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The Role of SHIP1 in the Innate Immune System During an Influenza A Viral Infection

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THE ROLE OF *SHIP1* IN THE INNATE IMMUNE SYSTEM DURING AN INFLUENZA A VIRAL INFECTION

By

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Abstract:

The *SHIP1* gene is a member of the inositol polyphosphate-5-phosphate (INPP5) and its expressed protein functions as a negative regulator of myeloid cell proliferation, survival, and migration. Mutations in this gene are associated with various defects and cancers of the immune system. Previous studies have shown that in response to wound formation, ship1-deficient zebrafish have increased neutrophil motility while overexpression of ship1 resulted in decreased neutrophil migration (Lam et al. 2012). From this research, it is suggested that SHIP1 is a key brake that limits neutrophil motility through a PI3K signaling-dependent pathway. While the role of SHIP1 during a wound response has been categorized, its function during a viral infection has been left uncharacterized. The goal of this present study is to examine the role of ship1 during an innate immune response to an influenza infection.

Using the zebrafish, *Danio rerio*, as an *in vivo* model organism, we hope to reveal the role of SHIP1 in the innate immune response to a viral infection. It was initially discovered that upon viral infection, SHIP1 is upregulated, promoting further research into the role of SHIP1 in the antiviral innate immune response. Morpholino-mediated *SHIP1* knockdown resulted in increased zebrafish survival upon influenza infection, suggesting that SHIP1 is a critical part of the antiviral immune response. Furthermore, SHIP1-KD in zebrafish exhibited decreased production of ROS, indicating that SHIP1 plays a role in pathogen killing.

Acknowledgements:

I would first like to thank my advisor Dr. Carol Kim for allowing me to perform this research in her laboratory for the past year. I also want to thank Dr. Paul Millard, Dr. Jean MacRae, Dr. Melissa Maginnis, and Dr. Edith Elwood for serving on my thesis committee and providing assistance and reassurance throughout this process. I would also like to thank members of the Kim Laboratory, especially Campbell Miller, for their continuous support and guidance; without their help, this thesis would not have been possible. I lastly and most importantly want to give thanks to my friends and family for their encouragement, inspiration, and belief in me during the past four years.

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INTRODUCTION

In order for an organism to defend itself from invading pathogens, the two components of the immune system: the innate immune system and the adaptive immune system must both be functioning properly. The innate immune system is the first line of defense and is present among all organisms, making it the subject of a wide range of research. The innate immune system is comprised of numerous cells including neutrophils and macrophages, involved in the phagocytosis and eradication of foreign organisms. Important steps in microbial killing are migration and phagocytosis, which in neutrophils, is tightly regulated by the phosphoinositide 3-kinase (PI3K) pathway and the control of the key signaling molecule, phosphatidylinositol (3,4,5) triphosphate (PIP3).

Viruses are intracellular pathogens that utilize the host cell machinery to replicate viral particles to cause infection. There are around 200 different viruses that are known to infect humans but despite their prevalence, vaccines are only available for 15 of the diseases caused by these viruses, necessitating the need for new antiviral treatments (Small and Ertl, 2011).

Many viruses have managed to evade microbial killing by disrupting the innate immune system. Recently, our lab has discovered that a key enzyme in neutrophil migration, phagocytosis, and microbial killing, SHIP1, is upregulated during a viral infection. SHIP1 degrades the key signaling molecule, PIP3, to phosphatidylinositol (3,4) diphosphate (PIP2) in the PI3K pathway, which inhibits important cell functions such as cell motility, protein synthesis, and actin polymerization. Further research into the role of SHIP1 during a viral infection could aid in the development of new and effective antiviral treatments.

BACKGROUND

Those who are infected with influenza, also known as the flu, experience the symptoms of fever, cough, congestion, aches, and fatigue. These symptoms are a result of a systemic viral infection which is acquired primarily through inhalation of airborne droplets from another infected individual.

Viral infections are common in the human populations. As obligate intracellular organisms, viruses are able to control and employ the host cell machinery to replicate and further cause infection. More than 90% of human illnesses may be caused by viral infections, which are generally systemic (Norkin et al., 2009). To combat viral infections, organisms utilize both innate and adaptive immune responses. The innate immune system is the first line of defense and is conserved among all organisms (Parkin, 2001). A critical cell in the innate immune response is the neutrophil. Neutrophils are phagocytic cells that migrate from the blood stream into infected tissues via chemotaxis. Once inside tissues, neutrophils are able to recognize foreign material through pathogen recognition receptors (PRRs) and the binding of pathogen associated molecular patterns (PAMPs)(Akira et al. 2006). Upon recognition of viral material, neutrophils are able to phagocytose and kill the invading pathogen via both oxygen dependent and independent mechanisms. A key cellular pathway in cellular motility, phagocytosis, and microbial killing is the phosphoinositide 3-kinase (PI3K) pathway. The key signaling molecule generated by this pathway, phosphatidylinositol (3,4,5) triphosphate (PIP3), mediates myriad cellular functions including protein synthesis, glucose metabolism, and actin polymerization at the cell surface. The phosphatase, SHIP1 has been characterized as an essential negative regulator of PIP3, inhibiting an overactive immune response and,

preventing damage (Lam et al., 2012). In zebrafish, it has been revealed that upon an influenza infection, SHIP1 is positively regulated, raising questions regarding the influence that the virus has on the host's machinery.

Due to issues accompanying the use of a variety of conventional animal models, and the clear ethical concerns surrounding human testing, zebrafish are commonly employed as models for human disease. Zebrafish are an established instrument for research into development, genetics, cancer, immunity, and infection. Advantages of the zebrafish versus its counterparts, the mouse (*Mus musculus*) and the fruit fly (*Drosophila melanogaster)*, are its short generation time, optical clarity, and its cellular and molecular similarity to humans (Lieschke and Currie, 2007). The zebrafish genome is fully sequenced and demonstrates high conservation between the human genome, making it a useful model for human immunity and disease.

Previous studies have shown that the knockdown of the SHIP1 gene leads to increased neutrophil migration, while overexpression of SHIP1 saw decreased neutrophil motility (Lam et al.,2012). Viral infection led to an initial upregulation of SHIP1 in these studies. SHIP1 may play an important role in neutrophil activity, specifically upon a viral infection. Understanding the mechanisms behind neutrophil activity, particularly the role of SHIP1, will aid in developing new and effective antiviral treatments.

VIRUSES

Viruses are categorized as intracellular pathogens that are composed of proteins and genetic information in the form of either DNA or RNA encompassed in a capsid. In order for the virus to replicate its genetic information and increase its pathogenicity, it must utilize host cell enzymes and other machinery (Garcia-Sastre and Biron, 2006). When a virus comes into contact with a host cell, it can insert its genetic information into the cell and then manipulate the host cell machinery to replicate its genome and viral proteins. Upon accumulation of viral proteins, the virus exits the cell to infect other cells, usually resulting in the death of the host cell (Garcia-Sastre and Biron, 2006).

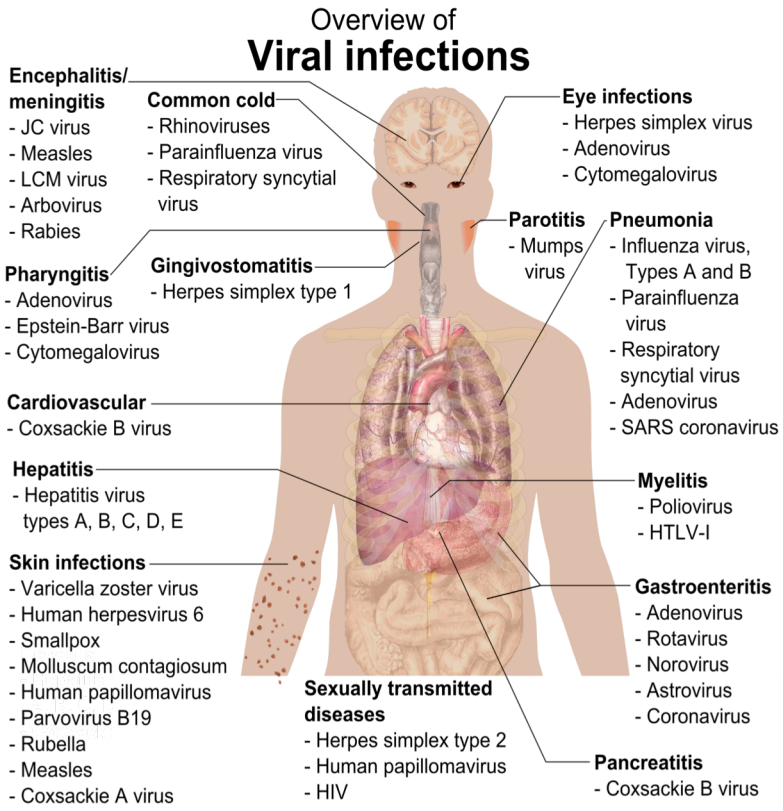


Fig. 1 Overview of viral infections in humans (Harvey et al., 2007)

Viruses are prevalent throughout the world and are responsible for countless infections and diseases (**FIG.1**). Over time, viruses have developed processes to evade the host cells' immune system, further increasing their pathogenicity. Understanding the

processes by which viruses are able to evade the immune system will provide insights into diagnosis, treatment, and elimination of viral diseases.

