot analyses of total protein lysates from 293T cells transiently transfected with plasmids expressing a Nitr9 isoform and EGFP (indicated above). Plasmids encode either a FLAG-tagged Nitr9 (*pFLAG-Nitr9L/EGFP*; *pFLAG-Nitr9S/EGFP*; *pFLAG-Nitr9SS/EGFP*) or an endogenous isoforms of Nitr9 (*pNitr9L/EGFP*; *pNitr9S/EGFP*; *pNitr9SS/EGFP*). The primary mAbs utilized are shown on the left and the molecular weights of identified bands are shown on the right. The anti-FLAG mAb serves as a positive control for Nitr9 detection and the anti-GFP mAb indicates transfection efficiency of each plasmid. Note the total protein loaded (bottom) for the Nitr9L isoform is ten times less than that for Nitr9S and Nitr9SS plasmids. Exposure times for chemiluminescence are indicated in each panel. (**B**) Nitr9L and Nitr9S are glycosylated. Western blot analyses of endoglycosidase-treated total protein lysates from 293T cells that were transfected with the endogenous Nitr9 plasmids (indicated on top). The anti-Nitr9 mAb, 90.10.5, bound to all three Nitr9 splice variants of the predicted size (right). (**C**) Detection of Nitr9 protein from zebrafish tissues. Western blot analyses of 50 μg of endoglycosidase treated total protein from intestine, spleen and kidney tissues of adult


A
pFLAG-Nitr9L/EGFP
pNitr9L/EGFP
pFLAG-Nitr9S/EGFP
pNitr9S/EGFP
pFLAG-Nitr9SS/EGFP
pNitr9SS/EGFP
pIRES2-EGFP
Anti-Nitr9 mAb 90.10.5 (1:1000)
1 min
30 sec
30 sec
Anti-FLAG mAb (1:2500)
20 min
1 min
20 min
Anti-GFP mAb (1:1000)
5 min
2 min
2 min
50
43
38
30
20
$M_r \times 10^3$
EGFP
2.0 2.0 20.0 20.0 20.0 20.0 20.0
Total protein per lane (μg)
B
pNitr9L/EGFP
pNitr9S/EGFP
pNitr9SS/EGFP
pIRES2-EGFP
Anti-Nitr9 mAb 90.10.5 (1:1000)
10 sec
10 sec
10 sec
34
30
20
$M_r \times 10^3$
Anti-GFP mAb (1:1000)
10 sec
10 sec
10 sec
10 sec
EGFP
20.0 20.0 20.0 20.0
Total protein per lane (μg)
C
Intestine
Spleen
Kidney
293T
Anti-Nitr9 mAb 90.10.5 (1:1000)
34 (Nitr9L)
30 (*)
20 (Nitr9SS)
$M_r \times 10^3$
10 min
10 min
Anti-H2A pAb (1:100)
H2A
1 min
1 min
50.0 50.0 50.0 20.0
Total protein per lane (μg)

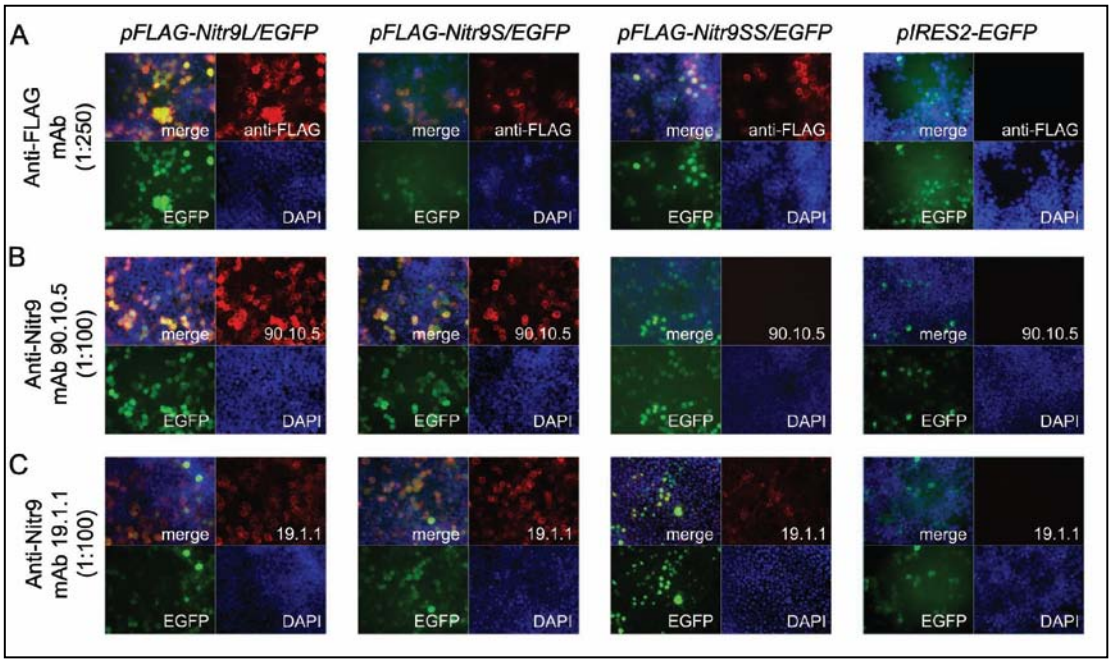


FIGURE 3. Detection of FLAG-tagged isoforms of Nitr9 from transfected cells by immunohistochemistry.

293T cells were transfected with plasmids encoding FLAG-tagged Nitr9 isoforms and EGFP as indicated on top of the panels. FLAG-tagged Nitr9 proteins were detected with (**A**) an anti-FLAG mAb, (**B**) the 90.10.5 anti-Nitr9 mAb or (**C**) the 19.1.1 anti-Nitr9 mAb and a PE conjugated secondary antibody (red). Transfected cells can be identified by EGFP expression (green). DAPI labels the nuclei of all cells (blue). The pIRES2-EGFP parental plasmid was included as a negative control.

