Spring 5-2016

The Role of Neutrophil Cytosolic Factor 1 in the Innate Immune Response to Influenza A Virus

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THE ROLE OF NEUTROPHIL CYTOSOLIC FACTOR 1 IN THE INNATE IMMUNE RESPONSE TO INFLUENZA A VIRUS

by

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A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biology)

The Honors College
University of Maine
May 2016

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Abstract

Influenza A Virus (IAV) causes over 21,000 deaths annually in the United States alone. The innate immune response to IAV includes the production of reactive oxygen species (ROS) via NADPH oxidase. ROS are known to impact signaling pathways and cellular processes in order to eliminate IAV, but can cause permanent damage to lung epithelial cells in the process. One gene involved in the production of ROS is Neutrophil Cytosolic Factor 1 (*ncf1*), which codes for a subunit of NADPH oxidase. Mutations in *ncf1* have been correlated with chronic granulomatous disease, chronic inflammation, and autoimmunity. Studying *ncf1* in response to IAV infection could potentially lead to the discovery of novel therapies for viral disease.

Acknowledgements

I wish to thank my advisor, Dr. Carol Kim, and Jacob Longfellow for their guidance and support throughout this process. I would also like to thank the rest of the Kim lab members and my committee members: Dr. Paul Millard, Edith Pratt Elwood, Dr. Con Sullivan, and Dr. Len Kass.

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Introduction

Innate Immune System

The innate immune system is the host's first line of defense and is responsible for recognizing foreign particles. It is comprised of physical barriers, such as skin, and chemical barriers, such as the low pH of sweat (Wilson et al., 2011). These barriers prevent pathogens from infecting the host's tissues. If the barriers are breached, the innate immune system will respond to the threat using proinflammatory cytokines and chemokines. Inflammation allows migration of leukocytes from the blood vessels into the damaged or infected tissue (Owens et al., 2013).

The majority of the cells released into the tissue in response to infection are phagocytic cells, like neutrophils and macrophages. These cells can engulf a pathogen, destroy it, then either present the fragments to other leukocytes or release the fragments (**Fig 1**). Both macrophages and neutrophils will produce a respiratory burst, which generates reactive oxygen species (ROS). The magnitude of this respiratory burst is indicative of the innate immune health of an organism, since the production of ROS is a necessity in killing phagocytized pathogens. Once the body is clear of foreign cells, cytokines will be released to trigger apoptosis of leukocytes and halt the inflammatory

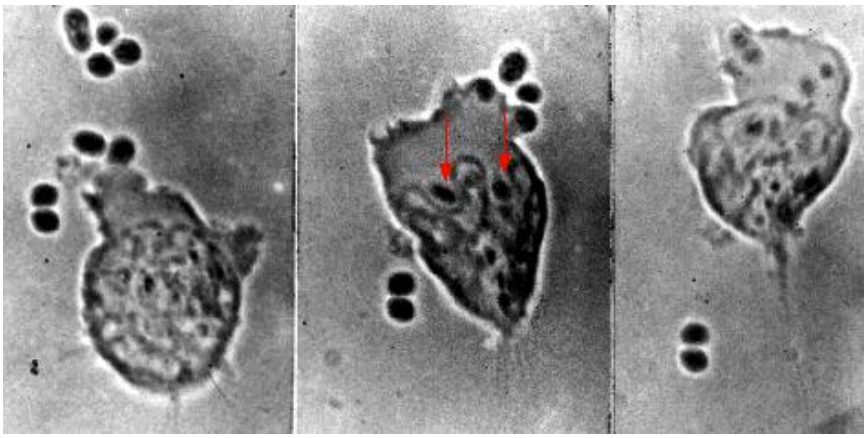

Fig 1 A neutrophil engulfing bacteria. The center panel shows two bacteria (red arrows) fully engulfed by the neutrophil. (Wood et al., 1946)

response (Owens et al., 2013). Studying the cells of the innate immune system can lead to discovery of the roles of specific cells and proteins in response to viral infection.

Viruses

Viruses are obligate intracellular parasites that typically enter host cells through cell-surface receptors that are recognized by viral surface proteins. Once the virus has entered the cell, it will shed its protein coat and utilize the host's machinery, to some degree, to replicate its genome. This replication step is prone to error, generating mutations in the genome of the new viral particles. Fully assembled viral progeny exit the cell and are able to infect surrounding host cells and continue the infection cycle. Viruses often kill the host cell during this final stage, damaging the tissue and causing some of the common viral symptoms (Owens et al., 2013). Viruses can cause a plethora of infections and diseases in humans and have evolved numerous ways to evade the human immune system (**Fig. 2**). One of the common viral infections in humans is the seasonal flu, caused by Influenza viruses.