INFLUENZA VIRUS

Influenza, commonly known as the seasonal flu, is a virus that can infect many types of animals including birds, horses, pigs, and humans and is characterized by seasonal epidemics and pandemics (Schaechter et al 2013). In the United States, influenza ranks among the major public health threats, causing an annual average of about 20 million respiratory illnesses, 100,000 hospitalizations, and more than 20,000 deaths (Schaechter et al 2013). Influenza type A and B are responsible for most infections in humans. Influenza enters the host through inhaled airborne respiratory droplets that then infect the upper and lower respiratory tract. Symptoms of an influenza virus are fever, headache, and overall fatigue.

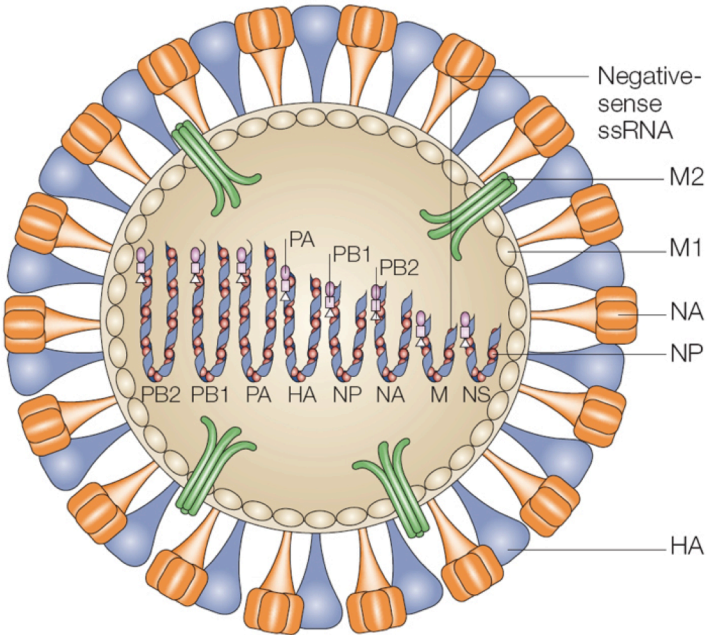


Fig. 2 Structure of the influenza virus (Clancy. 2008)

All influenza viruses contain eight negative sense, single stranded RNA segments surrounded by the matrix (M) protein (**Fig 2**). Each RNA segment is encapsulated by the viral nucleoprotein (NP) and the three virus-encoded polymerase proteins (PA, PB1, and

PB2) (Schaechter et al 2013). The matrix protein is surrounded by a host-derived lipid bilayer membrane with surface glycoproteins. The two glycoproteins, neuraminidase (NA) and hemagglutinin (HA), undergo antigenic drift or antigenic shift resulting, in changes in their composition. These changes allow the virus to evade the immune system and persist in the respiratory tract. Influenza infection of cells begins with the binding of HA to the sialic acid-containing glycolipid or glycoprotein viral receptors on the cell surface. Upon attachment, the cell then engulfs the virus through an endocytic vesicle (Schaechter et al 2013). The virus is then uncoated and its RNA segments are released into the cytoplasm. The RNA enters the nucleus where mRNA is replicated, resulting in the formation of new viral particles. The viral particles then exit the cell through lysis to further infect other cells. This present study employs influenza as a model virus to examine the immune system in these zebrafish.

ZEBRAFISH AS A MODEL ORGANISM

Due to ethical issues surrounding the use of biological testing on humans, animals have been employed to research infectious diseases in the human. *In vivo* animal testing is preferred over *in vitro* testing involving cell or tissue culture, as animals are complete biological systems and their use can provide insight into the complex cellular interactions, three-dimensional cellular geometry, and homeostatic regulation. Biomedical research depends on the use of animal models to study to the pathologies of human diseases at the cellular and molecular level (Lieschke and Currie 2007). Mammals, such as the mouse (*Mus musculus*), have been successful models for human disease as they have close genomic similarity as well as anatomy, cell biology and physiology. However, due to financial and physical restrictions on the use of mammalian

models, invertebrate models have been preferred for genetic studies (**Fig.3**). Despite their advantages, invertebrates lack organ systems similar to those that are involved in human disease pathogenesis, thereby limiting their use in viral infection studies. This is where the use of zebrafish (*Danio rerio*), has come into play.

Attribute of disease model	Model organism			
	Fly	*Zebrafish*	*Mouse*	*Rat*
Practical issues				
Husbandry infrastructure	$	$	$$$	$$$
Cost per animal per year	$	$	$$$	$$$
Characterized inbred strains	+	-	++++	+++
Outbred laboratory strains	+	+++	++	++
Anatomical similarity	-	+	++	++
Molecular or genetic similarity	+	++	+++	+++
Pathological similarity	-	++	+++	+++
Storage; for example, freezing sperm	No	Yes	Yes	Yes
Molecular biology tools				
Transgenesis*	++	++	++	++
Targeted gene modification*	+	-	++++	+
Transient *in vivo* assays*	++	++++	+	+
Allelic series from TILLING*	+++	++++	++	+
Feasibility of large-scale screens‡	++++	+++	++	+
Affordability of large-scale screens‡	++++	+++	+	-
Sequencing progress§	+++	++	+++	++
Annotation progress§	++	++	++++	++
Cell-biology tools				
Cell lines and tissue culture	++	+	++++	+
Antibody reagents	++	+	++++	++

*Reverse-genetics approach; ‡forward-genetics approach; §genome sequence; -, not relevant, or not a strength; $, $$, $$$ and +, ++, +++, relative cost ($) and strength (+) of the model in each category; ++++, outstanding strength of the model; TILLING, targeting induced local lesions in genomes.

Fig 3. While there are many advantages and disadvantages to employing various model organisms for human diseases, the zebrafish has been established as an excellent model organism for human diseases (Liescke and Currie, 2007)

Since 1930, zebrafish have been employed as an embryological and developmental model (Lieschke and Currie 2007). In the 1980s and 1990s, the development of genetic techniques such as cloning, mutagenesis, transgenesis, and mapping approaches established the zebrafish as a standard for developmental biology research. Zebrafish are vertebrate organisms that share striking anatomical and physiological homologies with higher organism counterparts, while maintaining the advantages of a lower organism (Goldsmith and Jobin 2012). Zebrafish have many characteristics that make them useful for studying human pathologies. They are highly

fecund with over 100 embryos per clutch, larvae that are functional for experiments 3 days after fertilization. Zebrafish are transparent up to 7 days after fertilization. They can also be genetically manipulated, contain a genome that is fully mapped, and possess organ and genetic homology to humans. Additional benefits include: external fertilization (allowing access to the various developmental stages), short generation time (3-4 months), egg size (0.7mm in diameter), and their short developmental period, i.e., all major organs are developed within 36 hours of fertilization (Spence et al. 2008) (**Fig 4**).

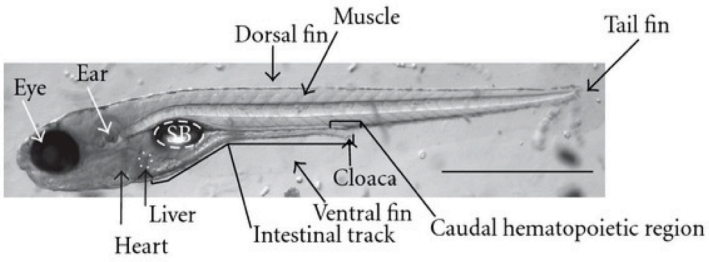


Fig 4. Image of the anatomy of the zebrafish taken using brightfield microscopy 6 days post fertilization. Scale bar is 1mm. Benefits of using zebrafish are its small size and transparency. (Goldsmith and Jobin, 2012)

Zebrafish possess an innate immune system comprised of neutrophils, NK cells, and monocyte/macrophage that are functional at 48 hours post fertilization (Goldsmith and Jobin 2012). The innate immune system is functional at 2 days post fertilization, while the adaptive immune system does not become fully functional until 4-6 weeks post fertilization. This time difference allows for studies that involve the innate immune system specifically. The adaptive immune system is highly analogous to that of mammals, containing T cells and B cells, as well as a nearly complete set of Toll-Like Receptors (TLRs) and associated innate signaling proteins, such as myeloid differentiation primary response 88 (MyD88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Goldsmith and Jobin, 2012). Studies using

zebrafish-human homolog genes of the immune system are made possible through the constant updating of an online database containing extensive genomic information (Goldsmith and Jobin, 2012).