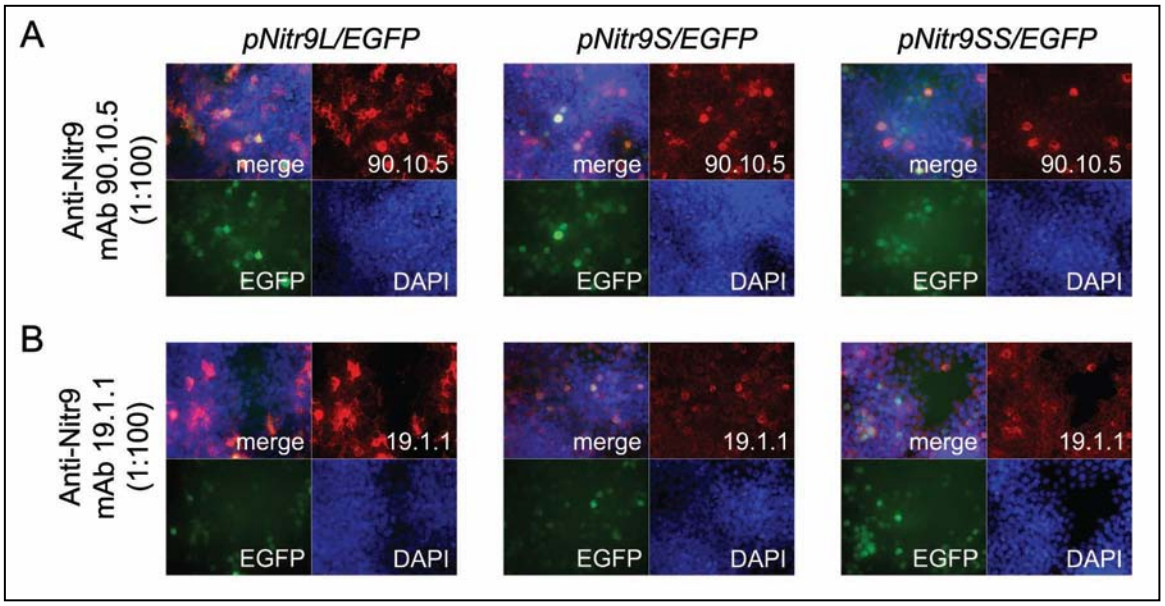


FIGURE 4. Detection of endogenous isoforms of Nitr9 from transfected cells by immunohistochemistry.

293T cells were transfected with plasmids encoding endogenous isoforms of Nitr9 and EGFP as indicated on top of the panels. Nitr9 proteins were detected with (**A**) the 90.10.5 anti-Nitr9 mAb or (**B**) the 19.1.1 anti-Nitr9 mAb and a PE conjugated secondary antibody (red). Transfected cells can be identified by EGFP expression (green). DAPI labels the nuclei of all cells (blue).

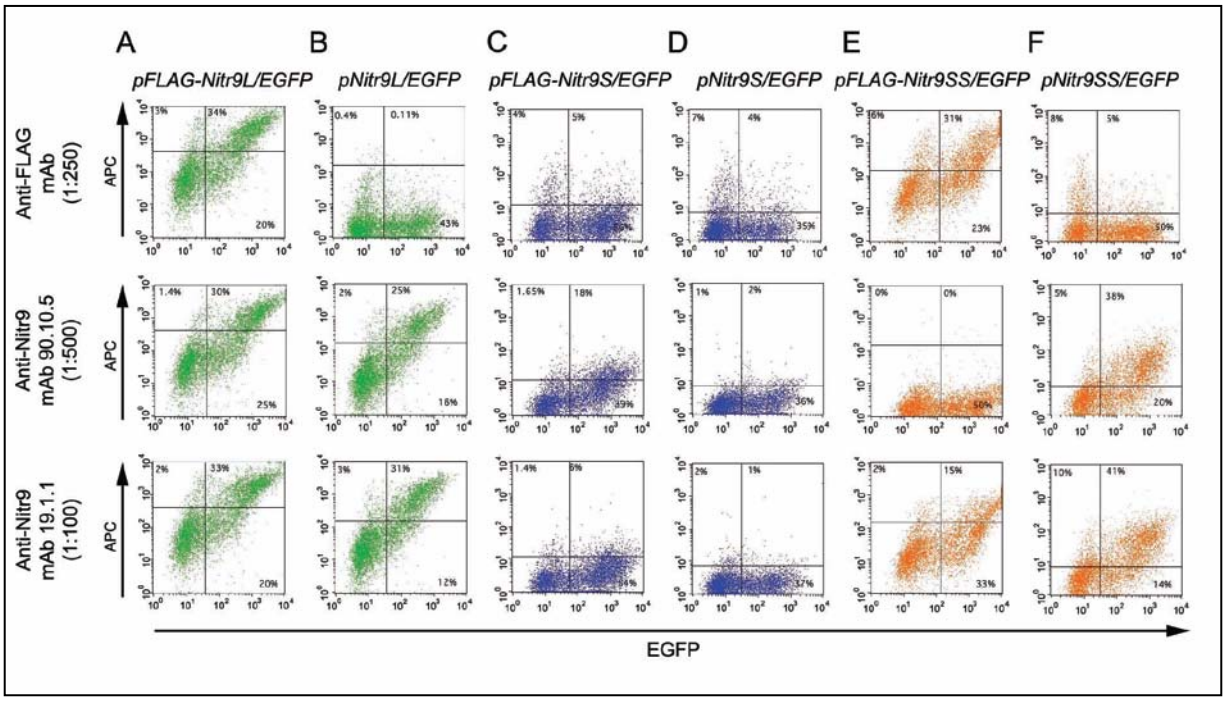


FIGURE 5. Detection of Nitr9 isoforms by flow cytometry.

293T cells were transfected with plasmids encoding FLAG-tagged (**A, C, E**) or endogenous (**B, D, F**) isoforms of Nitr9 and EGFP as indicated above the panels. Cells were labeled with an anti-FLAG mAb (top row), the 90.10.5 anti-Nitr9 mAb (middle row) or the 19.1.1 anti-Nitr9 mAb (bottom row) and an APC conjugated secondary antibody. Flow cytometric analyses were employed to detect GFP positive (X axis) and APC positive (Y axis) cells. Isotype matched mAbs were evaluated as controls for both anti-Nitr9 mAbs and displayed no labeling of transfected cells (data not shown).