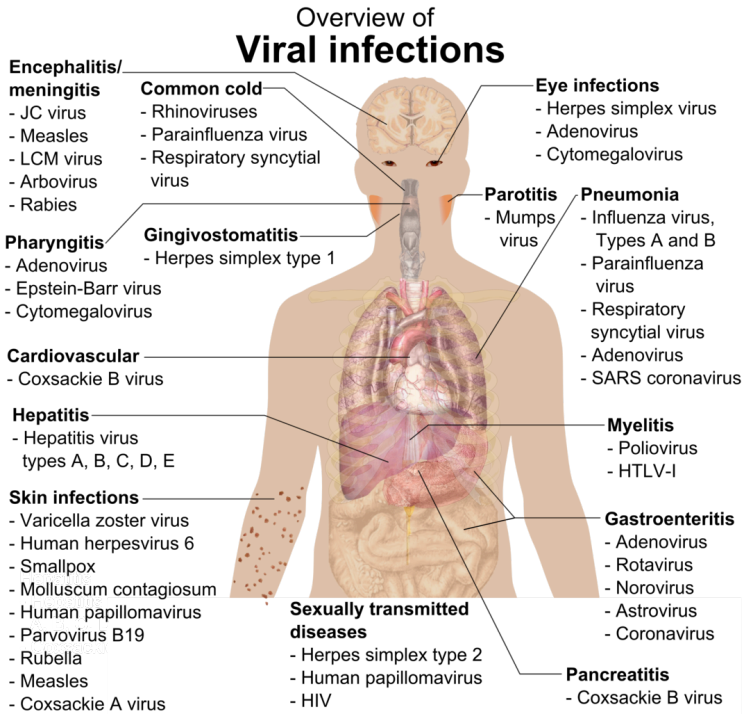

Fig. 2 Overview of viral infections in humans (Harvey et al., 2006)

Influenza A Virus

Influenza A virus (IAV) is responsible for 21,000 deaths and over 170,000 hospitalizations annually in the United States alone and has been known to give rise to pandemic outbreaks, primarily due to a genome that can mutate rapidly (Owens et al., 2013). IAV infections cause different host responses, depending on the age and overall health of the host. Disease symptoms can range from asymptomatic to a severe form of febrile respiratory disease, depending on the age and overall health of the host. Enhanced disease severity and high mortality is common in infants, the elderly, and in immunocompromised individuals (Pulendran and Maddur, 2014).

Influenza is a negative sense single-stranded RNA virus that belongs to the *Orthomyxoviridae* family. It infects the respiratory tract, specifically alveolar epithelial cells, of humans and several other animal species, sometimes causing permanent damage to alveolar tissue (Pulendran and Maddur, 2014). Two types of surface glycoproteins are found on the viral surface: hemagglutinin (HA) and neuraminidase (NA). HA trimers aid in the attachment of the virus to the host cell by binding to sialic acid groups on the surface of the host cell's plasma membrane. NA is an enzyme that cleaves sialic acid from naïve viral glycoproteins to aid in viral budding from an infected host cell. New strains of Influenza are named based on different antigenic subtypes of HA and NA (Owens et al., 2013).

IAV has been a focal point of biomedical research due to the potential of its rapidly mutating antigens to result in pandemic outbreaks (Trumpey et al. 2005). There are two mechanisms behind antigenic variation in HA and NA. The first, antigenic drift, occurs through a series of spontaneous point mutations that result in minor changes to

HA and NA over time. The other mechanism, antigenic shift, results in a new subtype of influenza with markedly different HA and NA structures (Owens et al., 2013). Due to antigenic shift and antigenic drift, changes in IAV strains are random and hard to predict. A more complete understanding of the host's immune response to IAV may result in new treatments and therapies.

Innate Immune System in Response to Influenza

The human body has several mechanisms for resisting or eliminating IAV, the first being the mucosal layers found in the nasopharyngeal and respiratory tracts. The mucosal membrane can effectively trap the virus and expel it through sneezing, coughing, or swallowing of mucus (Owens et al., 2013). If IAV breaches this barrier, innate immune cells resist the infection of respiratory alveolar cells. These cells generate proinflammatory cytokines and chemokines that promote recruitment of other innate immune cells.

One of the first innate immune cells to arrive at the site of infection are neutrophils. Multiple studies have shown that neutrophils are recruited to the upper and lower respiratory tract during IAV infection and play a critical role in limiting virus replication (Pulendran and Maddur, 2014). Lethal dose infection of IAV in mice depleted of neutrophils produced increased virus titers in the lungs with increased mortality when compared to lethal dose infections in wild-type mice (Tate et al., 2008, 2011). Neutropenia in mice infected with influenza also led to exacerbated pulmonary inflammation, edema, and respiratory dysfunction. Additionally, depletion of neutrophils can allow a mild influenza infection to progress to a severe clinical disease state (Tate et al., 2009). The mechanisms behind the role of neutrophils in the innate immune response

to IAV are largely unknown. One emerging target of innate immune research has been the production of reactive oxygen species (ROS) by neutrophils in response to infection. The goal of this study is to determine the role one protein that is partially responsible for the production of ROS in response to IAV.

Reactive Oxygen Species

ROS are generated by the NADPH oxidase (NOX) protein complex in order to destroy phagocytized pathogens (Owens et al., 2013). The source of ROS in the lung has been an intensive area of study, revealing NADPH oxidase 2 (NOX2) as the NOX primarily responsible for the respiratory burst observed in phagocytic cells. Upon activation of NOX2, phosphorylation and translocation of the cytosolic regulatory subunits, $p47^{phox}$ (*ncf1*), $p67^{phox}$ (*ncf2*), and $p40^{phox}$ (*ncf4*) occur. These subunits associate with the membrane subunits of NOX2, allowing electron transfer from NADPH and ultimately reducing O_2 to O_2^-. Studies of NOX2 in IAV-infected mice have shown that loss of NOX2 leads to increased clearance of IAV, reduced inflammation, and improved lung function (Grandvaux et al., 2015).