One of the most important qualities of the zebrafish for research is its transparency. The optically clear embryos allow real time imaging of critical cellular processes such as phagocytosis and chemotaxis during an immune response. Using transgenic strains of zebrafish that express fluorescently labeled immune cells or pathogens, *in vivo* imaging of the interactions between the host and the pathogen is possible (Meijer and Spaink, 2011).

Although using zebrafish as a model for human pathologies has many advantages, there are limitations to its uses. Because the use of zebrafish is relatively new, there are fewer available strains compared to with the mouse model. Zebrafish also have numerous duplicate genes, which complicates forward-reverse genetic manipulation (Goldsmith and Jobin, 2012). The environmental conditions for maintaining zebrafish differ significantly from those of humans. Zebrafish also require 28°C water containing specific ion concentrations. These requirements present certain limitations to the use of zebrafish for providing a complete representation of human biological processes.

To date, zebrafish have played a critical role in biomedical research. Zebrafish have been shown to be susceptible to bacterial, protozoan, and viral infections, which is important for modeling human diseases (Goldsmith and Jobin, 2012). At the University of Maine, it has been shown that zebrafish can be infected with the gram negative bacterium, *Pseudomonas aeruginosa* in Dr. Carol Kim's Lab, the fungus *Candida albicans* in Dr.

Robert Wheeler's Lab, and also the virus, Influenza also in Dr. Carol Kim's Lab. These discoveries provide the foundation for further biomedical research on human pathologies.

IMMUNITY

As stated earlier, zebrafish exhibit an immune system that is similar to that of humans. Both branches of immunity, innate and adaptive, are present in the zebrafish, which allows it serve as a model for human disease. More importantly, the adaptive immune system is not developed until 4-6 weeks post fertilization, allowing studies that are specific to the innate immune system (Goldsmith and Jobin, 2012).

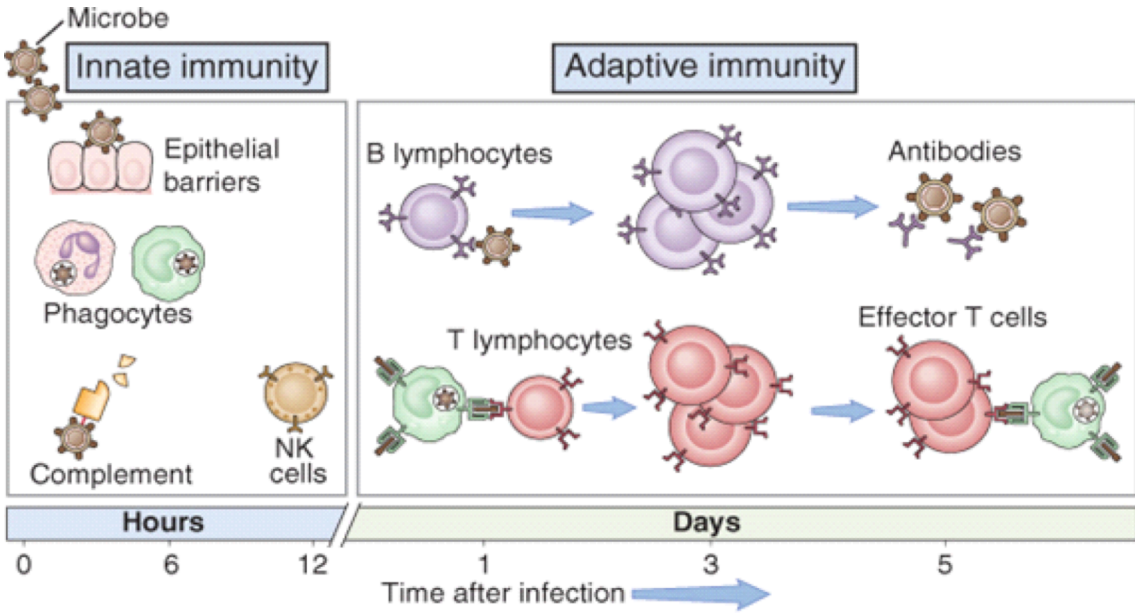


Fig 5. Host immunity is composed of two components: innate immunity and adaptive immunity. The innate immune response is first line of defense against infection, while adaptive immunity provides a highly specific response, but takes longer to activate (Townsend et al., 2008).

The immune system is divided into 2 broad components: innate immunity and adaptive immunity (**Fig 5**). In order for an organism to successfully recognize and eliminate a pathogen, the two components must function properly. In order to function efficiently, the host's immune system must fulfill three requirements: (1) recognize a wide, but

diverse array of pathogens, (2) rid the host of these pathogens once they are recognized by the immune system, and (3) differentiate between self and non-self (Beutler et al. 2004). The interactive network responsible for facilitating the immune response is comprised of lymphoid organs, cells, humoral factors, and cytokines. Improper functioning of these components can result in an underactive or overactive immune system, resulting in immunodeficiency, autoimmunity, or allergies (Parkin 2001).

The innate immune system is the host's first line of defense against infections. It reacts immediately to infection, with a comprehensive, broad range response that targets most pathogens. There are physical and chemical barriers, such as the skin and mucous membranes, and cellular components such as neutrophils, macrophages and natural killer (NK) cells. The interaction of these mechanisms in the body with the invading microbes often leads to a constellation of responses called inflammation (Schaechter et al. 2013). The innate immune response is present among all metazoan, plant, invertebrate, and vertebrate organisms. Vertebrate animals, such as humans and zebrafish, developed a second line of defense called adaptive immunity.

While the innate immune system employs genetically encoded receptors that detect and recognize foreign materials, the adaptive immune system uses a different subset of leukocytes called lymphocytes. This elite set of leukocytes known as B-cells and T-cells have the capacity to generate a large number of antigen-specific cell surface receptors by random rearrangement (Schaechter et al. 2013). Despite taking days or weeks to develop, the adaptive immune system is able to clear the pathogen as well as generate immunologic memory. Creating immunologic memory allows the host immune system to elicit a rapid and specialized response when encountering the same pathogen in the

future (Bonilla and Oettgen, 2009). Although the adaptive immune response is more effective at eliminating specific pathogens, its activation requires signaling from the innate immune system.

The immune system is able to discriminate between foreign pathogens and harmless microbes through the use of pattern recognition receptors (PRRs). One family of PRRs is called toll-like receptors (TLRs). TLRs recognize many essential microbial molecules, including essential cellular components such as lipopolysaccharide, lipoproteins, lipoteichoic, peptidoglycan, flagella, bacterial DNA, fungal cell walls, and viral RNA (Schaechter et al. 2013). For example, in the case of an influenza infection, TLR7 and TLR8 recognize single stranded viral RNA, activating viral inhibitory proteins, MyD88 and NF-κB, as well as interferons to activate macrophages and NK cells.

Of all the innate antimicrobial defenses in the body, the most potent is the cellular response which consists of neutrophils, eosinophils, and monocytes into infected tissues (Schaechter et al. 2013). Neutrophils are phagocytic cells that are recruited to the site of infection and are crucial for a proper immune response. In order for neutrophils to be effective, they must first migrate to the site of infection. Infected or damaged tissues and other immune cells elicit cytokines that attract neutrophils. Cytokines are small secreted proteins that play a crucial role in the interaction and communication between cells. Cell surface receptors on neutrophils allow for the detection of chemical gradients from cytokines guiding them to the site of infection via chemotaxis.

Once neutrophils have migrated from the blood stream into tissues they recognize foreign pathogens through surface receptors such as TLRs, C-type lectin receptors, and

nucleotide-binding oligomerization protein 1 (NOD1) (Mantovani et al. 2011). Early signaling events, such as the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) through the PI3K pathway induce polymerization of actin and remodeling of the localized membrane, which is essential for particle ingestion (Lee et al. 2003). After engulfing the pathogen, the neutrophil develops machinery that is necessary to kill it. To eradicate the pathogen, the neutrophil employs contents of intracellular granules. The largest of these, the azurophilic granules contain various antimicrobial substances to kill invading pathogens. Azurophil-type granules contain peptides such as α-defensin and phospholipase A_2, and enzymes such as lysozyme, elastase, and myeloperoxidase enzymes, which produce hypochlorous acid (HOCl) and Cl^- to kill the pathogen. Specific-type granules contain peptides such as cathelicidin and lactoferrin, and enzymes such as lysozyme and NADPH oxidase, which are used to eradicate the pathogen (Schaechter et al. 2013). Once the microbe is killed, the phagolysosomes accumulate in the cytoplasm and the neutrophil eventually lyses and the resulting debris are phagocytosed by macrophages.

Neutrophils play a critical role in the immune response. It has been demonstrated that neutrophils also act as an important bridge between the innate and adaptive immune response as they are important mediators of T helper cells and activators of B cells (Mantovani et al. 2011). Organisms that suffer from congenital or acquired defects in neutrophil life cycle and function can experience chronic infections and other life threatening conditions, demonstrating the importance of the neutrophil in the immune response.