TABLE 1. Oligonucleotide primer sequences

Purpose	Primer Name	Primer Sequence
PCR amplify Nitr9 I domain for bacterial expression construct	Nitr9-I-For	ATGGAAAAGCACACTGTAGTA[1]
	Nitr9-I-Rev	TTATTTAGAGCCATTCCTGTCC[2]
PCR amplify Nitr9L for FLAG-tagged expression cassette	Nitr9L-For	CACCCAAATGCACCACCTGTGTTTGTTAAAC[3]
	Nitr9-rev-NotI	gactgcggccgcTTACTGCTGGTTAGAAAC[4]
PCR amplify Nitr9S for FLAG-tagged expression cassette	Nitr9S-For	CACCCAAATGCACCACCTGTG[c]
	Nitr9-rev-NotI	gactgcggccgcTTACTGCTGGTTAGAAAC[d]
PCR amplify Nitr9SS for FLAG-tagged expression cassette	Nitr9-SS For	CATGATTTAATTCCATCCCA[c]
	Nitr9-rev-NotI	gactgcggccgcTTACTGCTGGTTAGAAAC[d]
PCR amplify endogenous Nitr9L, Nitr9S and Nitr9SS for endogenous expression cassettes	Nitr9_ORF_WT_For	gatcggatccgacATGATCAACTTTTGGATTT[5]
	Nitr9_ORF_Rev	gatcgaattcTTACTGCTGGTTAGAAACCGAG[6]

[1] An artificial start codon is underlined
[2] An artificial stop codon is double-underlined
[3] These primers are designed for blunt PCR cloning into the *Eco*RV site of pLF.
[4] Overhang (5′) sequences are in lower case text and include a *Not* I site for cloning into pLF.
[5] Overhang (5’) sequences are in lower case text and include a *Bam*HI site for cloning into pcDNA3.
[6] Overhang (3’) sequences are in lower case text and include an *Eco*RI site for cloning into pcDNA3.

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CHAPTER 3: METHODS TO BOOST NITR9 EXPRESSION *IN VIVO*

Contributions by our Collaborators:

- Genotyping for the homozygous rag1$^{-/-}$ and heterozygous rag1$^{+/-}$ zebrafish and tissue collections was performed in Dr. Nikolaus Trede's laboratory at University of Utah.

- *Snakehead rhabdo* virus infections and tissue collection was performed in Dr. Carol Kim's laboratory at University of Maine.

3.1 ABSTRACT

Of the 14 NITR gene families in zebrafish, Nitr9 is the only receptor that has been proposed to be similar to activating mammalian NKRs. With reference to mammalian activating NKRs it is hypothesized that zebrafish Nitr9 is expressed on NK or NK-like cells in zebrafish and functions in the innate immune response to clear virally infected and/or tumor cells from the host. The goal of this study was to identify a zebrafish model in which *nitr9* expression is elevated, so that it could be utilized as a means to characterize and purify Nitr9 expressing cells with the mAbs described in chapter 2. Quantitative real-time PCR (qRT-PCR) was carried out to determine if Nitr9 is expressed and/or elevated in the absence of the adaptive immune response, in *rag1*$^{-/-}$ mutant zebrafish and if *nitr9* is transcriptionally responsive to viral (Snakehead rhabdovirus, SHRV) infection in wild-type fish. Results of qRT-PCR analyses suggest that relative levels of *nitr9* gene expression in the intestine of homozygous *rag1*$^{-/-}$ mutant zebrafish tissues is higher than that of heterozygous *rag1*$^{+/-}$ zebrafish. However; transcription of *nitr9* was relatively unchanged in tissues of virally infected zebrafish (as compared to mock infected control animals). These data suggest that the *rag1*$^{-/-}$ zebrafish may be useful for identifying Nitr9 expressing cells.

3.2 INTRODUCTION

As described in chapter 2, attempts to detect Nitr9 expressing cells and purify them with the anti-Nitr9 mAbs was unsuccessful possibly due to the low levels of Nitr9 expression. Hence in order to identify Nitr9^{+} cells, it is necessary to increase either the number of cells expressing Nitr9 or the level of Nitr9 expression in individual cells. It is hypothesized that Nitr9 is an activating receptor, and is expressed on NK or NK-like cells that function as part of the innate immune system to eliminate virally infected and/or tumor cells. Based on this hypothesis two experimental systems were utilized to evaluate if the expression of *nitr9* can be manipulated *in vitro.* The first system employs zebrafish lacking the functional adaptive immune response and the second system employs zebrafish that were virally infected.

Through the process of random ENU-induced point mutation a recessive nonsense mutation in the zebrafish *rag1* gene has been reported. This mutation results in the translation of a truncated Rag1 protein that leads to defective V(D)J recombination, and a block of immunoglobulin and TCR gene assembly [2, 3]. Animals homozygous for this mutation (*rag1*$^{-/-}$) are viable and fertile in standard, nonsterile aquarium conditions, although these fish are more susceptible to an injected dose of *Mycobacterium marinarum* [4, 5]. It has been proposed that, to survive in nonsterile conditions, the innate immune response of these Rag1 deficient zebrafish may be

augmented to compensate for the absence of functional adaptive immune cells. To this effect, transcriptional profiling studies of tissues from *rag1*$^{-/-}$ zebrafish revealed increased transcription of several innate immune genes, including a putative interferon stimulated gene and multiple genes of the coagulation and complement pathway [4]. Thus, it was hypothesized that *nitr9* gene expression levels may be elevated in the *rag1*$^{-/-}$ zebrafish and this model was utilized to determine relative levels of *nitr9* expression in tissues from *rag1*$^{-/-}$ zebrafish compared to those in *rag1*$^{+/-}$ zebrafish by quantitative real-time PCR.