NOX have also been shown to promote chemotaxis of phagocytic cells, such as macrophages and neutrophils, during *C. albicans* infection. Time-lapse imaging of NOX-blocked zebrafish infected with *C. albicans* showed a lower number of neutrophils recruited to the infection site at early and late stages of infection (Brothers et al., 2011, 2013). Taken together, it has been shown that recruitment of phagocytic cells and their production of ROS is an essential component of the immune response.

While ROS play a vital role in eliminating pathogens during the innate immune response, collateral damage to surrounding tissue can occur due to excessive or

prolonged inflammation (Segal et al., 2012). The production of ROS from phagocytic cells is responsible for many acute and chronic lung inflammatory diseases including acute lung injury, acute respiratory distress syndrome, asthma, and COPD (Grandvaux et al., 2015). The balance between the production of ROS and limiting the injury from excessive inflammation is critical in host survival (Segal et al., 2012). Studying how the components of NOX work to achieve this balance can lead to a more complete understanding of the impact of ROS during IAV infections. Studying how the components of NOX work to achieve this balance can lead to a more complete understanding of the impact of ROS during IAV infections.

Neutrophil Cytosolic Factor 1

Ncf1, previously known as $p47^{phox}$, codes for a cytosolic subunit of NOX2 and has been shown to be vital in the elimination of pathogens. Knockdown of *ncf1* causes a loss of phagocyte recruitment comparable to that of NOX2 knockdown, a result that suggests *ncf1* is required for sufficient phagocyte recruitment (Brothers et al., 2013). Additionally, *ncf1* knockout mice were shown not to produce superoxide anion and were unable to effectively kill staphylococci. In these studies, mice developed lethal infections and granulomatous inflammation similar to that in human chronic granulomatous disease (CGD), an inherited disease characterized by the failure to mount an innate defense against bacterial and fungal infections (Jackson et al., 1995). Taken together, these studies show that *ncf1* has a critical role in both phagocyte recruitment and in phagocyte activity.

Zebrafish as a Model Organism

Animal models are used in biomedical research to study the pathogenesis of human diseases at the organismal, cellular, and molecular levels. Zebrafish (*Danio rerio)* bridge the gap between invertebrates, such as *C. elegans* and *Drosophila melanogaster*, and mammals, such as mice, rats, and sheep (**Fig. 3)**. Zebrafish share a number of physiological, anatomical, and genetic characteristics with humans while still maintaining the ease of use of a lower organism (Goldsmith and Jobin, 2012). This model has been used since the 1930s, when it was introduced as a developmental and embryological model. The unique combination of optical clarity and embryological manipulability made zebrafish a classic model in this field of research. In the 1980s, their use extended to cloning, mutagenesis, transgenesis, and genetic mapping. (Lieschke and Currie, 2007).

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 A comparison of model organisms commonly used in the study of human pathophysiology. The zebrafish is a cheap, effective model. (Lieschke and Currie, 2007)

Since its introduction as a research model, the zebrafish has been utilized due to its larval transparency (through 7 days post fertilization), high fecundity (100 eggs per clutch), amenability to genetic manipulation, and overall genetic and organ system homology to humans (**Fig. 4**). Other benefits of using the zebrafish as a model organism include external fertilization, which allows access to all developmental stages, egg size (0.7mm in diameter), which allows for hundreds of eggs to be utilized in one experiment, and rapid development, with all major organs fully developed within the first 36 hours post fertilization (Spence et al. 2008).

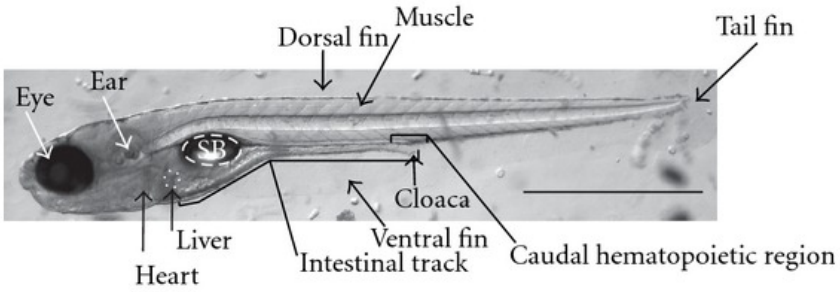

Fig. 4 Diagram of zebrafish anatomy at 6 days post fertilization. Larval transparency and small size are two benefits associated with using the zebrafish. SB=swim bladder. Scale bar is 1mm (Goldsmith and Jobin, 2012)

Additionally, zebrafish have many characteristics that make them an ideal model for the study of the innate immune response to Influenza virus. The innate immune system of a zebrafish is very similar to that of the human; it contains many of the same cell types including neutrophils, Natural Killer cells, monocytes, and macrophages, as well as cytokines and their associated signaling molecules and pathways. The innate immune system of a zebrafish is fully functional within 48 hours post fertilization (hpf), while the adaptive immune system takes 4 – 6 weeks to develop. This allows for easy observation of the innate immune system without interference from the adaptive immune system (Goldsmith and Jobin, 2012). Larval transparency aids in the examination of the

host immune system's interaction with the pathogen *in vivo* and in real time. Because zebrafish are amenable to genetic manipulation, strains have been developed with fluorescently marked phagocytic cells to further aid in the visualization of the host-pathogen interaction (Lieschke and Currie, 2007).