PI3K SIGNALING PATHWAY

Neutrophils are vital to an effective immune response. Upon exposure to inflammatory signals neutrophils perform several specialized functions including chemotaxis, phagocytosis, and microbial killing (Moraes and Downey, 2003). In order to be successful in eradicating invading pathogens neutrophils rely on the phosphatidylinositide 3-kinase (PI3K) pathway. Phosphatidylinositide 3-kinases are a group of enzymes that convert plasma membrane phosphatidylinositol-4,5-biphosphate (PIP2), to phosphatidylinositol-3,4,5-trisphosphate (PIP3) upon exposure to signals in the form of growth factors, chemokines, and cytokines on the cell surface. PIP3 is the key messenger in the PI3K signaling pathway. Formation of PIP3 initiates numerous cellular functions such as phagocytosis, chemotaxis, microbial killing, and apoptosis through activation of numerous other pathways (**FIG 6**). If the PI3K pathway is overactive, resulting in the overproduction of PIP3, immune cells can produce a detrimental immune response which leads to excessive inflammation and damage of tissues. To control the level of PIP3, phosphatases PTEN and SHIP1 degrade PIP3 to its inactive form, PIP2. It is well established that in neutrophils, chemoattractant signaling via G protein-coupled receptors induces an increase in the PIP3/PIP2 ratio at the leading edge during chemotaxis *in vivo* (Yoo et al., 2010). Activation of PI3K results in rapid polarized F-actin polymerization, which is crucial for cellular motility (Barberis and Hirsch, 2008). Without the key PIP3 molecule, immune cells cannot migrate to the site of infection and perform specialized functions such as phagocytosis, microbial killing, and apoptosis (Moraes and Downey, 2003).

The PI3K family of enzymes is divided into 3 classes, I, II and III, according to their primary structure and substrate specificity (Moraes and Downey, 2003). Class II and III kinases are expressed in all cells, but their mechanisms of action are not well understood. Class I kinases are further divided into two subgroups, I_A and I_B. The catalytic subunit, p110 has four recognized isoforms: p110-α, β, δ, in class I_A and γ in class I_B. Class I_A p110 subunits are activated predominantly by receptor tyrosine kinases (RTK). P110α and β are ubiquitously expressed in tissues while p110δ and γ of class I_B are primarily found in leukocytes (Moraes and Downey, 2003).

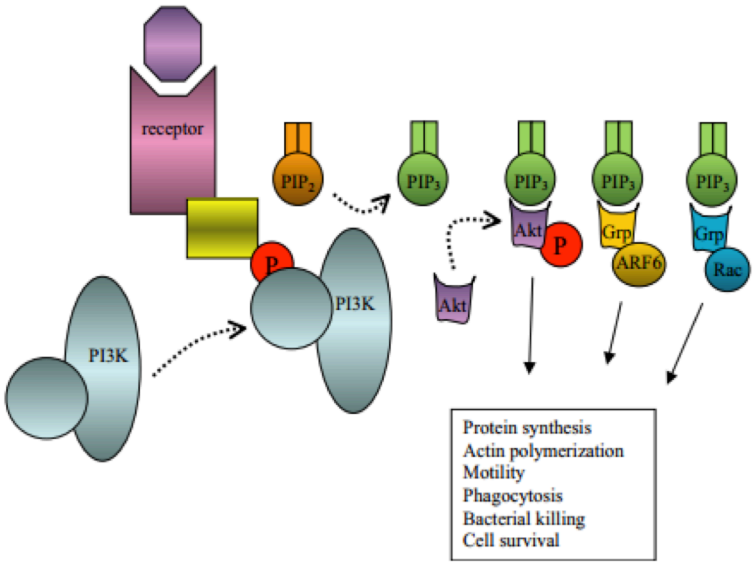


Fig 6. Overview of the PI3K signaling pathway. The key signaling molecule PIP3 which is produced via this pathway mediates various cell functions such as protein synthesis, cell motility, and phagocytosis (Moraes and Downey, 2003).

The PI3K pathway is initiated by the activation of the PI3-kinase enzymes. Through receptor tyrosine kinases (RTKs), integrins, B and T cell receptors, cytokine receptors, G-protein-coupled receptors and many others. Binding of extracellular growth factors to plasma membrane-bound RTK results in dimerization and autophosphorylation. Dimerization and autophosphorylization at the tyrosine residues allows the RTKs to interact with SH2 domain containing molecules, such as SHIP1 phosphatases (Castellano

and Downard, 2011). Lipid kinase PI3K is then recruited to the plasma membrane binding site and PI3K is activated. PI3K then converts membrane bound PIP2 to its active form, PIP3. PIP3 drives various downstream pathways such as Akt, Rac, and Arf6 to regulate numerous cellular functions such as cell migration, phagocytosis, cell survival and microbial killing. These cytosolic proteins have pleckstrin homology (PH) domains that bind to PIP3, leading to their activation (Moraes and Downey, 2003). Numerous studies have shown that in neutrophils, the PI3K pathway plays a central role in endothelial adhesion and transmigration into infected tissues, chemotaxis, phagocytosis, microbial killing, and apoptosis (Moraes and Downey, 2003).

AKT/PKB PATHWAY

The serine/threonine kinase, Akt/PKB, and its isoforms consist of a conserved domain structure: an amino terminal pleckstrin homology (PH) domain, a central kinase domain, and a carboxyl-terminal regulatory domain (Song et al. 2005). The PH domain of the protein kinases interacts with PIP3 that is produced by PI3-kinase. All Akt isoforms have two regulatory phosphorylation sites, Thr308 in the activation loop within the kinase domain and Ser473 in the C-terminal regulatory domain. Activation of Akt requires a PI3-kinase and a PH domain translocation, as well as phosphorylation of both sites (Song et al. 2005). While phosphorylation of the Thr308 site partially activates Akt/PKB, full activation requires phosphorylation of both sites. The PH domain is required for the recruitment of Akt/PKB to the plasma membrane through the high affinity binding to PIP3 (Song et al. 2005). PIP3 does not activate Akt/PKB, but instead recruits it to the plasma membrane to be phosphorylated by the phospho-inositide-dependent kinase-1 (PDK1). Once at the plasma membrane, Akt/PKB is phosphorylated at two specific sites,

Thr308 and Ser473. Thr308 is the main site for phosphorylation, which is accomplished by PKB. Less is known about the phosphorylation of Ser473 but there is evidence suggesting that it can be auto phosphorylated or phosphorylated by the integrin-linked kinase (Song et al. 2005). The phosphorylated Akt/PKB can then go on to activate transcription factors, proteins and other signaling pathways that control apoptosis, cell growth, and glucose metabolism.

RAC PATHWAY

Other PH domain-containing proteins that are activated by PIP3 include guanosine diphosphate (GDP)-GTP exchange factors for Rac proteins (Cantley et al. 2002). Rac proteins are a subfamily of the Rho family of monomeric GTPases and are responsible for multiple cellular functions such as cell adhesion, transcriptional and translational activation, protein synthesis, as well as formation of reactive oxygen species by NADPH oxidase complex in neutrophils which is crucial for microbial killing (Welch et al. 2003). Rac proteins are molecular switches that are inactive when GDP is bound and active when bound to GTP (**Fig 7**). Rac-GDP complexes are attached to the membrane through its C-terminal prenylation, exposing

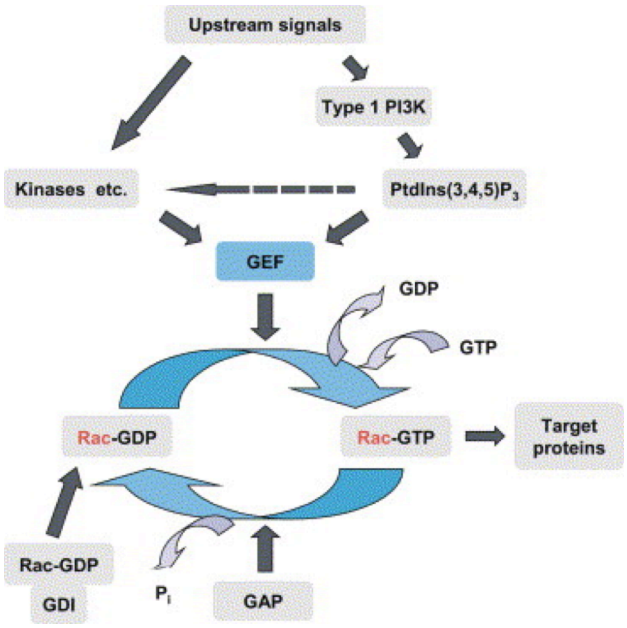


Fig 7. The Rac pathway is activated by PIP3 via their PH domain-containing proteins. Once activated, Rac GTPases are responsible for cell adhesion, protein synthesis and other cell functions (Cantley et al., 2002).

them to the activating enzyme, a guanine-nucleotide exchange factor, GEF (Welch et al. 2003). GEF is able to expose the nucleotide binding site of the GTPase, which facilitates the dissociation of GDP and binding of GTP, which is present at high concentrations in the cytosol of the cell. Rac activation can be accomplished in both PI3K-dependent and PI3K-independent pathways through the activation of GEF, but a large number of Rac-GEF families can be activated directly by PIP3. Once Rac proteins are activated, they can bind to their target proteins which are crucial to myriad functions in the cell.