The zebrafish has been adapted as an excellent model for the study of infectious disease and immunology [3, 6-9]. A variety of bacteria and viruses have been used to infect zebrafish to address questions on host-pathogen interaction [7, 10]. The infection kinetics and pathology following *Snakehead rhabovirus* (SHRV) have been well characterized in the zebrafish. SHRV is an enveloped, non-segmented, negative sense RNA virus that affects warm water fish with an optimal temperature range for replication suitable for zebrafish maintenance. Experimental infection with SHRV in adult zebrafish leads to acute infection associated with necrosis and upregulation of interferon [9, 11, 12]. Since it is likely that *nitr9* plays a role in clearing virally infected cells, qRT-PCR was performed to determine the response of *nitr9* expressing cells to SHRV infection.

3.3 MATERIALS AND METHODS

- Animals and Infection

Adult zebrafish were housed on a 10-h light / 14-h dark cycle in recirculating water at 28 °C and fed twice daily. For removal of organs, zebrafish were euthanized in a buffered solution of 0.02% Tricaine methanesulfonate (Finquel MS-222; Argent Chemical Laboratories, Redmond WA). The abdominal wall was opened and the spleen, and intestine tissues were removed in TriZOL (Invitrogen, Carlsbad, CA). After all intra-abdominal organs were removed the kidney was visualized ventral to the spinal canal and removed in TriZOL (Invitrogen, Carlsbad, CA).

All homozygous *rag1*$^{-/-}$ and heterozygous *rag1*$^{+/-}$ zebrafish were maintained in Dr. Nikolaus Trede's laboratory at University of Utah, and genotyped as described [4]. The fish were dissected and tissue samples were shipped in TriZOL (Invitrogen, Carlsbad, CA) on dry ice.

The infection experiments were carried out in Dr. Carol Kim's laboratory at University of Maine. Adult zebrafish were anethesized with 0.016% tricaine (Sigma) and divided into three groups of SHRV infected, mock injected with PBS and uninjected fish (10 fish per group at each time point). The infected fish were injected intraperitoneally (i.p.) with 10^5 $TCID_{50}$ (Tissue Culture Infectious Dose) of SHRV/ml of PBS [12]. Fish were euthanized at 2, 8, 24, 48, and 72 hours post infection (hpi)

and tissue samples collected in TriZOL (Invitrogen, Carlsbad, CA) and shipped on dry ice. Fish were handled according to the guidelines from Institutional Animal Care and Use Committee.

- RNA

Tissues from adult zebrafish were collected directly into Trizol (Invitrogen, Carlsbad, CA), homogenized using a Tissuemiser Homogenizer (Fisher Scientific, Pittsburgh, PA), stored at -80 °C, thawed within 2 months and total RNA was purified as described by the manufacturer. The quality and quantity of the resulting RNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

- Quantitative Real Time PCR

Total RNA from tissues (2 µg) was annealed to oligo dT primers, reverse transcribed with Superscript III (Invitrogen), diluted 5-fold with dH_2O and utilized for quantitative PCR. Custom Taqman probes (20X Gene Expression Assay Mix, Applied Biosystems), for *nitr9*, with forward primer sequence 5'-

GTCAAAGGGACAAGGCTGATAGTT-3' reverse primer sequence 5'-GTTCAAAACAGTGCATGTAAGACTCA-3' and reporter probe sequence 5'-CAAGGTTTGGAAAAGCAC-3' were used for qRT-PCR of zebrafish transcripts. All PCRs were performed using 2 µl of diluted cDNA, in a 25 µl reaction volume (2X Taqman Universal Master mix, Applied Biosystems). All reactions were performed in a 96 well plate and monitored with a BioRad iCycler and iCycler iQ Optical System Software, version 3.0a. Thermal cycling parameters were followed as per manufacturer's instructions including an initial holding temperature of 50 °C for 2 min, followed by denaturing at 95 °C for 10 min and 60 cycles of: denaturing at 95 °C for 15 s, annealing at 60 °C for 1 min. Reactions were completed in triplicate and the average relative levels of expression were calculated by normalizing to levels of *β actin* as described [1]. A negative control (no cDNA) for each primer set was utilized to exclude sample contamination.

3.4 RESULTS AND DISCUSSION

- Relative *nitr9* gene expression in mutant homozygous *rag1*$^{-/-}$ zebrafish tissues

It was reported that multiple innate immune genes are transcriptionally elevated in the *rag1*$^{-/-}$ zebrafish [4]. Thus, it was hypothesized that the expression of *nitr9*, a

possible functional equivalent of an activating mammalian NKR would be increased in the *rag1*$^{-/-}$ zebrafish. The relative levels of *nitr9* was determined in *rag1*$^{-/-}$ homozygous zebrafish intestine, spleen and kidney tissues as compared to that in *rag1*$^{+/-}$ heterozygous zebrafish tissues. These tissues were selected since the zebrafish intestine is a major secondary lymphoid organ and a site for continuous microbial challenge, the spleen is also a secondary lymphoid organ that contains ~11% of the lymphocyte population when analyzed by flow cytometry and the kidney is the hematopoietic organ, the bone marrow equivalent, of zebrafish [4, 10, 13]. Western blot analyses have demonstrated that Nitr9 protein is expressed most highly in the intestine of adult zebrafish (Chapter 2). In support of the hypothesis, the activating *nitr9* gene was elevated by ~9 fold in the *rag1*$^{-/-}$ intestine as compared to the heterozygous zebrafish controls (Figure 1). In contrast, expression levels of *nitr9* in the *rag1*$^{-/-}$ zebrafish spleen was the same as that of the heterozygous controls (Figure 1). It is possible that there may be post-transcriptional regulation of an increase in Nitr9 activation and signaling in the splenocytes. RT-PCR analysis of lymphoid and myeloid populations from wild-type zebrafish kidney indicates that *nitr9* is exclusively expressed in the lymphoid population. However; it has been reported that there is a significant reduction in the number of lymphocytes in the kidney of *rag1*$^{-/-}$ mutants [14]. In addition, Nitr9 protein is expressed at lower levels in the kidney than those observed in the intestine and spleen (Chapter 2). Thus, the decrease in *nitr9* gene expression in the *rag1*$^{-/-}$ kidney as seen in Figure 1 may be

due to lower numbers of lymphocytes and/or low levels of protein expression in the kidney. These results suggest that the *nitr9* possibly participates in the innate immune response, with respect to the elevated transcript levels observed in the intestine tissue of *rag1*$^{-/-}$ fish, however; multiple biological samples need to be tested to confirm these preliminary observations.