Previous studies in the Kim Lab have shown that zebrafish are an exceptional model organism for the study of IAV. Zebrafish epithelial cells have the same α – 2,6 linked sialic acid residues found on human IAV receptor cells, permitting human IAV to attach to, and enter, zebrafish cells. It has been shown that IAV is able to replicate in zebrafish cells and cause a systemic infection, leading to mortality in the zebrafish host. Furthermore, infected zebrafish present a pathology phenotype that is parallel to that in IAV-infected humans (**Fig. 5**). By 24 hours post infection (hpi), zebrafish become lethargic with yolk sac and pericardial edema that worsens over time. Histopathological analysis of the infected zebrafish showed characteristic symptoms of IAV, such as necrosis and edema. Additionally, fluorescence imaging of host infection using a GFP-expressing strain of IAV demonstrated the multiplication of IAV in the infected zebrafish (Gabor et al., 2014).

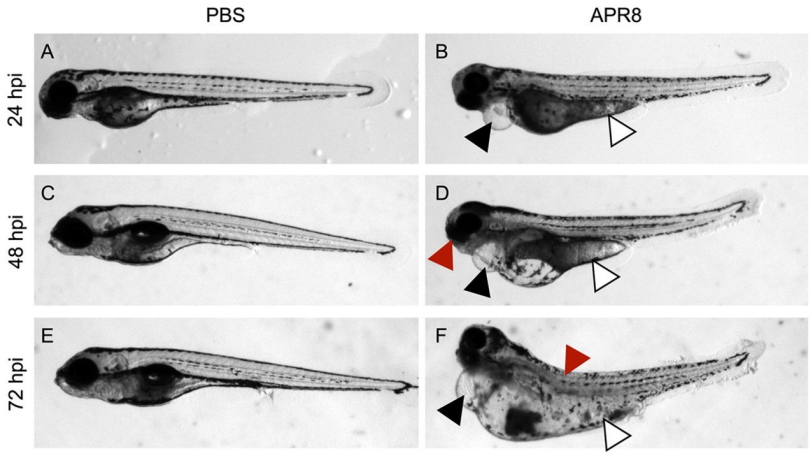

Fig. 5 IAV infection causes phenotypic changes in zebrafish. Infection caused pericardial edema (black arrowheads), yolk sac edema (white arrowheads), craniofacial abnormalities (red arrowhead in D), and arched backs (red arrowhead in F). (Gabor et al., 2014)

This study utilizes zebrafish as a model organism to

study the role of *ncf1* in the innate immune response to IAV. Zebrafish were used in the study to show the effects of *ncf1* knockdown on the innate immune system with and without IAV infection. Determining the role of *ncf1* in the innate immune response to IAV could lead to novel viral therapies for viral diseases.

Materials and Methods

Zebrafish Care and Maintenance

Zebrafish embryos were collected at the one-cell stage of development from the spawning of wild-type (AB) zebrafish. The embryos were stored in egg water (deionized water and 60mg/L of Instant Ocean, Spectrum Brands, Madison, WI) at 28°C. The egg water was changed every 24 hours and dead zebrafish were collected and disposed of daily. Unused zebrafish were euthanized by immersion in a lethal dose of tricaine and were disposed of properly.

Respiratory Burst Assay

1.0 mg of H_2DCFDA was dissolved in 1 mL dimethyl sulfoxide (DMSO). This stock solution was stored at -20°C until use. 1mg phorbol myristate acetate (PMA) was dissolved into 1mL of DMSO to produce a 1mg/mL stock, which was stored at -80°C until use. A working solution of H2DCFDA was made by adding 20 µL of H2DCFDA to 20 µL of DMSO in a 1.7 mL microcentrifuge tube wrapped in aluminum foil. A working solution of PMA was made by adding 10 µL of PMA to 490 µL of nuclease free water in a 1.7 mL microcentrifuge tube.

A dosing solution of H2DCFDA was made by adding 4990 µL of egg water and 10 µL of H2DCFDA working solution to a 15 mL conical tube labeled “H”. A dosing solution of H2DCFDA and PMA was then created by adding 4890 µL of egg water, 10 µL of H2DCFDA working solution, and 100 µL of PMA working solution into a 15 mL conical tube labeled “H+P”. Both dosing solutions were wrapped in aluminum foil and kept on ice until use.

Zebrafish were added individually to a 96-well microplate along with 100 µL of egg water. Columns 1-4 contained control zebrafish while columns 5-8 contained *ncf1* MO zebrafish. The H2DCFDA dosing solution was poured into a 25 mL reservoir. 100 µL of H2DCFDA dosing solution was added to columns 1-4, one channel at a time. The H2DCFDA and PMA dosing solution were then poured into another 25 mL reservoir. 100 µL of the H2DCFDA and PMA dosing solution were added to columns 5-8, one column at a time.

The microplate was then covered with aluminum foil and placed on a shaker for 20 seconds at 150 rpm to ensue the homogeneity of the mixture. The microplate was read at time = 0 hr in a microplate reader set to read fluorescence: Excitation – 485 nm, Emission – 528 nm, Optics position – top 510 nm, Sensitivity – 65, and a 5 second shaking before the read. The microplate was also read at time = 4 hr.