ARF6 PATHWAY

The ADP-ribosylation factors (Arfs) belong to a family of Ras-related GTP-binding proteins. Much like Rac proteins, Arfs alternate between the inactive GDP-bound complex and the active, GTP-bound complex. Arf6 has been the subject of recent studies because of its effects on membrane trafficking and actin cytoskeleton at the plasma membrane (Donaldson, 2003). Arf6 activation is facilitated by guanine nucleotide exchange factors (GEFs), which allows the binding of GTP. While it is well understood that Arf6 plays a significant role in control of the actin cytoskeleton to affect the cell shape, it also is required for cell migration (**Fig 8**). Arf6 has been recently shown to be crucial in leukocyte chemokine-stimulated migration

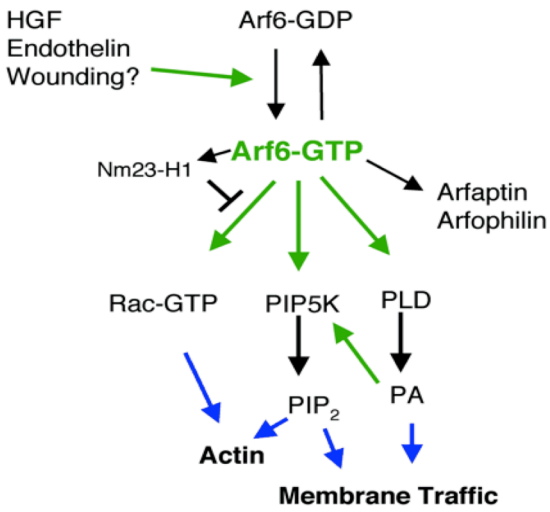


Fig 8. Arf6 are activated by PIP3 through their PH domain. Activation of Arf proteins results in the remodeling of cytoskeleton actin, changing cell shape (Donaldson, 2003).

across epithelial cells (Donaldson, 2003). Actin cytoskeleton restructuring and cellular migration are key components to an effective immune response.

SHIP1

The SHIP1 phosphatase enzyme serves as a negative regulator of the key signaling molecule, phosphatidylinositol (3,4,5) triphosophate. SHIP enzymes contain 1190 amino acids, have a molecular mass of 133kD, and contain several motifs that are important for protein-protein interactions (Rohrschneider et al. 2000). The central amino acid domain contains genetic information for the enzymatic activity of the SHIP enzyme, while the carboxy-terminal domain encodes two NPXY motifs and multiple PxxP motifs, and the SH2 domain is found at the N-terminus (**Fig 9**). Upon tyrosine phosphorylation of NPXY motifs, proteins with a phosphotyrosine binding (PTB) domain, like the tensin proteins, are able to interact with SHIP at these sites. Phosphorylation of the NPXY motifs also provides potential interaction sites for SH2 domain-containing proteins (Rohrschneider et al. 2000). Several PxxP motifs are present on the carboxyl terminus and can serve as binding sites for proteins containing SH3 domains. Together, these structural features comprise a unique and important protein that has enzymatic and signaling properties. SHIP is expressed in almost all cells of the bone marrow and blood cells express at least one form of the SHIP protein (Rohrschneider et al. 2000). *SHIP* mRNA has been found at the earliest stages of hematopoietic cell development in mouse

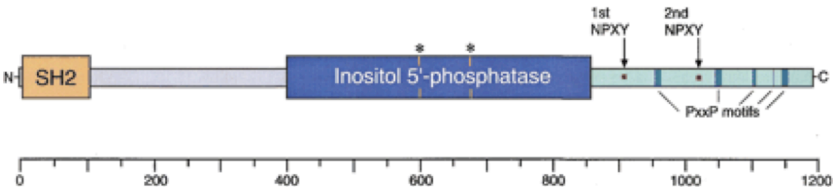


Fig 9. This is the SHIP1 gene containing SH2 domain, the two NPXY domains, and the multiple PxxP binding domains (Rohrschneider et al. 2000).

embryos and protein expression has been detected in all blood cell lineages including macrophages and granulocytes. SHIP homologues, SHIP1 and SHIP2 are expressed in all mammals, with SHIP1 expression limited to the hematopoietic lineage and while SHIP2 is expressed broadly (Lam et al 2012). Zebrafish express one copy of SHIP1 and two copies of SHIP2: SHIP2a and SHIP2b. The central enzymatic domain of SHIP proteins has been identified by its similarity to inositol phosphates. This domain is highly conserved (96% identical) between murine and human SHIP proteins. SHIP enzymes remove the phosphate group from the 5' position on both phosphatidylinositol 3,4,5 – triphosphate [PI(3,4,5)P3] and 1,3,4,5-tetrakisphosphates (IP_4) (Rohrschneider et al. 2000). The 3' position of the inositol phospholipid must be phosphorylated before SHIP1 can act on it, suggesting that SHIP acts sequentially with phosphatidylinositol 3 kinases (PI3K). PI3K, which contains a 85-kD regulatory subunit and a 110-kD catalytic subunit, is responsible for PIP3 production. The p85 subunit of PI3K contains two SH2 domains, which are recognized by the p85 SH-2 domain in SHIP proteins. This p85/PI3K association with SHIP could represent an interesting complex of enzymes that convert PI(4,5)P2 to the key signaling molecule, PI(3,4,5)P3, then finally to PI(3,4)P2. The PI(3,4)P2/ PI(3,4,5)P3

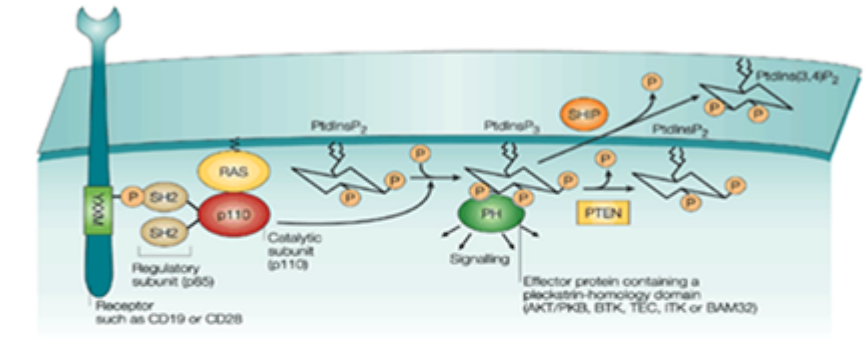


Fig 10. This image shows SHIP1 within the PI3K pathway. SHIP phosphatases control the levels of the key signaling molecule, PIP3 within the cell by converting it to PIP2. SHIP1 in zebrafish is a negative regulator of neutrophil motility (Okkenhaug and Vanhaesebroeck, 2003).

accumulation at the leading edge of neutrophils *in vitro* inspired research into the role of SHIP phosphatases during neutrophil motility *in vivo*. This work showed that in zebrafish, both SHIP1 and SHIP2 promptly localize to the leading edge and occasionally the tail of migrating neutrophils. Deletion of SHIP1 increased neutrophil migration while overexpression inhibited neutrophil motility (Lam et al. 2012). Since PI3K activity and leading edge PI(3,4,5)P3 is required for neutrophil motility *in vivo* (Yoo et al. 2010), it is suggested that SHIP1 hydrolyzes the key signaling molecule PI(3,4,5)P3 into PI(3,4)P2 in neutrophils, thereby limiting their motility. It has also been suggested that SHIP1 acts as a key brake in neutrophil migration to prevent a damaging, over active immune response via a PI3K signaling pathway.

MORPHOLINO OLIGONUCEOTIDES (MO)

Gene manipulation has long been a tool used in genetic research and developmental biology. Forward genetics focuses on the study of a specific phenotype and determination of the genetic basis for that phenotype (Lieschke and Currie, 2007). Reverse genetics allows for the study of the biological function of a gene by altering the expression of that gene during development and observing the effects.

The most widely used reverse genetics antisense technique in zebrafish involves morpholino oligonucleotides (MO), which are employed for gene knockdown. MOs are synthetic oligonucleotides composed of 25 morpholine bases [$O(CH_2CH_2)_2NH$] bound by a neutrally charged phosphorodiamidate backbone (Bill et al, 2009). Although similar in structure to DNA and RNA oligomers, MO oligomers are neutrally charged; making them more stable in the cellular environment and the altered backbone allows them to resist degradation by circulating endogenous endonucleases. Most importantly, the

morpholine bases can still participate in Watson-Crick base pairing allowing MOs to bind to specific cellular RNA sequences (Ekker and Akimenko, 2010). Developed by Dr. James Summerton, MOs were first designed to inhibit translation of mRNA *in vivo*.