- Relative *nitr9* gene expression following SHRV infection in zebrafish tissues

In order to determine the relative transcription levels of *nitr9* in response to viral infection, adult zebrafish were immune challenged with SHRV and the relative mRNA expression levels of *nitr9* were determined in infected zebrafish tissues by qRT-PCR. Figure 2A, B and C shows the transcriptional response of *nitr9* to SHRV infection at 2, 8, 24, 48 and 72 hpi in intestine, kidney and spleen respectively. No obvious transcriptional changes were observed for *nitr9* as compared to the mock infected PBS controls. It is possible that *nitr9* may have increased activation in response to infection that could not be measured at the transcriptional level. In addition, there may be a wide variation of *nitr9* expression among individual fish as observed in the case of *L. anguillerum* bacterial infection [15]. Multiple biological samples need to be tested to provide more definitive results about the transcriptional response of *nitr9* to viral infection.

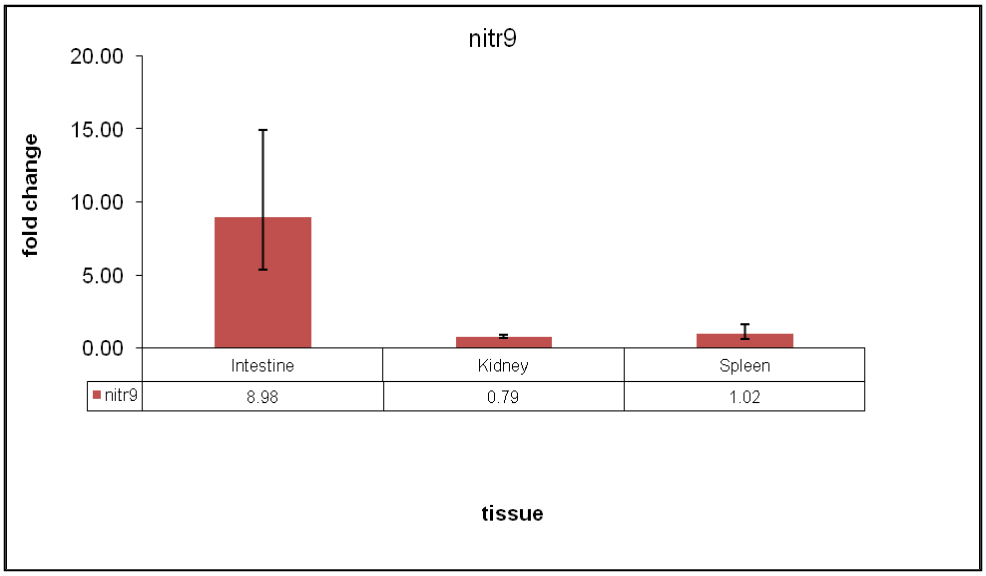
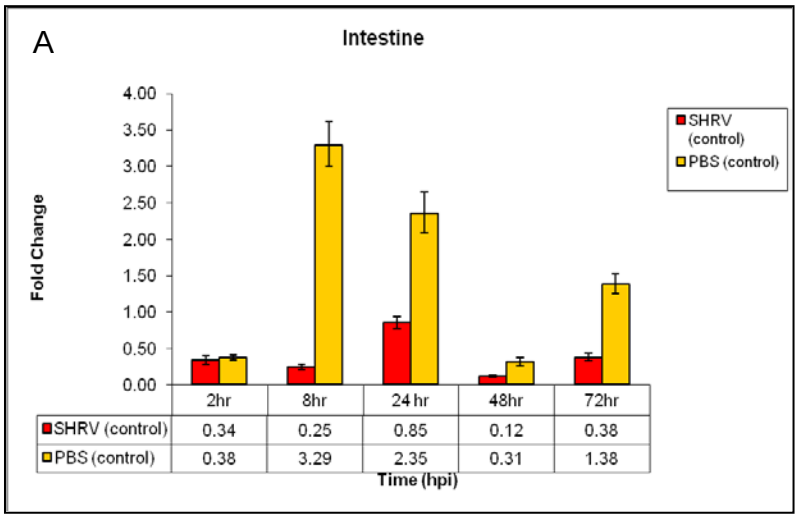


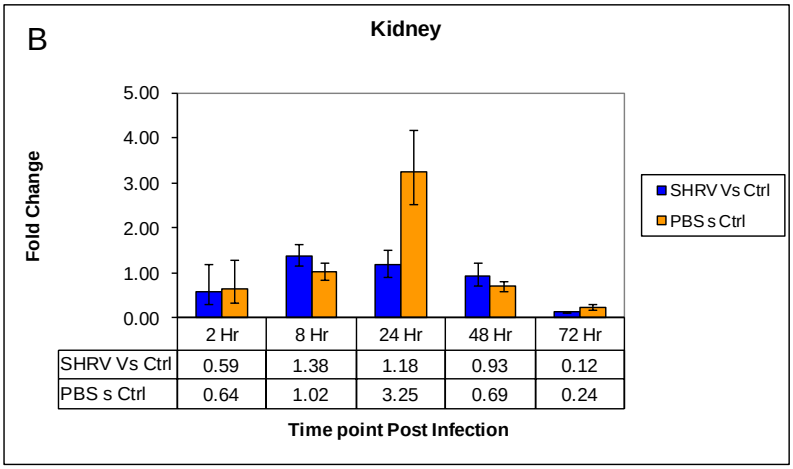
Figure 1: Relative levels of *nitr9* gene expression in zebrafish *rag1*$^{-/-}$ tissues.