The data were analyzed by subtracting the average fluorescence of the un-induced control group from the individual PMA-induced control group's florescence values. The same calculation was done using the experimental PMA-induced and un-induced groups. The data were organized into two columns: a control + PMA column and an experimental + PMA column. The mean and standard deviations were calculated from the normalized fluorescence values of the two columns. The means of the two columns were compared using an unpaired, two-tailed t-test. The means of the two groups, control + PMA column and experimental + PMA, were graphed with error bars to show the standard deviations (Goody et al., 2013); (Hermann et al., 2004).

Morpholino Injections

A morpholino (MO) is an oligonucleotide that binds to complementary RNA, temporarily knocking down expression of the targeted gene by blocking translation or pre-mRNA splicing (Draper et al., 2001). Most MOs work for 7 days, allowing ample time for experimental observations. Approximately 400 zebrafish embryos were collected at the one-cell stage of development and microinjected with 3ng/embryo of MO to knockdown *ncf1*. Approximately 400 embryos at the one-cell stage were injected with 3ng/embryo of control MO. The zebrafish were stored in a 28°C incubator.

Influenza A Virus Infection

The MO injected zebrafish were dechorionated manually using forceps at 2 days post fertilization (2dpf). Approximately 60 zebrafish were injected with APR8 at 1.5×10^4 EID50/embryo IAV into the duct of Cuvier to create a systemic infection (Fig. 6). Thirty *ncf1* MO zebrafish and 30 control MO zebrafish were injected with IAV while 30 additional *ncf1* MO zebrafish and control MO zebrafish were injected with a control. The control contained phenol red for visualization and Hank's Balance Salt Solution, a buffer used to maintain pH and osmotic balance. Zebrafish that were not properly injected were removed and disposed of

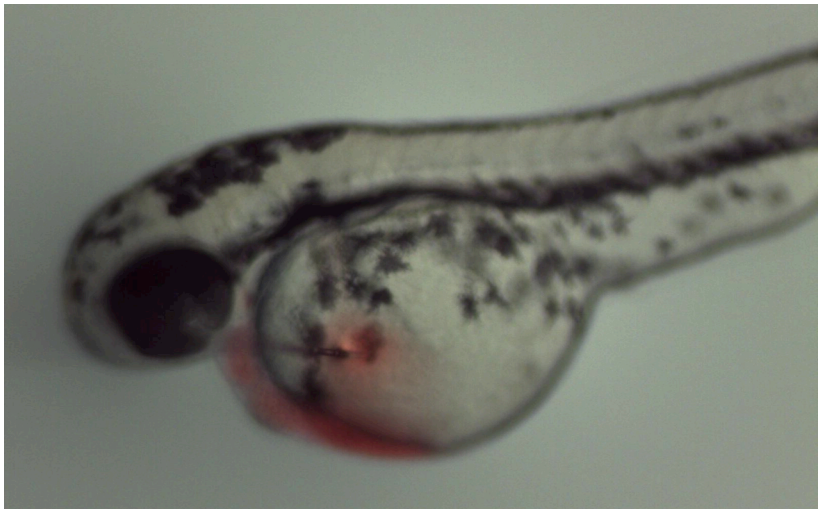

Fig. 6 The duct of Cuvier is a large vein found along the yolk sac in 2dpf zebrafish. It runs directly into the heart, creating a rapid systemic infection.

properly to ensure all animals included in the study were infected with either IAV or the control. After viral injection, the zebrafish were stored in 50mL of egg water at 33°C. Every 24 hours, dead fish were removed and the egg water was changed.

Fixing

At 4 dpi zebrafish were fixed with 4% paraformaldehyde, which kills the fish and crosslinks their proteins into a rigid structure, ensuring their durability for long-term storage and imaging. Fish were incubated at room temperature on the bench top for 1-2 hours then rinsed out of the fixative using PBS containing 0.1% Tween. The fish were washed 3 times with PBS 0.1% Tween then left in PBS 0.1% Tween in a dark 4° C incubator for up to one month before imaging.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR was used to quantify the amount of gene expression in real time. Zebrafish were infected with either IAV or a mock infection at 2 dpf with no MO. The zebrafish were fixed at 12 hpi, 24 hpi, 48 hpi, 72 hpi, and 96 hpi. cDNA was then extracted from the fixed fish following iScript protocol (BioRad, 2007). A master mix was made using 5.0 µL of PerfeCTa SYBR Green from Quanta BioSciences, 0.2 µL of 10uM forward and reverse primers, and 3.8 µL of NFW. 1.0 µL of cDNA was added to the master mix to create 10 µL total. This solution with cDNA from mock-infected fish was placed in rows A-C. The solution with cDNA from IAV-infected fish was placed in rows D-F. Column 1 contained 12-hpi cDNA, column 2 contained 24-hpi cDNA, column 3 contained 48-hpi cDNA, column 4 contained 72-hpi cDNA, and column 5 contained 96-hpi cDNA. Column 6 was left blank, and this pattern was repeated in columns 7-11. Rows 1-5 measured the expression of the gene of interest, *ncf1*, while rows 7-11 measured the

expression of a control gene, 18S. 18S is a housekeeping gene and will always show the same amount of expression.

During qPCR, the gene of interest is repeatedly amplified and the fluorescent nucleic acid stain, SYBR, binds to the accumulating DNA product, resulting in enhanced dye fluorescence. The fluorescence values are measured and are directly correlated to the amount of DNA produced during the amplification process.