There are two types of MOs: splice blocking and translational blocking morpholinos (**Fig 11**). Splice blocking MOs inhibit the function of the spliceosome, a protein complex that removes introns from pre-mRNA (Bill et al, 2009). Translational blocking MOs bind to complementary mRNA sequences within the 5' untranslated region (UTR) which inhibits ribosome assembly, hence obstructing translation. Procedures such as reverse transcription polymerase chain reaction (RT-PCR) can quantify the efficacy of a splice blocking MO, whereas a western blot assay can measure the efficacy of translation

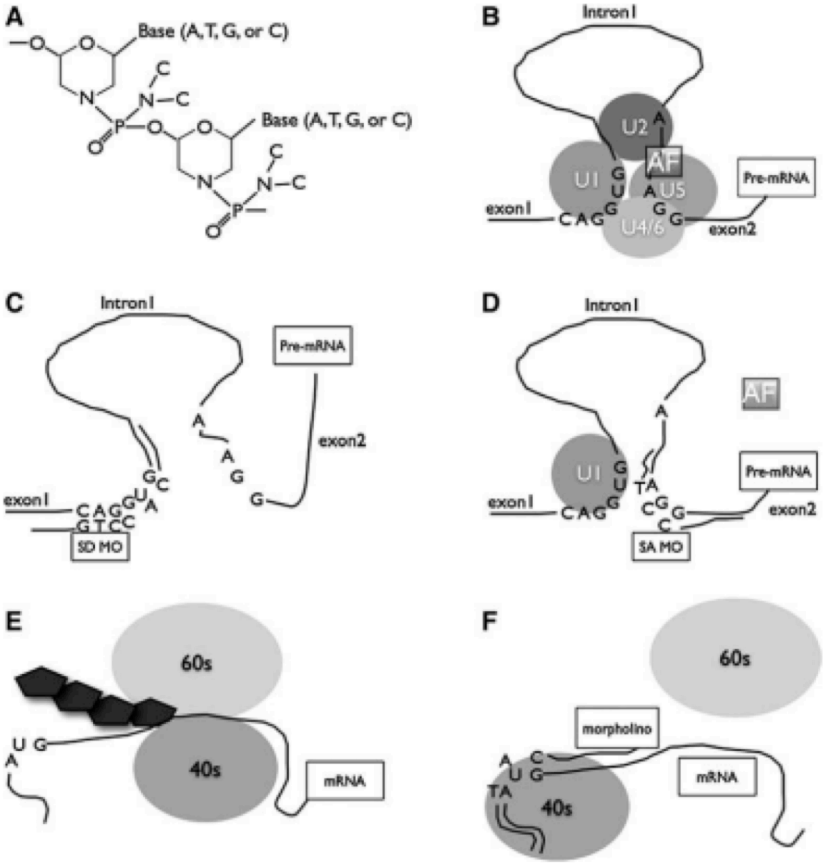


Fig 11. Gene knockdown technique employing MOs. (A) Structure of MOs, which is similar to DNA and RNA oligomers. (B,C,D) Splice blocking MO technique and (E,F) shows translational blocking MO mechanism (Bill et al., 2009).

blocking MOs. Typically, MOs are introduced into zebrafish embryos at the 1-8-cell-stage, allowing rapid, comprehensive delivery through the early embryonic cells (Bill et al, 2009).

Although the use of MOs is beneficial for genetic research, it is a relatively new practice and certain constraints should be recognized. First, in smaller embryos the repeated injection of specific volumes of MOs can be a source of error. Inconsistent MO administration among embryos can result in unreliable gene expression (Eisen and Smith, 2008). Second, MO injection can affect off-target genes. The MO can interfere with production of irrelevant gene products, thereby introducing the possibility that the newly observed phenotype is not the result of the targeted gene knockdown. The last concern is the administration of the control. The most reliable control is to attempt to "rescue" the phenotype by introducing the gene product of interest in a form that is unaffected by the MO (Eisen and Smith, 2008). An effective rescue technique is to inject mRNA at the 1-cell stage. In the case of a translational blocking MO, removing the 5' UTR of the mRNA or introducing silent mutations into the coding region should rescue the phenotype (Eisen and Smith, 2008). With a splice-blocking MO, injecting mRNA can also have the ability to rescue the phenotype (Eisen and Smith, 2008). The use of MO knockdowns and other genetic manipulation techniques have allowed research into gene identification, gene function, and the verification of specific mutant phenotypes (Bill et al., 2009).

MATERIALS AND METHODS

Zebrafish Maintenance, Antisense Morpholino Injection, Viral Injection, and Mortality:

Zebrafish Maintenance. Zebrafish embryos were held in 75 mL of egg water (60 mg/L of Instant Ocean, Spectrum Brands, Madison, WI) at 28°C. Once the embryos were injected with virus, zebrafish larvae were held at 33°C in the same egg water solution.

Antisense Morpholino Injection. Zebrafish embryos were injected with an antisense morpholino oligonucleotide at the 1 cell stage (0.5 to 1 hours post fertilization). One *SHIP1* splice-blocking morpholino (*SHIP1 MO*) was injected at 250 μM per embryo in a 3 nL injection. The control MO was injected at the same concentration and volume. The *SHIP1 MO* was designed using Gene Tools, LLC (**Table 1**).

Morpholino	**Sequence (5' – 3')**
SHIP1 MO	ATG ACT TAA GAC ATC TCA CCC ATG T
Control	CCT CTT ACC TCA GTT ACA ATT ATA A

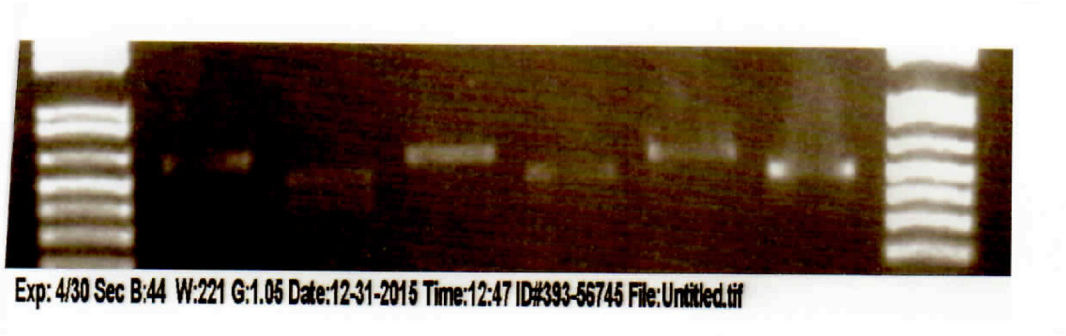
Table 1 The *SHIP1* morpholino sequence, designed by Gene Tools (Philomath, OR), was injected at the 1 cell stage.

Mopholino efficiency was tested using RT-PCR. After MO was injected, 6 fish were isolated at 1, 2, and 3 days post fertilization (dpf) for each treatment (control and SHIP1-KD). After injection of MO, the expression of SHIP1 was expected to be greatly reduced, or knock down (SHIP1-KD). RNA was extracted using the Trizol protocol (Ambion) and

cDNA was then synthesized using the iScript protocol. For PCR, the PCR MasterMix was used with the 2x kit protocol (Promega #9P1M750) with the following primers:

Primer	Sequence (5'-3')
SHIP1 Forward	GTC TCC TGG AGC TGG AAG ACT AAG
SHIP1 Reverse	GTC ATG TGG GAT TTG AGG GGC TGT G

The products were then analyzed on a 1% agarose gel. 10µL of PCR product was then loaded with 2µL of 6x Orange G dye.



[Ladder][LANE 1][LANE 2][LANE 3][Ladder]

Fig 12 Agarose gel results demonstrate that the SHIP1 MO at the concentration that was used efficiently knocked SHIP1 down through 3dpf. Shifted bands represent an exon skip caused by the MO interaction. Gel setup: 1dpf – 2dpf – 3dpf with ladder on each end.

Viral Injection. For survival studies: at 48 hpf, embryos were dechorionated with forceps and anesthetized in tricaine. Influenza A/PR/8/34 (H1N1, EID50 $10^{9.6}$) virus (Charles River, North Franklin, CT) was injected into the tail vein to elicit a systemic infection in both SHIP1 morphant and control embryos. Two, 2 nL injections were administered per

embryo with 12,720 Embryo Infective Dose, EID50 per fish. This is amount of virus particles required to infect 50% of the zebrafish. The virus injection contained 8 µL of virus stock, 0.5 µL of 5% phenol red, and 1.5 µL Hanks Balanced Salt Solution (HBSS). The control injection contained 9.5 µL HBSS and 0.5 µL of 5% phenol red solution.