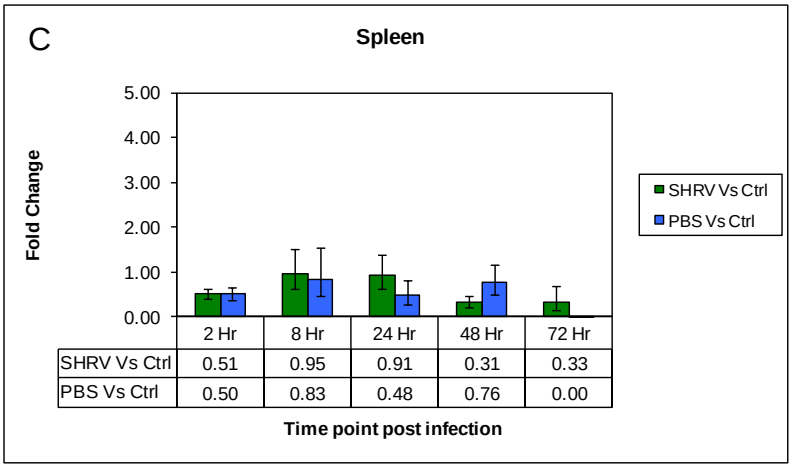
qRT-PCR of zebrafish NITR genes was carried out to determine relative *nitr9* expression in *rag1*$^{-/-}$ zebrafish intestine, kidney, and spleen. Ct values were normalized to zebrafish β *actin* 2 and the relative fold-difference (Y-axis) was calculated using the $2^{-\Delta\Delta CT}$ method as described [1]. The values represent normalized expression levels in *rag1*$^{-/-}$ zebrafish tissues relative to the *nitr9* expression in *rag1*$^{+/-}$ zebrafish tissues. The error bars indicate standard error between triplicate reactions.

Figure 2: Relative levels of *nitr9* gene expression in SHRV infected tissues of adult zebrafish tissues.

qRT-PCR of zebrafish *nitr9* was carried out to determine relative gene expression in the SHRV infected intestine (A), kidney (B), and spleen (C) at 2, 8, 24, 48 and 72 hours post infection (hpi). Ct values were normalized to zebrafish β *actin* 2 and the relative fold-difference (Y-axis) was calculated using the $2^{-\Delta\Delta CT}$ method as described [1]. Mock, PBS injected fish were used as a control for response to injection. The values represent normalized expression levels in SHRV infected zebrafish tissues and mock injected tissues relative to the *nitr9* expression in uninfected control zebrafish. The error bars indicate standard error between triplicate reactions.


A
Intestine
Fold Change
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
SHRV (control)
PBS (control)
2hr 8hr 24 hr 48hr 72hr
SHRV (control) 0.34 0.25 0.85 0.12 0.38
PBS (control) 0.38 3.29 2.35 0.31 1.38
Time (hpi)


B
Kidney
Fold Change
5.00
4.00
3.00
2.00
1.00
0.00
SHRV Vs Ctrl
PBS s Ctrl
2 Hr 8 Hr 24 Hr 48 Hr 72 Hr
SHRV Vs Ctrl 0.59 1.38 1.18 0.93 0.12
PBS s Ctrl 0.64 1.02 3.25 0.69 0.24
Time point Post Infection


C
Spleen
Fold Change
5.00
4.00
3.00
2.00
1.00
0.00
SHRV Vs Ctrl
PBS Vs Ctrl
2 Hr 8 Hr 24 Hr 48 Hr 72 Hr
SHRV Vs Ctrl 0.51 0.95 0.91 0.31 0.33
PBS Vs Ctrl 0.50 0.83 0.48 0.76 0.00
Time point post infection

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CHAPTER 4: DISSERTATION SUMMARY

4.1 SUMMARY

NITRs were identified more than 12 years ago, resulting from a search for genes that contain variable domains but do not undergo gene rearrangements in fish (1). Today, NITRs have been identified in at least 13 species of bony fish, and are likely present in all bony fish (2). NITRs are believed to play an important role in teleost immunity and may represent the primordial genes that gave rise to the rearranging genes of the adaptive immune system (2). The studies described here focus on increasing our understanding of the NITR gene family in zebrafish.

Of the 14 NITR gene families identified in zebrafish, I chose to study zebrafish Nitr9 since it is the only activating NITR and cells that express this molecule are hypothesized to play an important role in immune mediated destruction of virally infected cells and tumor cells (3-5). This dissertation encompasses work that was carried out to develop and employ a tool to identify and characterize Nitr9 to eventually understand its role in innate immunity.

Two mouse anti-Nitr9 monoclonal antibodies (mAbs) were developed using a recombinant Nitr9 intermediate domain. These mAbs facilitated the identification of endogenous Nitr9 protein expression in zebrafish tissues by Western blot. The Anti-Nitr9 Ab, 90.10.5, bound to all three isoforms of Nitr9 in Western analyses and provided the first evidence of an Nitr at the protein level in bony fish species. In

addition Western blot analyses indicated that the Nitr9 proteins are glycosylated and differentially expressed in different tissues of adult zebrafish possibly providing tissue specific functions.

Immunofluorescence and flow cytometric assays were standardized in a cell culture system that expressed all the Nitr9 isoforms, utilizing the anti-Nitr9-mAbs. Anti-nitr9 Abs bound efficiently and specifically to Nitr9L and Nitr9S expressing cells by immunofluorescence and both anti-nitr9 Abs recognized Nitr9L and Nitr9SS but failed to recognize Nitr9S by flow cytometry. These anti-nitr9 monoclonal antibodies can eventually be utilized to purify Nitr9 positive cells from zebrafish tissues that would enable the identification and characterization of these cells. In addition the anti-Nitr9 Abs can be utilized for activating (cross-linking) or blocking Nitr9 function in both cell culture and ex vivo functional assays.

Preliminary studies were carried out in order to evaluate the transcriptional response of *nitr9* in the absence of an adaptive immune response and during immune challenge. Quantitative real-time PCR analysis determined relative levels of *nitr9* gene expression in *rag1*$^{-/-}$ as compared to those in their heterozygous controls. Higher expression of *nitr9* was observed in the intestine of the mutant zebrafish, and these fish could possibly be utilized as a model to identify Nitr9 expressing cells. Further, the elevated level of *nitr9,* in the absence of an adaptive immune response, supports the hypothesis that Nitr9 may play a role in the innate immune response of

zebrafish. However; no conclusive results were obtained regarding the mRNA level of *nitr9* following viral infection.