Mounting

Before imaging was possible, zebrafish had to be mounted in agarose gel. Fish were individually placed into wells of a 24-well plate. All liquid was removed from the wells using a 3mL plastic transfer pipette. A 1% solution of agarose was poured into the well to cover the bottom of the well along with the entire fish. Before the gel could harden, the fish was positioned on its side for easy viewing in the inverted microscope. PBS was added on top of the hardened gel, the plate was wrapped in aluminum foil, and was stored in a 4°C incubator until needed for imaging.

Confocal Imaging

Confocal microscopy was performed to visualize morphological changes, such as edema, in the zebrafish. Laser scanning confocal microscopy allows for imaging of a specimen in a series of different focal planes to create a 3D image. The software program allows images to be compiled, saved, and processed.

Results

N*cf1* knockdown causes a decreased respiratory burst in zebrafish.

A respiratory burst assay of control zebrafish and *ncf1* MO zebrafish was done to determine the difference in the magnitude of the respiratory burst produced. The magnitude of a respiratory burst is indicative of the amount of ROS produced by phagocytic cells. Zebrafish with *ncf1* knockdown exhibited a significantly smaller respiratory burst than control zebrafish. This suggests that *ncf1* plays a critical role in the production of ROS. A decrease in the production of ROS can be either beneficial or detrimental to an organism. Some production of ROS is necessary to eliminate infections or heal wounds, but too much ROS production can cause damage to surrounding host cells (Segal et al., 2012). In the case of IAV infection, ROS can cause permanent damage to lung epithelial tissue (Grandvaux et al., 2015).

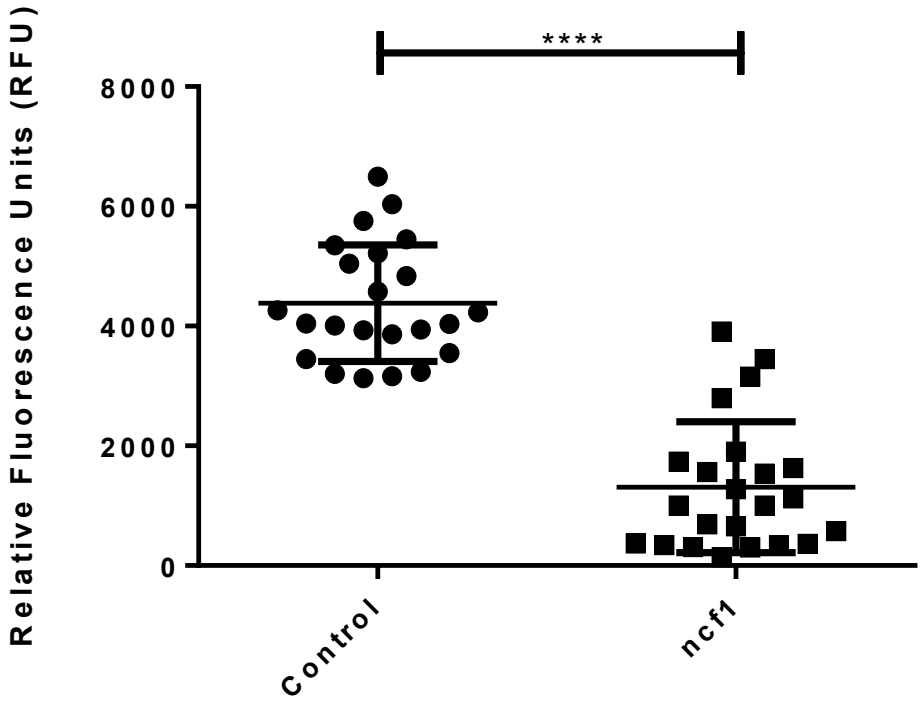

Fig. 7 Knockdown of *ncf1* in zebrafish significantly reduces the magnitude of the respiratory burst.

qPCR of *ncf1* after infection with IAV shows significant upregulation of *ncf1* at 48 hours post infection (hpi) and at 96 hpi.

A qPCR assay of *ncf1* in IAV-infected zebrafish was done to determine whether *ncf1* has any response, negative or positive, to IAV. The results of this study show that *ncf1* is significantly upregulated at 48 hpi and 96 hpi, while there is no change at 24 hpi and an insignificant amount of upregulation at 72 hpi. It cannot be determined from these data whether *ncf1* is being upregulated as part of the host's immune response to IAV or as a mechanism of IAV infection. Because *ncf1* is responsible for the recruitment and activity of phagocytic cells, one assumption could be that *ncf1* is upregulated to boost the host's immune response and recruit more phagocytic cells to the site of infection, leading to an increase in the production of ROS.