For qPCR (Injection performed by Denise Jurczyszak of the Kim Lab): Charles River Stock EID50 $10^{10.3}$/mL, an influenza virus stock was used in this experiment. 2 nL were injected into the tail vein of the zebrafish (12,500 EID50/embryo) to elicit a systemic immune response.

Mortality. Embryos injected with control or IAV were kept in 50 mL of egg water throughout the experiment. At 8 hours post infection (hpi), deceased embryos were removed and surviving embryos were counted. This represented the count at Day 0. At this stage, all deceased embryos were attributed to incidental trauma from injection rather than from IAV. In 24 hour intervals, surviving embryos were counted through 5 days and egg water was changed upon each counting.

RNA Extraction, cDNA Synthesis, qPCR, and Respiratory Burst Assay:

RNA Extraction. Embryos from SHIP1-morphant and control zebrafish were collected at 12, 24, 48, 72, 96, and 120 hpi. Five zebrafish were used per replicate and three replicates were done for each treatment at each time point. RNA was extracted using the Trizol protocol (Ambion). To quantify the RNA extracted, a NanoDrop spectrophotometer was used.

cDNA Synthesis. cDNA was synthesized from the extracted RNA from each treatment and time points, using the iScript cDNA synthesis kit (BioRad, Hercules, CA). The samples were the diluted to 50 ng/µL with nuclease free water.

qPCR. For a total of 10 uL per qPCR reaction, each reaction contained 5uL of SoFast EvaGreen mastermix (BioRad, Hercules, CA) or Quanta Green FastMix (Quanta, Gaithersburg, MD) as the fluorescent dye, 0.2 µL of forward/reverse target gene primer (0.2 µM final concentration) and 2uL of cDNA (10 ng/µL final concentration). 2.8 µL of nuclease free water was also added to bring the volume up to 10 µL. Quantification cycle (Cq) values were calculated using a CFX96 real-time detection system. Analysis of these Cq values was then completed in GraphPad Prism 6.

Primer	Sequence (5'-3')
SHIP1 Forward	GGC ACT TGG AAC ATG GGA AA
SHIP1 Reverse	CCT CCT GTG TCC CGA TTA CG
18s Forward	TCG CTA GTT GGC ATC GTT TAT G
18s Reverse	CGG AGG TTC GAA GAC GAT CA

Table 2 The SHIP1 forward/reverse primers were added during qPCR measurements. The 18s forward and reverse primers were used as the control. Primers were designed by Gene Tools (Philomath, OR). Primers are designed to bind to the SHIP1 DNA sequence during the annealing phase, resulting in amplification of the SHIP1 gene to determine its relative expression.

Respiratory Burst Assay (RBA). Zebrafish embryos were injected with MO as previously described. The RBA was the performed at 3 dpf as described in (Goody et al., 2013) - with the exception that the plate was not shaken immediately after adding phorbol myristate acetate (PMA), instead it was placed directly into a 28°C incubator. The PMA was added along with the fluorescent molecule, H2DCFDA, to the zebrafish. The PMA

involves the activation of protein kinase C (PKC) which induces a respiratory burst of phagocytes. Upon respiratory burst, the reactive oxygen species (ROS) that are produced inside the phagocyte react with the fluorescent molecule H2DCFDA. This reaction oxidizes H2DCFDA, causing it to fluoresce. The intensity of the fluorescence correlates to the amount of ROS produced within the phagocyte.

RESULTS

SHIP1 is upregulated upon infection by influenza A virus. To test the expression of SHIP1 in zebrafish during a viral infection, both control and SHIP1-KD zebrafish were injected with either influenza A virus or HBSS control. At various time points, RNA was extracted and then synthesized into cDNA. Real time qPCR was used to measure the levels of SHIP1. Upon infection, SHIP1 is upregulated within the first 24 hpi until 120hpi. Statistical significance was found at 24, 72, 96, and 120hpi (**Fig 13**). SHIP1 is a negative regulator of neutrophil activity, thereby decreasing the immune response. qPCR data show that SHIP1 is upregulated during the time frame during which the immune response would be strongest, suggesting that the influenza virus is able to manipulate SHIP1 expression, allowing the virus to evade the immune response.

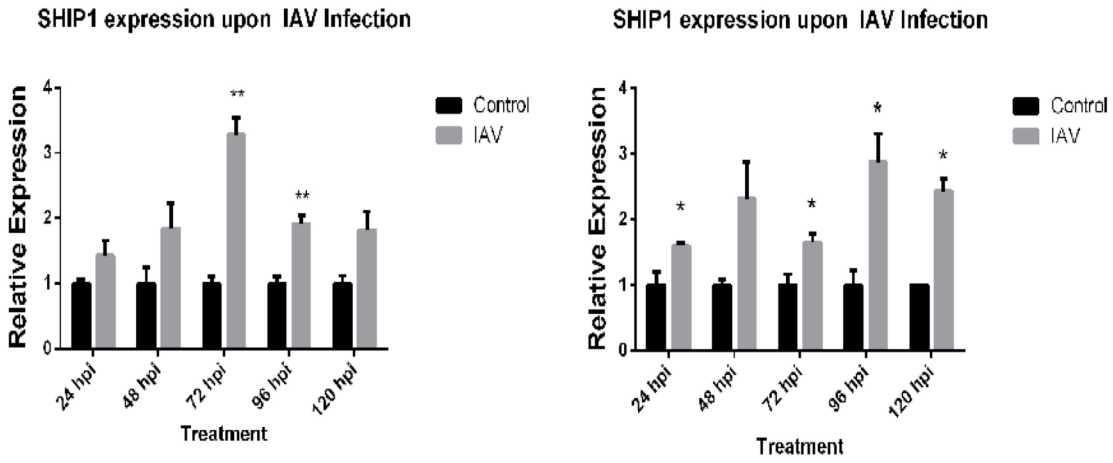


Fig 13. Relative expression profiles of SHIP1 in zebrafish after IAV infection. At various time points it can be seen that SHIP1 is upregulated upon IAV infection. Both graphs represent separate experiments and with one injection performed by Campbell Miller and the other performed by Denise Jurcyzsak, both of the Kim Lab.

SHIP1 plays a critital role in zebrafish survival during an IAV infection. To determine whether SHIP1 has a role in the innate immune response to a viral infection, H1N1 IAV was injected into 48 hpf, SHIP1-KD zebrafish. Every 24 hours, the number of surviving zebrafish was determined and the precent survival was calculated over the span of 5 days. At this early stage in development, only the innate immune system is present in zebrafish larvae and is responsible for clearing the viral infection. As anticipated, IAV infection in control zebrafish resulted in decreased percent survival. In SHIP1-KD zebrafish, however, IAV infection resulted in greater percent survival, suggesting that SHIP1 plays a role in the zebrafish innate immune response (**Fig 14**).

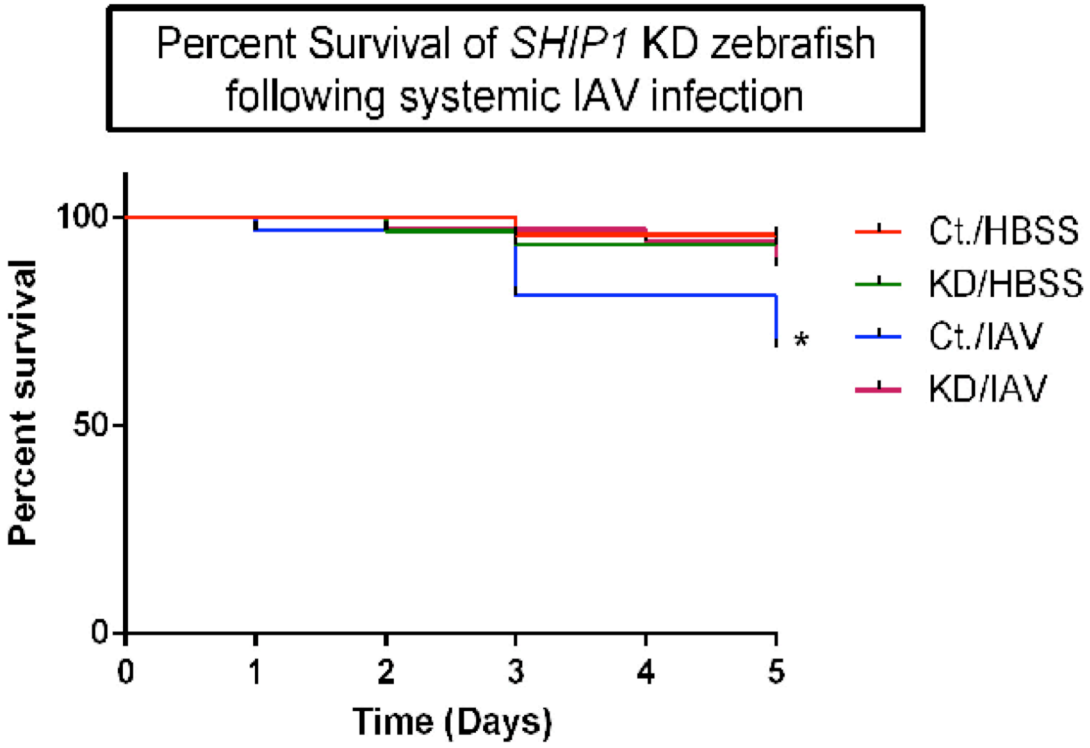


Fig 14. Percent survival curves of control and SHIP1-KD zebrafish that were injected with either HBSS control or IAV. The graph demonstrates that compared to control zebrafish infected with IAV, SHIP1-KD zebrafish infected with IAV has a greater percent survival that is statistically significant. Ct indicates zebrafish injected with control MO, while KD indicates zebrafish injected with SHIP1 MO. These fish were then infected with IAV or HBSS.