In conclusion, this research has established that Nitr9 is expressed *in vivo* and provided a tool to study Nitr9 function in zebrafish, which will pave the way to understand the significance of activating NITRs and possibly provide insight into the signaling pathways associated with the innate immune response in zebrafish. Such studies will shed light on the evolution of the innate and adaptive immune receptors in vertebrates.

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APPENDIX: PROTOCOLS FOR ANTI-NITR9 MONOCLONAL ANTIBODY SCREENING AND PURIFICATION

WESTERN BLOT ANALYSES USING THE MINIBLOTTER SYSTEM

293T cells transfected with FLAG-tagged Nitr9-L-pIRES2-EGFP plasmid were washed with 1x phosphate buffered saline (PBS) and lysed with Mammalian Protein Extraction Reagent (M-PER®: Pierce; Rockford, IL). Lysates were centrifuged to remove nuclei and cell debris and protein concentrations were determined (BCA Protein Assay: Pierce). ~100μg of total Nitr9-L protein from transfected 293T cells was resolved in the prep well of the 15% pre-cast SDS-PAGE gel from Bio-Rad (Hercules, CA) at 100V. The proteins were transferred to polyvinylidene difluoride membranes at 100 mA overnight. Membranes were washed four times in 1XTris-buffered saline with 0.1% Tween-20 (TBST) and blocked in 10mls of blocking buffer (100mM boric acid, 25mM Na-Borate, 75mM NaCl, 5% goat serum and 5% dry milk powder) for 1 h. Membranes were then rinsed with 1XTBST and placed on the miniblotter system. The membranes were dried using a vacuum pump and 50μl of hybridoma supernatants (UNC- Chapel Hill, Immunology Core Facility) were added to each channel. The membranes were incubated with the different supernatants for 45 minutes at room temperature without shaking. The anti-FLAG (M2) antibody obtained from Sigma-Aldrich was used as the positive control in one channel. The supernatants were then removed from the chambers using vacuum suction. The membrane was washed with 500ml of 1XTBST using the manifold provided with the miniblotter system and then removed from the miniblotter (after marking the different

channels on the membrane). The membrane was washed once more for 20 minutes in 1X TBST and incubated for 30 minutes with horseradish peroxidase conjugated anti-mouse IgG antibody (Roche) diluted 1:4000 in blocking buffer. The blots were washed four times in 1X TBST and binding patterns were visualized using Lumi-Light^{PLUS} Western Blotting substrate and detection system (Roche) as shown in Figure 1.

WESTERN BLOT ANALYSES OF SELECTED HYBRIDOMA SUPERNATANTS

293T cells transfected with FLAG-tagged Nitr9-L-pIRES2-EGFP plasmid were washed with 1x phosphate buffered saline (PBS) and lysed with Mammalian Protein Extraction Reagent (M-PER®: Pierce; Rockford, IL). Lysates were centrifuged to remove nuclei and cell debris and protein concentrations were determined (BCA Protein Assay: Pierce). 10μg of protein was resolved on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes for Western analyses. Membranes were washed in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated in blocking buffer (100 mM boric acid, 25 mM Na-Borate, 75 mM NaCl, 5% goat serum and 5% dry milk powder) for 1 hr. Membranes were incubated overnight with primary antibodies in blocking buffer at 4 °C. Primary antibodies include an anti-FLAG (M2) mouse mAb (Sigma-Aldrich; St. Louis, MO) at a 1:2500 dilution, and the two selected hybridoma supernatants at a 1:100 dilution. Membranes were washed in TBST, followed by incubation with blocking buffer and peroxidase-conjugated anti-mouse IgG secondary antibody (Roche) and final washes with TBST. The Lumi-Light^{PLUS} Western Blotting substrate and detection system (Roche) was used to visualize reactivity as shown in Figure 2.

MONOCLONAL ANTIBODY PURIFICATION

Amicon Ultra-15 tubes (Millipore) were used to desalt 3.0mls of the hybridoma supernatant diluted in 9.0mls of tris-buffered saline, pH 7.4 as per manufacturer's instructions. 1 ml of Protein A Agarose, Fast Flow beads from Upstate Cell Signaling Solutions (Temecula, CA) were packed in a glass column (Bio-Rad, Hercules, CA). The column was washed and equilibrated with 10 column volumes (10mls) of tris-buffered saline, pH 7.4. 10mls of the desalted supernatant was allowed to flow through the column, and was collected as 1.0ml fractions. The bound IgG was eluted in 10mls of glycine, pH 2.7 as 0.5ml fractions in tubes containing tris-HCl, pH 8.0 (neutralization buffer). The protein concentration of the flow through and eluted fractions was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The eluted samples with a higher protein concentration were selected and run on an SDS-PAGE gel and stained with Coomassie blue (Figure 2). The fractions containing the purified monoclonal antibodies were desalted and their concentration was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). 0.02% sodium azide and 1X protease inhibitors (Pierce) were added and the purified antibodies were aliquoted and stored at -80C.