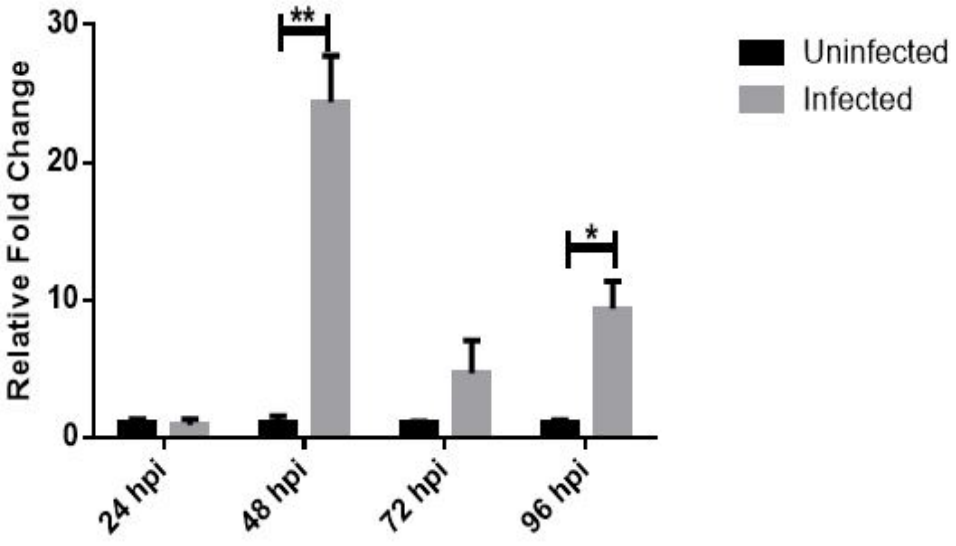

Fig. 8 *Ncf1* is significantly upregulated at 48 hpi and 96 hpi with IAV.

Ncf1 knockdown significantly decreases survival of IAV-infected zebrafish

Six trials were done (represented below in **Fig. 8**) to confirm that *ncf1* knockdown does not enhance the survival of IAV-infected zebrafish, contradicting previous studies done on IAV infections and IAV (Grandvaux et al., 2015). In fact, based on the data presented here, it appears that *ncf1* knockdown decreases the survival rate of IAV-infected zebrafish when compared to control IAV-infected zebrafish. These findings are consistent with previous studies done on *ncf1* in response to *C. albicans* infection in zebrafish (Brothers et al., 2013; Jackson et al., 1995). An interesting observation about these data is that only 90% of the *ncf1* MO zebrafish with mock infections survived while 100% of the control zebrafish with mock infection survived. This may indicate that *ncf1* aids in zebrafish survival with or without IAV infection. However, more research is necessary to confirm this hypothesis.

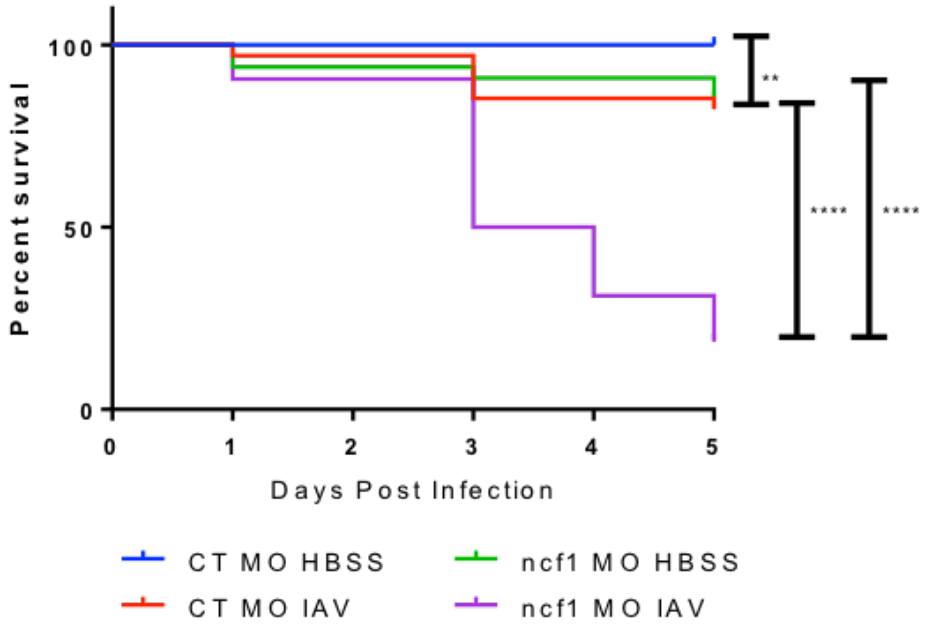

Fig. 9 Knockdown of *ncf1* does not enhance survival of IAV-infected zebrafish.

Discussion

IAV infections are responsible for over 21,000 deaths and 170,000 hospitalizations annually in the U.S alone, costing billions of dollars in healthcare. IAV is a particularly important focal point for research because it can give rise to pandemic infection, as it has in the past. Influenza vaccines have decreased the risk of Influenza infection in humans by 50-60%, but there are very few effective therapies for those who do acquire an infection (CDC, 2016). A more complete understanding of the host's immune response to IAV could lead to more effective therapies for this and other viral diseases.

The goal of this study was to understand more completely the role of *ncf1* in the innate immune response to IAV infection. qPCR was used to examine the effect of IAV infection on *ncf1*. It was found that IAV infection upregulated *ncf1* at 48 and 96hpi. *Ncf1* was then knocked down using a MO injection. A respiratory burst assay was performed to determine the effect of *ncf1* on the production of ROS and it was found that *ncf1* knockdown significantly decreases the production of ROS. Zebrafish were injected with *ncf1* MO or a control MO then injected with IAV or a mock injection to create four subsets of zebrafish. These fish were observed for 5 days post infection to determine the effect of *ncf1* knockdown on survival rates. These fish were also imaged using a confocal microscope to view the effects of *ncf1* knockdown on the development of pericardial and yolk sac edema. It was found that *ncf1* knockdown significantly decreased survival rates in IAV-infected zebrafish.