SHIP1 plays a role in reactive oxygen species (ROS) production. Having established that SHIP1 is upregulated during an IAV infection and plays a role in the zebrafish antiviral immune response, it was of interest to explore its function in phagocyte activity. A respiratory burst assay was performed to determine the role of SHIP1 in ROS production in phagocytic cells. SHIP1-KD and control zebrafish at 3dpf, were loaded onto microplates and PMA was added to induce a respiratory burst. It was determined that SHIP1-KD zebrafish produced fewer relative fluorescence units (RFU) compared to control fish, indicating fewer ROS species were produced (**Fig 15**). This data demonstrates that SHIP1 plays a critical role in ROS production.

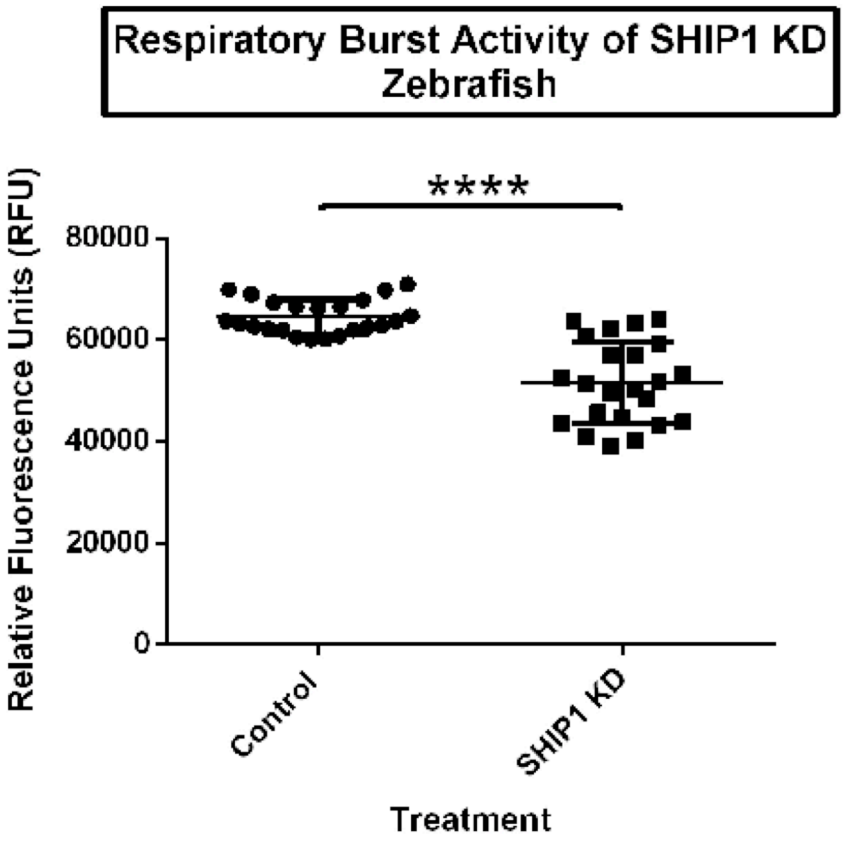


Fig 15. Respiratory burst assay (RBA) results of control and SHIP1-KD zebrafish. Results of the RBA demonstrate fewer RFU in SHIP-KD zebrafish compared to controls, indicating that SHIP1 plays a role in ROS production and microbial killing.

DISCUSSION

Viruses are common throughout the world and cause numerous diseases. We are interested in studying the innate immune system as it pertains to specific viral infections. Using zebrafish as model organisms, these studies provide insight into a variety of pathways and mechanisms that contribute to viral pathology. Understanding these mechanisms can contribute to the development of treatments for viral infections among humans. The gene *SHIP1* has yet to be characterized specifically during a viral infection, yet its role in the innate immune system during a wound response has been researched widely. In the current study, the role of SHIP1 during an innate immune response to an influenza virus infection was investigated *in vivo* using zebrafish.

Previous studies revealed that SHIP1 is upregulated during an influenza infection in zebrafish (Campbell Miller, unpublished results). SHIP1 enzymes act as negative regulators of neutrophil motility, possibly to prevent damage from an overactive immune response. More specifically, SHIP1 phosphatases degrade the key signaling molecule phosphatidylinositol (3,4,5) triphosphate (PIP3) at the plasma membrane to phosphatidylinositol (3,4) diphosphate (PIP2) in the phosphoinositide 3-kinase (PI3K) pathway. Neutrophils rely on the PI3K signaling pathway to induce chemotaxis, phagocytosis of pathogens, and microbial killing. These data suggest that the virus causes upregulation of SHIP1, allowing it to evade the host innate immune response. SHIP1 expression during a viral infection was established and so further research was performed to identify other effects SHIP1 might have during the antiviral innate immune response.

First, the role of SHIP1 in overall zebrafish survival was examined during an influenza infection. SHIP1 MO was administered to zebrafish embryos at the one cell

stage in order to knock down SHIP1 expression. The SHIP1-KD zebrafish and control zebrafish were then injected in the tail vein with HBSS control solution or IAV 48hpf to result in a systemic infection. As expected, the control zebrafish infected with IAV had a decreased percent survival (**Fig 14**). However, SHIP1-KD zebrafish had an increased percent survival, similar to that of control zebrafish injected with HBSS control solution. These data support the notion that the expression of SHIP1 during a viral infection is possibly mediated by the virus rather than the host to evade the immune response and cause further damage. Knock down of SHIP1 led to increased survival indicating that increased SHIP1 expression during a viral infection, which was established by previous qPCR data (**Fig 13**), leads to decreased survival among IAV infected zebrafish.

A respiratory burst assay (RBA) was then performed to determine the effect of SHIP1 on production of reactive oxygen species (ROS). ROS are chemically reactive molecules which are present in phagocytes and are responsible for microbial killing. RBA results demonstrated that SHIP1-KD zebrafish exhibited less fluorescence (relative fluorescent units - RFU) compared to control zebrafish. These data indicate that SHIP1-KD zebrafish produced lower levels of ROS compared to control zebrafish. These results suggest that SHIP1 plays a role in microbial killing through the production of ROS. Although upregulation of SHIP1results in higher levels of ROS, and subsequently more pathogen killing, this could have a damaging effect as well. Elevated levels of ROS, and their release upon phagocyte apoptosis, can damage host epithelial cells. During a viral infection, the upregulation of SHIP1 causes an increase in the production of ROS which, could further damage the host and be, in part, responsible for the decreased percentages survival values (**Fig 14**).

The data presented are consistent with the hypothesis that SHIP1 plays a critical role in the innate immune system during an influenza infection. Although SHIP1 serves as a negative regulator of neutrophil motility to prevent an over active immune response, aberrant and increased expression of SHIP1 during an influenza infection, can lead to increased host damage, decreased survival, and decreased ROS production., Further research is needed to fully characterize the role of SHIP1in the innate immune response as this could lead to the development of antiviral treatments, especially those directed toward influenza infections.

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AUTHOR'S BIOGRAPHY

Spencer E. Traxler was born on April 27th, 1994 in Baltimore, Maryland. In 2000, his family moved to Newburyport, Massachusetts where he grew up and graduated from Newburyport High School in 2012. In May 2016, Spencer graduated magna cum laude with High Honors from the University of Maine with a Bachelor of Science degree in Bioengineering. He was accepted into the New England College of Optometry in Boston, MA to pursue a career as an optometrist.

Outside of the classroom, he was a member of Engineers Without Borders, where he devoted time designing and fundraising for a water delivery project for La Y de la Laguna, Ecuador. In August, 2014, Spencer was fortunate enough to travel to the community as a translator. This trip inspired him to help others and to further pursue a career in the healthcare field. In his free time, Spencer enjoys spending time with close friends and family members as well as running, skiing, hiking and playing soccer. Upon graduation from the New England College of Optometry, Spencer plans to remain in New England and eventually open his own optometry practice.