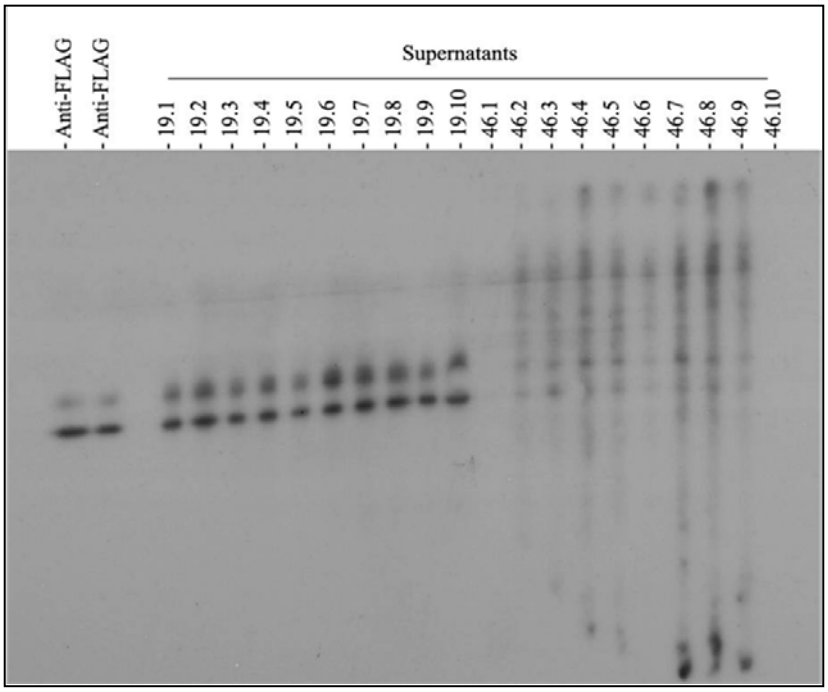

Figure 1: Screening hybridoma supernatants.

A representative blot showing the results of the second round of screening the hybridoma supernatants. The anti-FLAG antibody was used as a positive contol (first two lanes). The hybridoma supernatants 19.1 to 19.10 showed a similar binding pattern as that seen with the FLAG-Antibody.

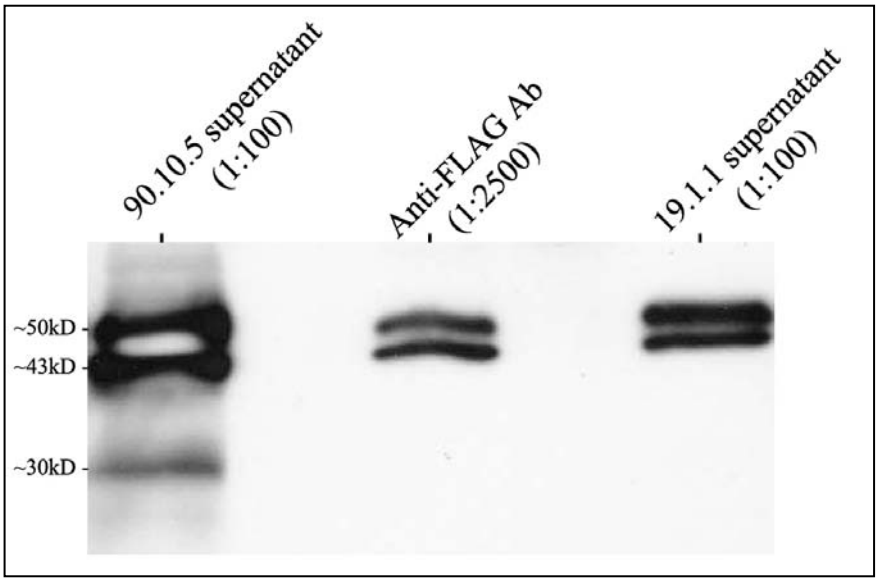

Figure 2: Western blot analyses of the two mAb supernatants – 90.10.5 and 19.1.1, selected after the third round of screening.

293T mammalian cells were transfected with the FLAG-Nitr9-L-IRES2-eGFP plasmid and total protein lysates were used for the Western blot analyses. The anti-FLAG antibody was used as a positive control. As shown, both supernatants gave the same sized double bands of 50kD and 43kD, as observed with the control. An additional band of ~30kD was observed with the 90.10.5 supernatant. The dilutions used are indicated above the blot. Even though the same pattern was observed as the FLAG-Ab, the bands were of higher molecular weight than the

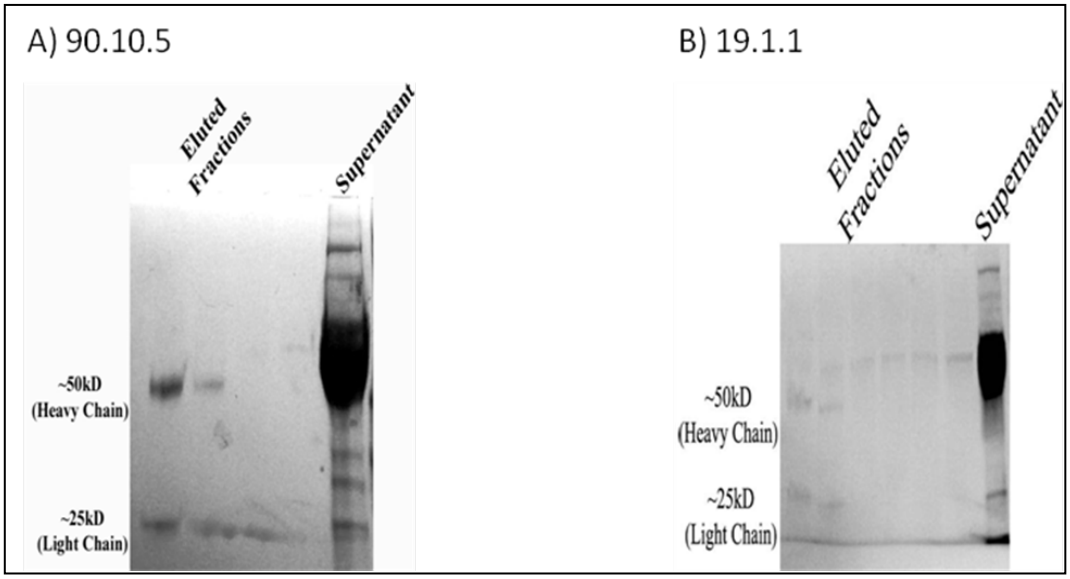

Figure 3: Anti-Nitr9 mAb purification.

The selected hybridoma supernatants, 90.10.5 (A) and 19.1.1 (B) were purified using Protein A agarose beads as described in the protocol. The eluted samples were separated on a 12% SDS-PAGE gel and stained with Coomassie blue. 10μl of the hybridoma supernatant was loaded in one well for comparison.