These data show that *ncf1* is critical in the host's innate immune response to IAV. Previous work shows that NOX2 (of which *ncf1* is a component) can cause permanent

damage to epithelial tissue during IAV infection due to the prolonged inflammatory response of immune cells that secrete proinflammatory cytokines (Grandvaux et al., 2015). However, it has also been shown that NOX2 and *ncf1* play a role in sufficient, not prolonged, phagocyte recruitment and activity (Brothers et al., 2014). These data emphasize the importance of the balance between exacerbated inflammation and insufficient inflammation, as pointed out by Segal et al., 2012. These studies also yielded critical information about the role of NOX2 and *ncf1* in the innate immune response to fungal and viral infections, but it is clear from the present study that future research on the topic should be aimed at determining the appropriate amount of inflammation for affectively eliminating an IAV infection while minimizing damage to surrounding epithelial cells.

The present study produced preliminary data that can lead to further investigations of the role of *ncf1* in the innate immune response to IAV. The data collected led to new questions about *ncf1*'s role in the innate immune system, as well as in the overall health of uninfected zebrafish. Previous work from the Kim lab (Gabor et al., 2014) showed that IAV-infected zebrafish exhibit phenotypic changes including pericardial and yolk sac edema (**Fig. 5**). It was observed during infection trials that *ncf1* MO fish, whether subjected to mock infection or infected with IAV, exhibited increased edema when compared to control zebrafish with mock infection or with IAV infection (**Fig 10**). Other work performed in the Kim lab has shown that the *ncf1* MO has no off-target effects and that any phenotypic changes are likely to be due to the loss of *ncf1* (Jacob Longfellow, personal communication).

Another question that arose during this study was how the knockdown of *ncf1* would affect an RBA with IAV infection. **Fig. 7** shows an RBA of control zebrafish compared to *ncf1* knockdown zebrafish, in the absence IAV infection. During this study, one RBA with IAV infection was performed, but the results were inconclusive. It would be beneficial to the overall understanding of *ncf1*'s role in an IAV infection to perform several RBA's of *ncf1* MO with IAV infection. Neutrophil response to viral infection is not understood completely. Since it is known that *ncf1* has a critical role in phagocyte recruitment and activity, it would be useful to know whether or not *ncf1* knockdown decreases the magnitude of ROS produced during an IAV infection. To test this, a neutrophil-specific RBA could be performed.

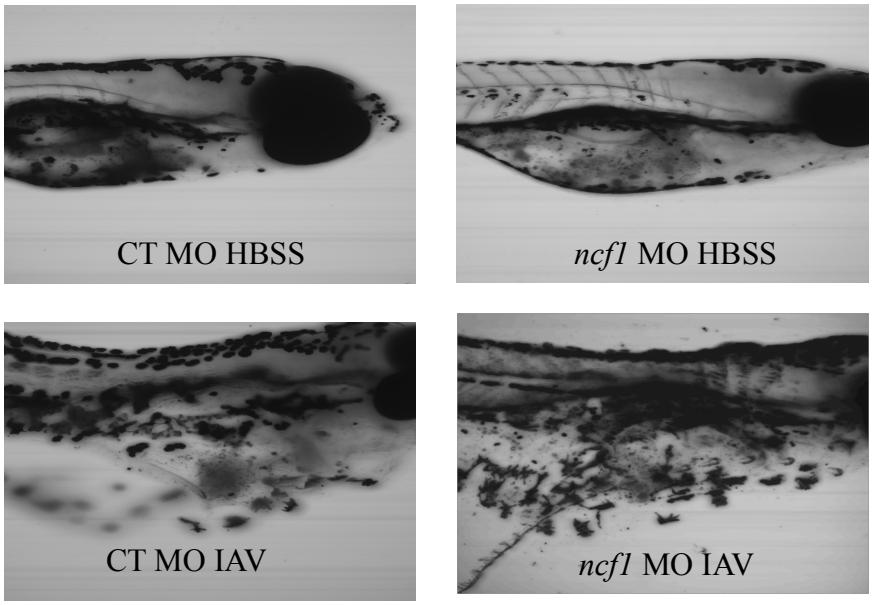

Fig. 10 Confocal imaging of control, *ncf1*-morphant, IAV-infected, and mock-infected zebrafish shows increased edema in IAV-infected zebrafish as well as *ncf1*-morphant zebrafish.

Cytokine profiling of *ncf1* MO zebrafish and control MO zebrafish could also yield valuable information. *Ncf1* is known to be partially responsible for the recruitment and activity of phagocytic cells, which, along with engulfing foreign particles, releases cytokines for further recruitment of immune cells. Cytokine profiling would determine whether or not *ncf1* plays a role in the release of cytokines from phagocytic or other cells

after IAV infection. Cytokine production is vital in the innate immune response and determining which genes are involved in their production would be a worthy endeavor.

Lastly, genetic manipulation of zebrafish to overexpress the *ncf1* message, and therefore protein, would add to what is already known about how *ncf1* responds to an IAV infection. Overexpressing a gene can make the gene's normal effects, such as increased edema or dysfunction in organ systems, easier to visualize. All of these studies would lead to a greater understanding of the role of *ncf1* in the innate immune response to IAV in zebrafish, with the hope of discovering novel therapies for viral diseases.

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After graduation, Lucy plans to work as an EMT in Cape Elizabeth, Maine and volunteer at Maine Medical Center. She will also be working at Sugarloaf Mountain as a ski and snowboard instructor for the third year. Lucy enjoys spending her free time outdoors with her friends and family.